Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (4)

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

Sport und Buch Strauß, Köln, 1997

S. HORNING, W. SCHÄNZER:
Steroid Screening Using GC/HRMS

In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke (eds.) Recent advances in doping analysis (4). Sport und Buch Strauß, Köln, (1997) 261-270

S. Horning and W. Schänzer

Steroid Screening Using GC/HRMS

Institute of Biochemistry, German Sports University,
Carl-Diem-Weg 6, D-50933 Köln, Germany

Gas chromatography/high resolution mass spectrometry (GC/HRMS) is a powerful analytical technique for the detection of anabolic steroids. In the preceding contribution, fundamentals of high resolution mass spectrometry and the use of electric field scanning for multiple ion detection (MID) were described. Here we report on the screening of steroids by GC/HRMS and illustrate the use of this technique for the detection of metabolites of metandienone and stanozolol.

As noted in the preceding contribution, the Finnigan MAT 95 GC/MS instrument is usually termed as simply a high resolution mass spectrometer (HRMS), but we have shown that it is properly characterized as a high resolution, high sensitivity, high precision and high stability instrument. In the Cologne laboratory, the MAT 95 has been used since 1993 for routine screening of anabolic steroids. For the HRMS screening analyses we use the per-TMS derivatized urine extract (combined fraction) prepared for analysis with the quadrupole (GC/MS) mass spectrometer, i.e., there is no special sample workup for the HRMS analysis, the sample is simply reinjected. Analyses are performed using electric field MID at 3,000 resolution and largely long-term excreted metabolites are monitored to increase the retrospectivity of steroid misuse. The analyses are automated and under computer control. The HRMS analysis is not a replacement for screening performed with the quadrupole mass spectrometer, rather it compliments the GC/MS screening procedure and allows for a more complete steroid screening analysis.

Several anabolic steroids and their metabolites are included in the HRMS screening analysis. Many are listed in the following table. In general, steroids and steroid metabolites which are excreted in high amounts are not monitored as they are easily detected in the quadrupole

SOME STEROIDS SCREENED USING HRMS "COLOGNE LABORATORY"

Substance	GC Index
3α-hydroxy-5α-estran-17-one	2438
3α-hydroxy-5β-estran-17-one	2487
17β-hydroxy-5β-androst-1-en-3-one	2451
clenbuterol	2164
3'-hydroxystanozolol	3218
4β-hydroxystanozolol	3218
methyltestosterone	2753
17β-methyl-5β-androst-1-ene-3α,17α-diol	2453
17α-methyl-5β-androst-1-ene-3α,17β-diol	2604
3α-hydroxy-1α-methyl-5α-androstan-17-one	2604
17β-methyl-5β-androstane-3α,17α-diol	2442
17β-methyl-5α-androstane-3α,17α-diol	2458
17α-methyl-5α-androstane-3α,17β-diol	2608
17α-methyl-5β-androstane-3α,17β-diol	2614
epitestosterone	2614
18-nor-17,17-dimethyl-5β-androst-1,13-dien-3α-ol	2265
18-nor-17,17-dimethyl-5α-androst-13-en-3α-ol	2271
testosterone	2660
3α-hydroxy-2α-methyl-5α-androstan-17-one	2553
3α-hydroxy-1-methylen-5α-androstan-17-one	2576
9-fluoro-17α-methylandrost-4-ene-3α,6β,11β,17β-tetrol	2856
4-chloro-3α-hydroxyandrost-4-en-17-one	2691
17α-ethyl-5β-estrane-3α-17β-diol	2693
4-chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-en-16-one	2997
epioxandrolone	2666

GC indices of steroids TMS derivatives (MSTFA/TMIS) determined using a 17 m Ultra 1 column, 0.2 i.d., 0.11 μm film. Temperature program from 185°C at 5°C/min to 310°C.

HRMS SCREENING ANALYSIS


```
CHRO:  60222005                          22-Feb-96   Elapse:  07:03.0     575
Samp:  Vial 1 std met 1+                             Start :  19:12:53   1566
Comm:  185/5/240/20*SGE1(12)18*P16*SP20*S220*P250
Mode:  EI +VE + LMR  ESCAN LR
Oper:  METHYL2*1.75*R3000                            Inlet :          Vial   1
Peak:  1.00  mmu        Label wndw:   525 > 589      Masses:  317 > 660
Area:  1, 0.00, 0        Baseline  :   15, 2          Label :  1, 3.00
Disp:  Height
```

NORAND

m/z:405.2645 — 6:50 3760585; 7:00 2607; 7:03 1280; E+06 3.761

m/z:420.2879 — 6:50 1686790; 6:43 792; 7:00 22184; 7:06 3987; E+06 1.687

m/z:422.2308 — 6:47 72402; 7:02 1126; 7:06 32585; E+04 7.240

m/z:432.2880 — 6:59 885810; 6:47 9525; 6:51 6608; 7:07 3160; E+05 8.858

6:45 6:50 6:55 7:00 7:05

CLENB

m/z:335.0695 — 3:30 3630302; 3:37 14837; E+06 3.630

m/z:336.0586 — 3:30 2256261; 3:26 6799; 3:38 27249; E+06 2.256

m/z:337.0665 — 3:30 2514352; 3:25 51867; 3:37 7872; E+06 2.514

3:25 3:30 3:35 3:40

STAN METAB

m/z:520.3462 — 14:06 558838; 14:09 37816; 14:13 1731; 14:14 2077; 14:16 10597; E+05 5.588

m/z:545.3415 — 14:10 1045291; 14:06 25663; 14:15 44528; E+06 1.045

m/z:560.3650 — 14:10 1340627; 14:06 5197; 14:15 37926; E+06 1.341

14:04 14:06 14:08 14:10 14:12 14:14 14:16 14:18

MeT ISTD

m/z:448.3192 — 10:43 1547894; 10:34 415534; 10:27 4469; 10:51 2255; 11:00 193512; E+06 1.548

10:30 11:00

screening analysis. Many endogenous steroids are also monitored in the HRMS analysis since they are useful 'markers' to assist in reading the data, but normally no quantification is performed. Some steroid TMS derivatives, especially 17-methyl steroids, are not ideally suited for MID analysis (nor GC/MS or GC/MS/MS analysis, for that matter) as they predominantly form low mass fragment ions, e.g., the *m/z* 143 ion. Interestingly, this ion ($C_4H_6OSi(CH_3)_3$, *m/z* 143.0892) is not useful for trace level steroid analysis in urine, e.g., for the metabolites of methyltestosterone or stanozolol at low (1 ng/mL) concentration, as there is usually a high background signal which hampers the identification of the forbidden substance. Some steroids are more easily identified in the free fraction due to the reduced biological background, e.g., oxandrolone and formobolone, or as their non per-TMS derivative, e.g., 6β-hydroxymetandienone bis-TMS. As in any GC analysis, careful consideration of the biological background must be taken into account and proper GC derivatization conditions (catalyst selection), GC columns (polarity, film thickness, length), GC temperature programs, and sample injection volumes and methods (split or splitless injection) must be chosen.

An example of the output generated from the HRMS steroid screening analysis is shown on the previous page for a urine spiked with a synthetic mixture of steroids and steroid metabolites. Inspection of the ion chromatograms reveals the presence of forbidden substances. Ion traces for endogenous species are plotted to simplify the reading of the ion chromatograms, e.g., the *m/z* 520.3462 ion (top ion trace) in the traces for 3'- and 4β-hydroxystanozolol is used as a marker of the elution time of the stanozolol metabolites (STAN METAB, upper 3 ion traces on right hand side of figure). The header information reveals the significant analysis parameters, such as the sample name, vial position and the data file name (60222005, *ymmddxxx*, where *xxx* is the sequence sample number (005), *dd* the date (22), *mm* the month (02) and *y* the year). The MID analysis file name METHYL2, the multiplier voltage, 1.75 kV and the resolution, 3,000, are also shown, as is the GC program, GC column, split ratio and temperatures. All of the information listed in the header is automatically appended to a file after each analysis to document the analysis and simplify data archiving and searches. The data are saved on magnetic tape (DAT) and on magnetic optical disk (MOD).

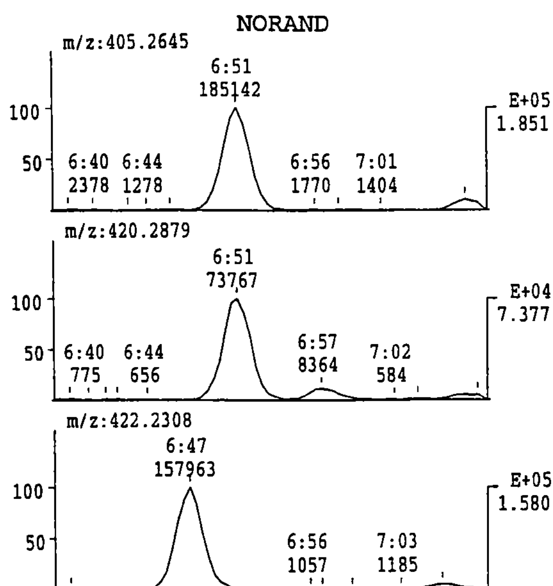
GC analyses are performed in the split injection mode (2 μL, 1:20 split, from a 2 mL urine extract derivatized with 100 μL). This is advantageous as less material is deposited onto the column (longer column life) and the ion source is kept cleaner. To perform GC analyses in the splitless mode, the sample must be specially prepared (HPLC fractionation or immunoaffinity purification [1]) or diluted, as excessive sample material leads to unstable ion source conditions. Several GC columns have been tested, among the best were an Ultra 1, 0.11 μm film thickness (Hewlett Packard), a BPX5, 0.25 μm film thickness (SGE) and a DB5-MS, 0.25 μm film thickness (J&W); however, the best overall separation of TMS derivatives of anabolic steroids and their metabolites from endogenous substances is obtained using the Ultra 1 column. With the polar columns there was poorer separation from coeluting background material for several steroids, including the metandienone metabolite, 17β-methyl-5β-androst-1-ene-3α,17α-diol, and the stanozolol metabolites, 3'- and 4β-hydroxystanozolol. The polar columns, however, allow for good separation between the 18-nor metandienone metabolite, 18-nor-17,17-dimethyl-5β-androsta-1,13-dien-3α-ol, and stearic acid TMS. Slow and rapid GC temperature ramps were also tested and the best overall separation was found using an initial temperature ramp of 5°C/min. until the elution of methyltestosterone, at which time a more rapid temperature ramp of 20°C/min. is employed, as this greatly enhances the elution profile (sharper peak) of the stanozolol metabolites. A slower initial temperature ramp, e.g., 3°C/min., is not advantageous as some anabolic steroid metabolites coelute with endogenous material. The analysis time is 14:30 min. and a new analysis is started every 19 minutes.

Ions are formed via electron impact ionization at 65 eV electron energy. The ion source temperature is kept at 240°C. The degree of fragmentation of TMS steroids varies with the ion source temperature. At lower source temperature the molecular ion intensity is greater; however, the ion source (tuning) is less stable. Since the instrument was installed in 1993, the ion source has been cleaned on only one occasion, June 1995, after analysis of more than 10,000 samples. From the time of the last ion source cleaning to present (March, 1996) nearly 6,000 samples have been analyzed. The electron multiplier is operated at 10^6 gain for MID analyses at 3,000 resolution and care is taken to be sure the multiplier voltage (or accelerating voltage) is set to 0 V between analyses when it is not needed. MID steroid screening analyses are performed at 3,000 resolution as this has proven to provide the best compromise between sensitivity and selectivity,

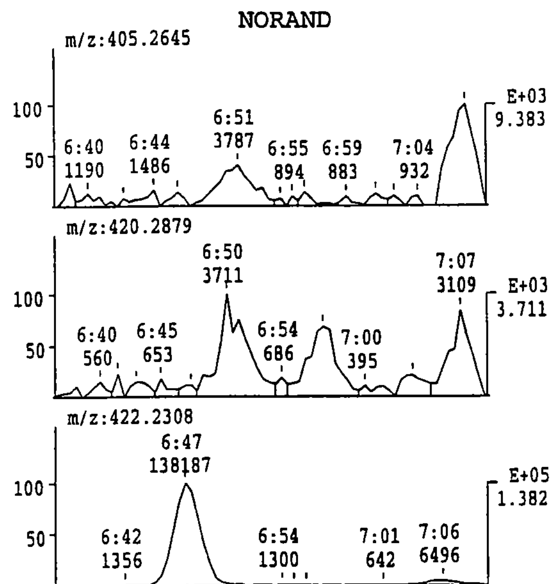
NORANDROSTERONE SPIKED URINE

NORMAL AND 1/100 CONCENTRATION

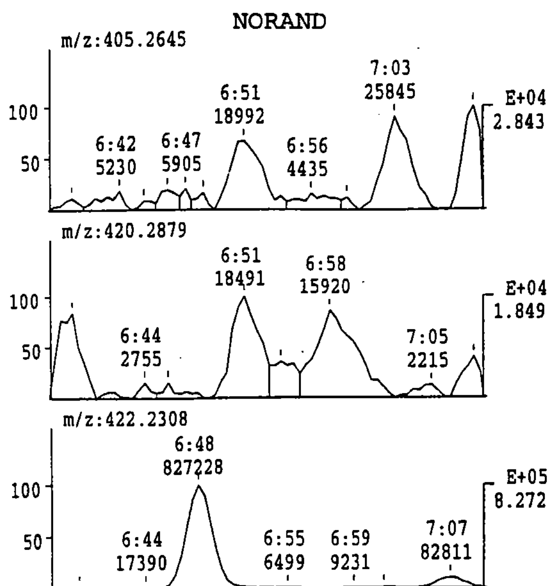
2 ng/ml (4 pg on column)
3000 Resolution



20 pg/ml (40 fg on column)
3000 Resolution



20 pg/ml (40 fg on column)
1500 Resolution

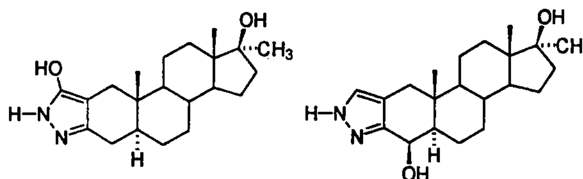


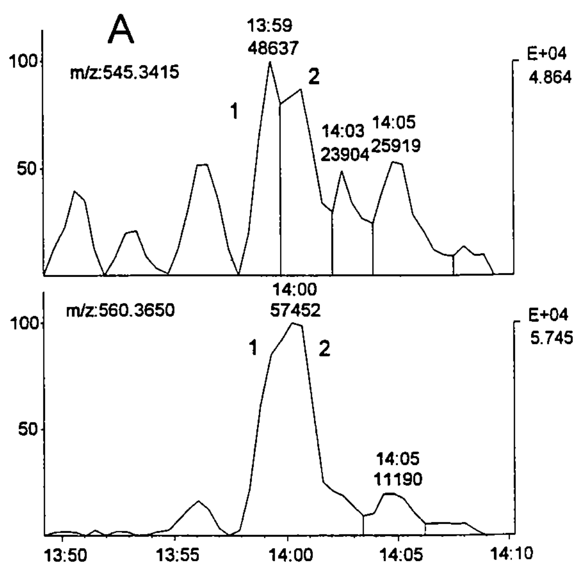
i.e., discrimination of background noise. At higher resolution, 5,000 or even 10,000, there is not a large enhancement in selectivity for most of the steroid metabolites, an exception is the stanozolol metabolites, but there is a loss in sensitivity. At lower resolution there is an increase in sensitivity but at a loss of specificity. This is shown in the analysis of a sample (2 mL urine) spiked with norandrosterone measured at 3,000 and 1,500 resolution. In the upper left corner ion traces are depicted for the sample spiked at 2 ng/mL and measured at 3,000 resolution in the split injection mode (normal operating conditions). When the norandrosterone concentration is diluted 100 fold, figure in upper right corner, the traces for norandrosterone are greatly diminished. The intensity of the ion *m/z* 422. 2308 (eluting at 6:47 min.) does not change. An improvement in the signal intensity can be achieved by lowering the resolution to 1,500, as shown in the bottom trace, but there is a loss of selectivity, e.g., in the ion trace *m/z* 405.2645 a signal appears at 7:03 min. that was not seen at 3,000 resolution. Note that at 1,500 resolution there is no contribution from the vitamin E metabolite in the ion traces *m/z* 405.2645 or 420.2879.

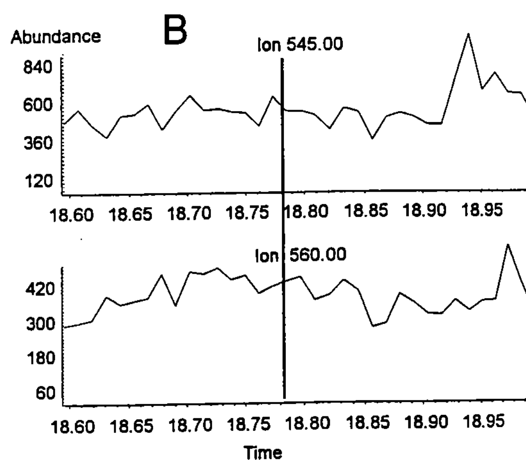
The final section of this contribution shows examples which illustrate the enhanced performance of the MAT 95 as compared to a conventional quadrupole mass spectrometer (Hewlett Packard, 5971A) for screening of urine samples for anabolic steroids. Analyses with the 5971A were performed in the split injection mode (3 μL, 1:10 split, or a factor of 3 times more sample injected than with the MAT 95). The following figure depicts the results of a routine screening analysis for a urine sample found positive for metabolites of stanozolol. Note that there is not complete separation of the stanozolol metabolites, 3'- (**1**) and 4β-hydroxystanozolol (**2**); however, the broadened peak assists in the identification of these metabolites. When a suspicious sample is identified, it is reanalyzed using gel immunoaffinity chromatography preparation to isolate the steroids from background material [1,2]. The second example shows results of screening analyses for one of the long-term excreted metabolites of metandienone, 17β-methyl-5β-androst-1-ene-3α,17α-diol (epimetenediol or EMD). The long-term retrospectivity following metandienone administration is due to the low level of background signal in the *m/z* 358.2692 ion trace. Additionally, a second metabolite, 18-nor-17,17-dimethyl-5β-androsta-1,13-dien-3α-ol (18-Nor-EMD), is used to detect metandienone administration, as is illustrated in the screening analysis report shown for a urine sample found positive for metandienone metabolites. Ion traces are shown for the two metabolites in the upper left-hand corner and bottom right-hand corner of

COMPARISON OF MAT 95 TO MSD QUADRUPOLE MASS SPECTROMETER

Detection of stanozolol metabolites

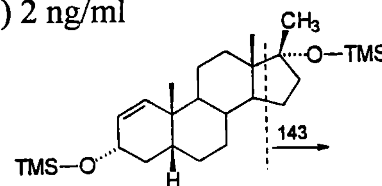


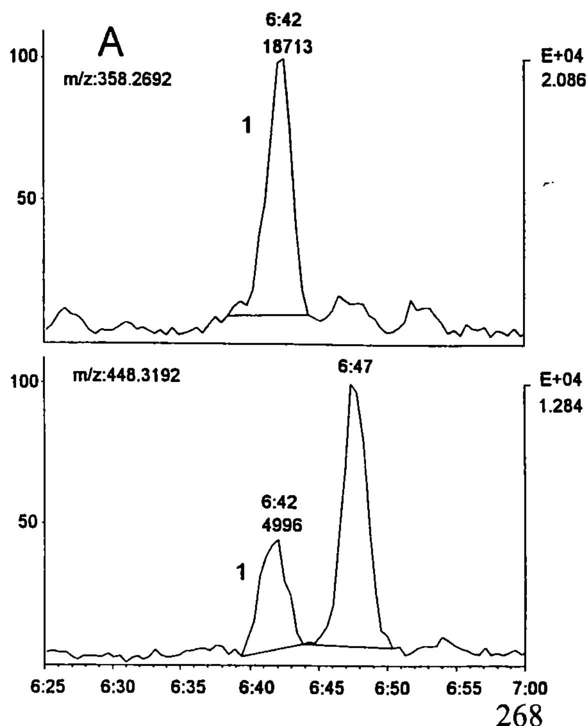


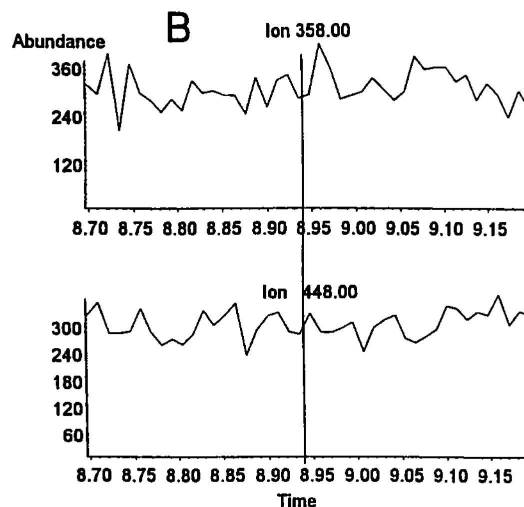


COMPARISON OF MAT 95 TO MSD QUADRUPOLE MASS SPECTROMETER

Detection of epimetenediol (17β-methyl-5β-androst-1-ene-3α,17α-diol) 2 ng/ml (4 ng/100 μl or 4 pg on column MAT 95, 12 pg on column MSD)







Screening Analysis of Sample Containing EMD and 18-Nor-EMD

EpiMetendiol (EMD)

THMT

Metdiol/Mester M

18-Nor-EMD

ANABOLIC STEROID POSITIVES

Cologne Laboratory, 1995 Jan-Sep

Number of Positives

MSD normal Procedure

MAT High Resolution

Metandienone Stanozolol Nandrolone Testosterone DHCIMT other Steroids

the screening report. In the upper portion of the figure, the ions *m/z* 448.3192 (M^+) and 358.2692 (M^+-90) eluting at 7:04 min. correspond to the epimetenediol (EMD) metabolite. In the lower portion of the figure, the ions the ions *m/z* 358.2692 (M^+) and 253.1956 (M^+-105) correspond to the 18-Nor-EMD metabolite. The major peak eluting at 4:40 min. in the ion trace *m/z* 358.2692 for 18-Nor-EMD (middle trace) corresponds to stearic acid TMS. Shortly thereafter, the 18-Nor-EMD metabolite is eluted (4:43 min.). To confirm the presence of either metabolite, the sample is reanalyzed using HPLC fractionation [1,3]. After isolation, a mass spectrum can be recorded which unambiguously demonstrates the presence of the metandienone metabolite.

Since the employment of the MAT 95 for routine screening analysis, there has been a dramatic increase in the number of positive steroids cases. Of the 116 positive cases reported last year (1995), 41 were detected using conventional GC/MS analysis. The other 75 positives were identified only by GC/HRMS screening. This enhanced performance is closely coupled with improved sample workup procedures which have been developed here in Cologne. GC/HRMS screening has had it's greatest impact on the detection of metabolites of metandienone and stanozolol. Detection of clenbuterol is also greatly enhanced, although here further improvements in the sample preparation must be made. Other analytical techniques, especially GC/MS/MS, performed either with a triple-quadrupole or an ion trap instrument, e.g., GCQ (Finnigan MAT), might also offer enhanced performance and it will be interesting to see how the use of these alternative techniques progresses.

References

1. W. Schänzer, P. Delahaut, H. Geyer, M. Machnik and S. Horning **Long-term detection and identification of metandienone and stanozolol abuse in athletes by gas chromatography-high resolution mass spectrometry** *J. Chromatogr. B.* **687**, 93 (1996).

2. M. Machnik, P. Delahaut, S. Horning and W. Schänzer **Purification and concentration of anabolic steroids by immuno affinity chromatography (IAC)** in W. Schänzer (Editor), *Recent Advances In Doping Analysis (4) - Proceedings of the 14th Cologne Workshop on Dope Analysis 1996*, Sport und Buch Strauß, Köln 1997.

3. A. Gotzmann, H. Geyer and W. Schänzer **HPLC clean-up for urine samples with disturbing background for confirmation of 17β-methyl-5β-androst-1-ene-3α,17α-diol (epimetenediol)** in W. Schänzer (Editor), *Recent Advances In Doping Analysis (4) - Proceedings of the 14th Cologne Workshop on Dope Analysis 1996*, Sport und Buch Strauß, Köln 1997.