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17,17-Dimethyl-18-nor-5ß-androst-1,13-dien-3α-ol (18-Normetenol) in Longterm Detection and Confirmation of Positive Metandienone Cases

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Introduction

Several publications on the metabolism of metandienone (Fig.1) have shown the complex metabolic pathways of this synthetic steroid. This began with the first publication in 1963, when 6ß-hydroxylation of metandienone was reported by Segaloff and Rongone [1]. In 1971 the occurence of a 17-epimeric metabolite was confirmed by MacDonald et al. [2].

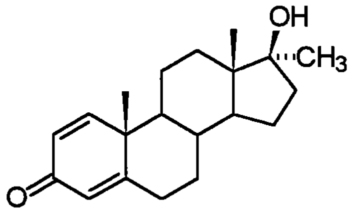

Fig. 1 Structure of metandienone

The mechanism for the formation of the 17-epimetandienone was investigated in 1989 by Edlund et al. [3] who proved the formation of a 17ß-sulphate which spontaneously decomposes in aqueous solution to several dehydration products and to 17-epimetandienone (Fig.2). Additional data to this unexpected chemical reaction pathways was published in 1992 [4, 5] and shows this rearrangement process occurs for nearly all 17ß-hydroxy-17α-methyl anabolic androgenic steroids. Additional studies on the metabolism of metandienone were presented by Dürbeck et al. [6] who only investigated the unconjugated urine fraction. Dürbeck first confirmed a 17,17-dimethyl-18-nor product of metandienone which is generated during the epimerization process (Fig.2). He also reported the formation of a 6,7-dehydro product of metandienone whose mechanism of generation is still unknown. In 1991 Schänzer et al. [7] presented data on the excretion of reduced conjugated metabolites of metandienone. The structure of most of these metabolites were confirmed by chemical synthesis.

For optimum detection of anabolic steroid misuse a strategy was developed to confirm and to identify these metabolites which could be detected for the longest time period after application. This has been reported for metandienone [7, 8] for the following three metabolites: 17,17-dimethyl-18-nor-5ß-androst-1,13-dien-3α-ol **I** (18-normetenol), 17ß-methyl-5ß-androst-1-ene-3α,17α-diol **II** and 17α-methyl-5ß-androstane-3α,17ß-diol **III**. All excreted as conjugates which can be liberated with ß-glucuronidase from E.coli. Both metabolites **I** and **II** are generated from the same precursor, 17α-methyl-5ß-androst-1-ene-3α,17ß-diol, which is assumed to decompose as its 17ß-sulphate, as presented in Fig.3.

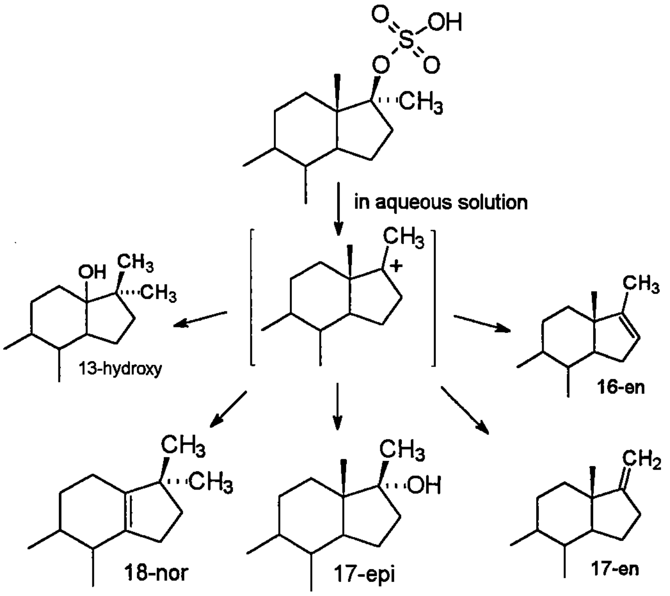

Fig.2 Rearrangement of 17ß-sulphated 17ß-hydroxy-17α-methyl steroids. Figure obtained from reference [10].

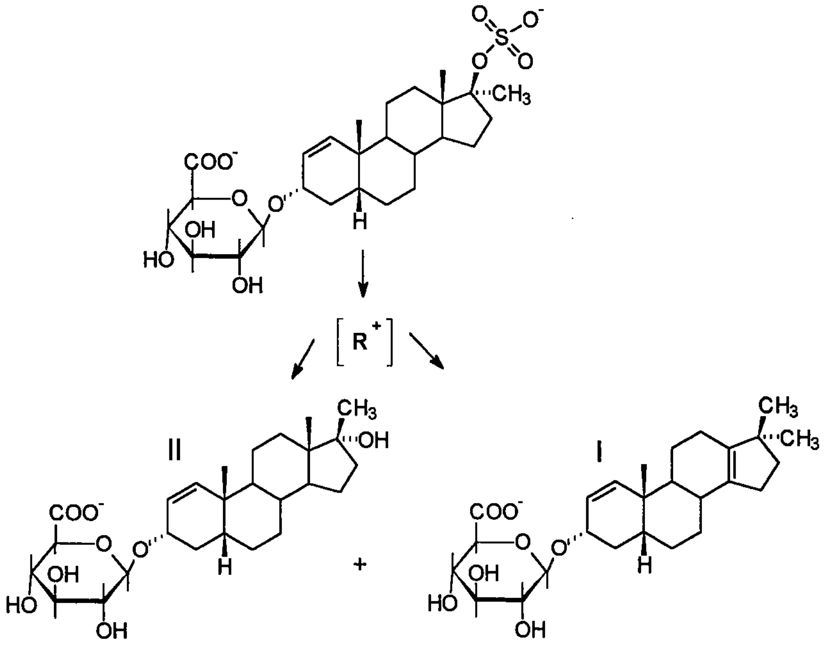

Fig.3 Mechanism for the formation of metandienone metabolites **I** and **II**. Rearrangement in urine. Figure obtained from reference [10].

In GC/MS analysis metabolites **I** and **II** elute from the GC column earlier than metabolite **III.** The detection of **I** and **II** is enhanced compared to **III** and they allow the detection of metandienone misuse for a long period of time after its last application [9, 10].
In 1997 analysis of positive metandienone samples showed that metabolites **I** and **II** varied in intensity in different positive urine samples. In several positive samples only 18-normetenol **I** was detected and indentified. This result was surprising as it was assumed that both metabolites are generated in urine via a 17ß-sulphate of the precursor 17α-methyl-5ß-androst-1-ene-3α,17ß-diol (Fig.3) and that the rearrangement process should yield both steroids in a constant ratio of intensities.
Here we describe the actual procedure to confirm positve metandienone cases by identification of only metabolite **I**, 18-normetenol. The detection of metabolites **I** and **II** with different intensities was also investigated and a reasonable explanation for this phenomenon is presented.

Results and Discussion

Synthesis of 18-normetenol andits generation as a GC rearrangement product of epimetendiol

18-Normetenol was synthesized in small amounts as a reference substance [9] starting with 5 mg of 17α-hydroxy-17ß-methyl-5ß-androst-1-en-3-on (synthesis of this product see reference [7]) solved in 1 ml of acetonitrile (HPLC grade).

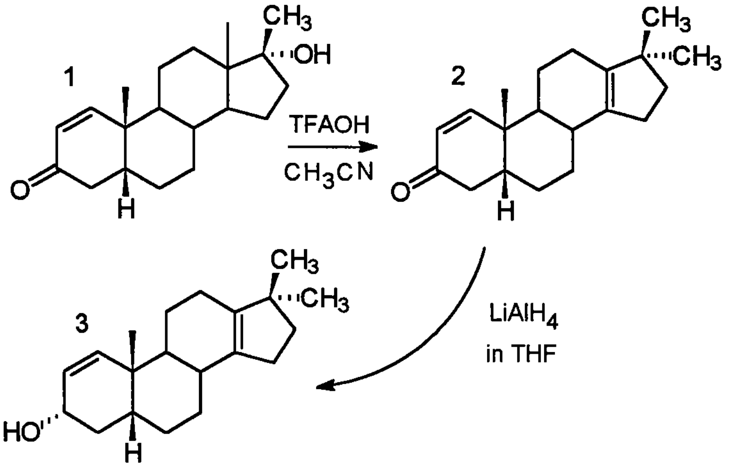

Fig.4 Synthesis of 18-normetenol.

The mixture was acidified with 0.1 ml of trifluoroacetc acid and heated for 1 h at 60°C. The reaction mixture was dried under reduced pressure, solved in 1 ml of tetrahydrofuran to which 50 mg of lithium aluminium hydride are added. After 10 min at room temperature 2 ml of water was added to remove the excess of the hydride. The reduced steroid was extracted with 5 ml of t-butylmethyl ether, dried over sodium sulphate and evaporatred to dryness. The reaction yielded in high amount 18-normetenol. 18-Normetenol TMS is also partly generated from epimetendiol bis-TMS via gas chromatography when active insert liners are used (Fig.5). This side reaction should be avoided in general and the extent of gas chromatographic rearrangement was controlled by GC/MS analysis of a epimetendiol bis-TMS calibration standard.

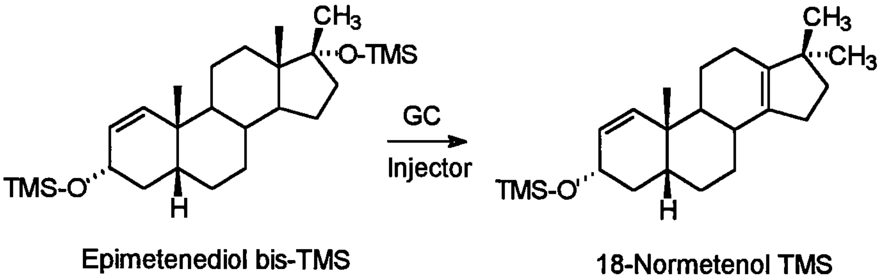

Fig.5 Formation of 18-normetenol TMS by GC rearrangement of epimetenediol bis-TMS

Rearrangement of trimethylsilylated 17-hydroxy-17-methyl steroids has been observed in gas chromatography only with 17α-hydroxy-17ß-methyl steroids and not with 17ß-hydroxy-17α-methyl steroids (unpublished results). Therefore it is assumed that the 17α-hydroxy-17ß-methyl configuration is less stable than the epimeric form.

Detection of 18-normetenol as a main metabolite in positive cases

Since the introduction of high resolution mass spectrometry (HRMS) in dope analysis the detection of anabolic steroid misuse has clearly improved. Table 1 shows the increase from 1994 to 1995 in the number of positive A-samples for metandienone in doping control confirmed by the Cologne laboratory. In 1996 and 1997 the number of metandienone positives decreased, which can be discussed as a reaction of athletes, who have reduced anabolic steroid misuse because they became aware of the improved detection of anabolic steroids. On the contrary, it was obvious that a change in the detectability of metabolites **I** and **II** occured. Most positive cases showed in comparison to positive cases in the years before very weake signals for epimetenediol (**II**), often it was not detected, whereas an abundant signal for 18-normetenol (**I**) was observed (Fig.6). In screening (Fig.6) for metabolites **I** and **II** two diagnostic ions are registrated (Table 3). With the ion 432.2880 peaks coeluting with epimetendiol-bis-TMS are

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controlled. For the assessment of 18-normetenol TMS only one ion 253.1956 is exploitable as the ion 358.2692 is extensively influenced by partly coelution with stearic acid TMS. The ion 358 is the isotopic ion ($M^+ + 2$) of stearic acid TMS and at a resolution of 3.000 (used in screening) it is detected in the 18-normetenol trace (M^+) 358.2692. A complete separation is achieved at a resolution of 8.000 (see confirmation) which is not the resolution of choice for screening.

Table 1 Positive metandienone cases in the Cologne laboratory from 1994-1997

Year	Total Number of Analyzed Samples	No. of Positves Total (HRMS*)	Confirmation Epimetenediol	Confirmation 18-Normetenol
1994	8005	4	4	-
1995	6717	78 (63)	78	-
1996	7171	28 (21)	28	-
1997	6053	31 (28)	7	24

* only detected in HRMS screening and not with GC/Low Resolution MS instruments

Table 2 Retention times of metabolites **I**, **II** and stearic acid as TMS derivatives

Substance	HRMS[1] Time [min]	HRMS[2] Time [min]	ITMS[2] Time [min]
Stearic acid TMS	4.68	6.77	6.00
18-Normetenol TMS (**I**)	4.72	6.80	6.04
Epimetenediol bis-TMS (**II**)	7.07		7.57
17α-Methyl-5ß-androstane-3α,17ß-diol bis-TMS (**III**)			8.75

1) GC conditions HRMS screening: Column: Ultra 1 crosslinked methyl silicone (OV 1) capillary, length 17 m, I.D. 0.2 mm, film thickness 0.11 µm, helium, 12 psi, flow 1 ml/min, split 1:10; temperature program: 0 min 180°C, + 3°C/min,0 min 229°C, + 40°C/min, 2 min 310°

2) GC conditions HRMS confirmation: same column as in 1), splitless mode, temperature program: 0 min120°C, + 20°C/min, 0 min 200°C, + 3°C/min, 0 min 218°, + 40° C/min, 2 min 320°C

3) GC conditions Ion Trap MS/MS: same column as in 1), helium, 13 psi, flow: 1 ml/min, splitless mode; temperature program: 0,5 min 100°C, +40°C/min, 0 min 180°C, + 10°C/min , 0 min 250°, + 40° C/min, 2 min 320°C

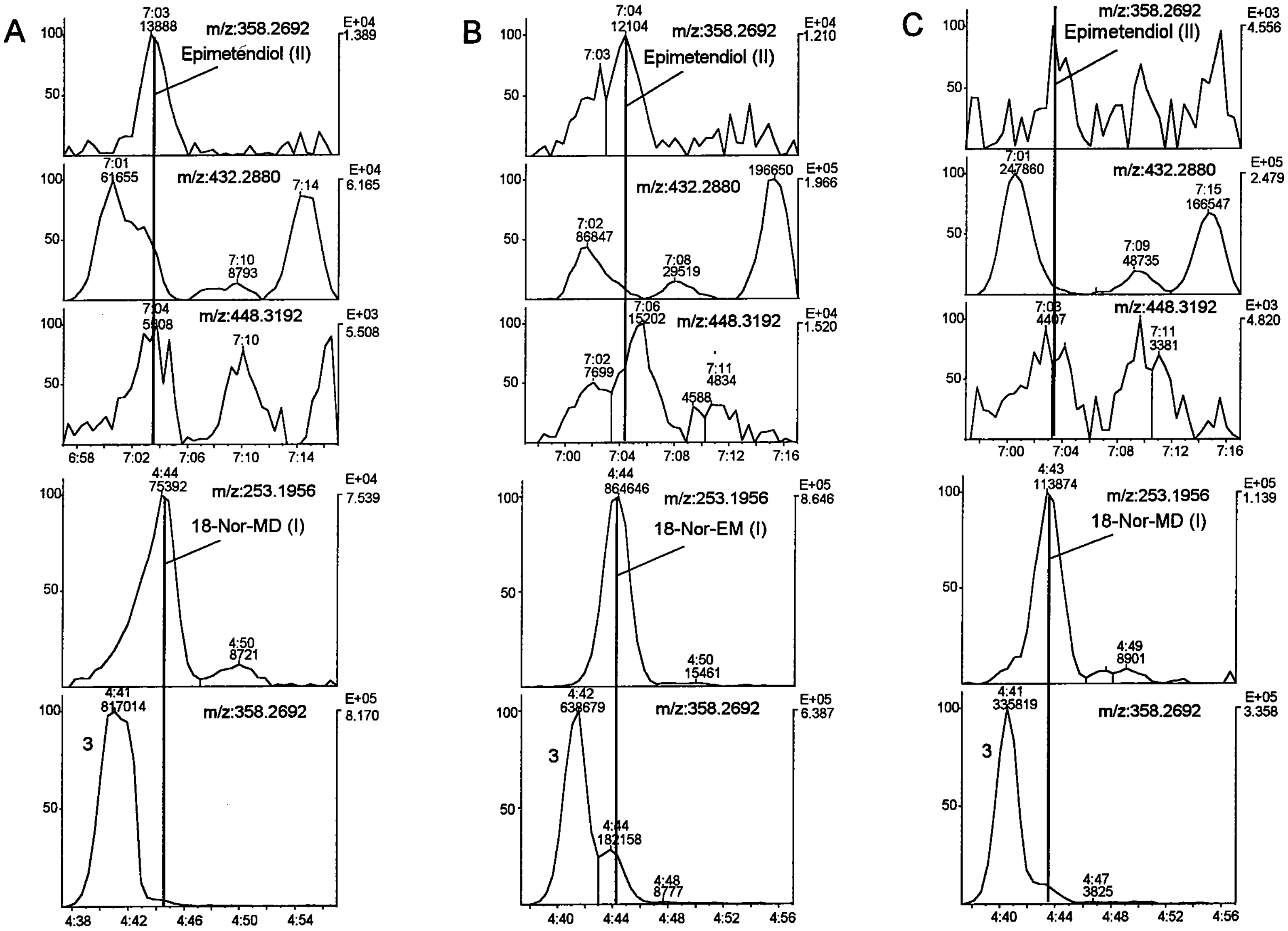

Fig.6 GC/HRMS screening of 18-normetenol (**I**) and epimetenediol (**II**), GC-conditions see Table 2; A) Control urine containing 2ng/ml of **I** and **II**, B) and C) Positive urines, with less intense of **II** but abundant signals of **I**; stearic acid TMS (3).

GC/HRMS confirmation of 18-normetenol (I) obtained from urine of positive samples,

For GC/HRMS confirmation metabolites **I** and **II** were isolated by HPLC [9]. In most positive cases confirmation was only possible for metabolite **I** since the MS criteria for confirmation of **II** were not fulfilled. This is in agreement with the screening results that metabolite **I** was more distinctly detected as compared to metabolite **II**.
A gas chromatographic separation problem in the confirmation of metabolite **I** TMS is the gas chromatographic co-elution of stearic acid TMS (Table 2). Stearic acid is permanentely present in all samples and a complete separation by sample clean-up is difficult to achieve. A sufficient separation of **I** from stearic acid is possible using (i) a different derivative, for example a t-butyldimethylsilyl (TBS) derivative, (ii) using HRMS at a resolution of 8.000 or (iii) Ion Trap MS/MS techniques with registration of daughter ions obtained by selected reaction monitoring (SRM) of a single fragment ion produced in the EI-ionization process.

In this paper we describe the use of TMS derivatives and separation by two different MS techniques. For HRMS confirmation a resolution of 8.000 was used to separate stearic acid completely from metabolite **I**. The exact masses used for the HRMS screening and confirmation analysis are listed in Table 3.

Table 3 Elemental composition and exact masses of analysed derivatives

Substance	Elemental Composition	Exact Mass
18-Normetenol TMS	$C_{23}H_{38}OSi$ (M^+)	358.2692
	$C_{22}H_{35}OSi$ (M^+ - CH_3)	343.2457
	$C_{20}H_{28}$	268.2191
	$C_{19}H_{25}$	253.1956
	$C_{16}H_{24}$ (Fig.7)	216.1878
	$C_{15}H_{21}$ (Fig.7)	201.1643
Epimetenediol bis-TMS	$C_{26}H_{48}O_2Si_2$ (M^+)	448.3192
	$C_{23}H_{38}OSi$	358.2692
Stearic acid TMS (M^+ 356)	$C_{20}H_{41}O_2Si$ + 2,0016 (isotope) (M^+ - CH_3 + 2)	343.2892

For HRMS confirmation of 18-normetenol the major ion fragments of the TMS derivative generated under EI ionization at 70 eV (see mass spectrum Fig.7) are selected. The ion fragmentation can be explained as m/z 358 (M^+), 343 (M^+ - CH_3), 268 (M^+ - TMSOH), 253 (M^+ - TMSOH - CH_3), 216 and 201 see Fig.7.

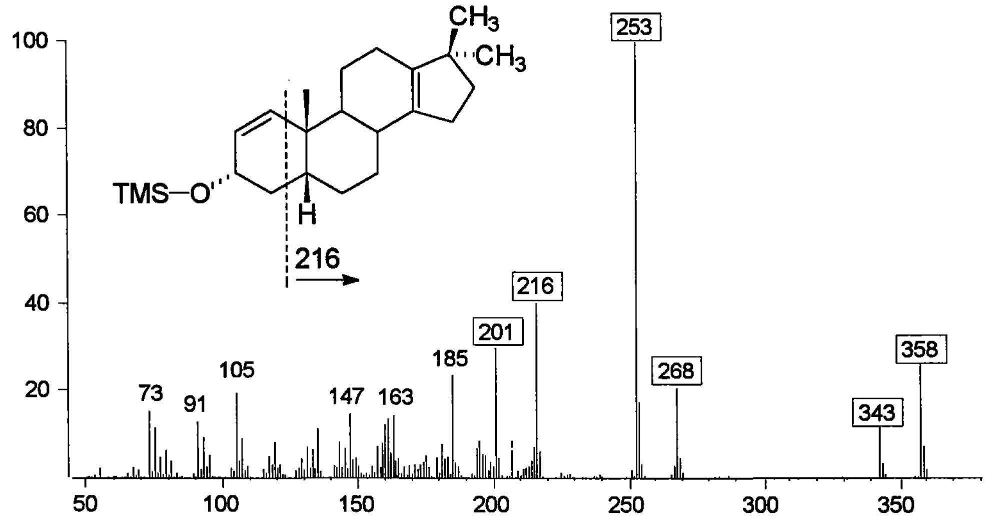

Fig.7 EI mass spectrum of 17,17-dimethyl-18-nor-5ß-androst-1,13-dien-3α-ol TMS (18-normetenol) M^+ 358, registration with HRMS instrument

For confirmation it is recommended to introduce an additional HPLC clean-up for isolation of 18-normetenol from urine to obtain improved GC/MS chromatograms for the selected ions. This enables the identification of metabolite **I** even at concentrations of 2ng/ml of urine and lower.

Fig.8 displays the HRMS confirmation for 18-normetenol using 6 substance specific ions at a resolution of 7000. The low intense baselines of the blank urine (chromatogram B) and the complete separation of stearic acid TMS (m/z 343.2892) from the ion trace 343.2457 for 18-normetenol TMS demonstrate the advantage of this method.

Ion Trap MS/MS confirmation of 18-normetenol obtained of positive urine samples

While HRMS is an expensive technique it was investigated whether the GCQ ion trap from Finnigan with MS/MS option can be used to confirm positve metandienone samples with low concentrations of 18-normetenol **I**. For this purpose daughter ion experiments of **I** were performed by selected reaction monitoring with abundant and characteristic ions generated via EI-ioniziation in the ion trap (EI mass spectrum, see Fig.9).
Screening of metabolites **I** and **II** with the ion trap was less sufficient compared to the HRMS screening.

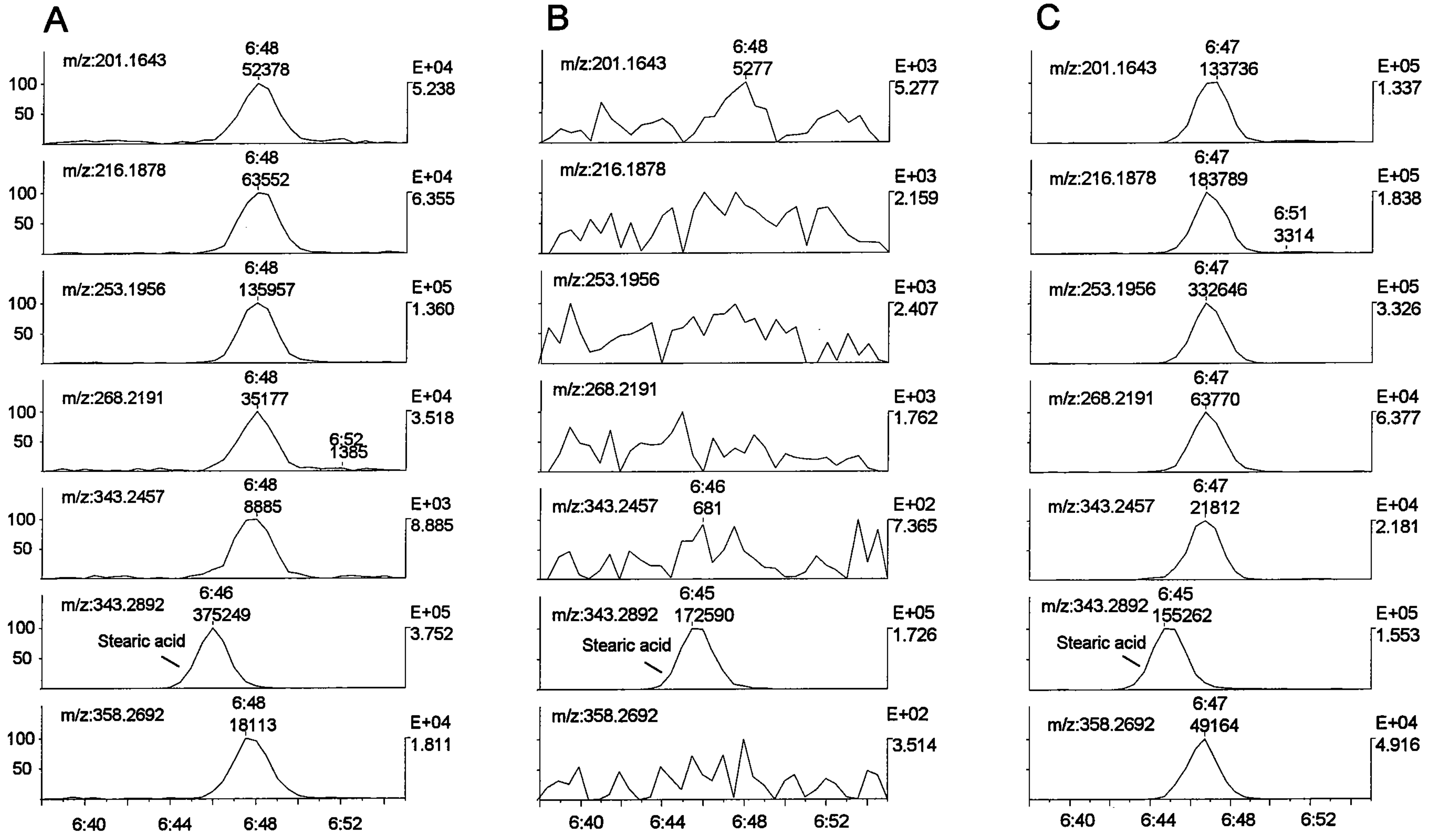

Fig.8 GC/HRMS confirmation of 18-normetenol (I) following HPLC separation, derivatisation with MSTFA/TMIS; A) Standard with 2ng /ml urine, B) Blank urine, C) Positive control, GC conditions see Table 2

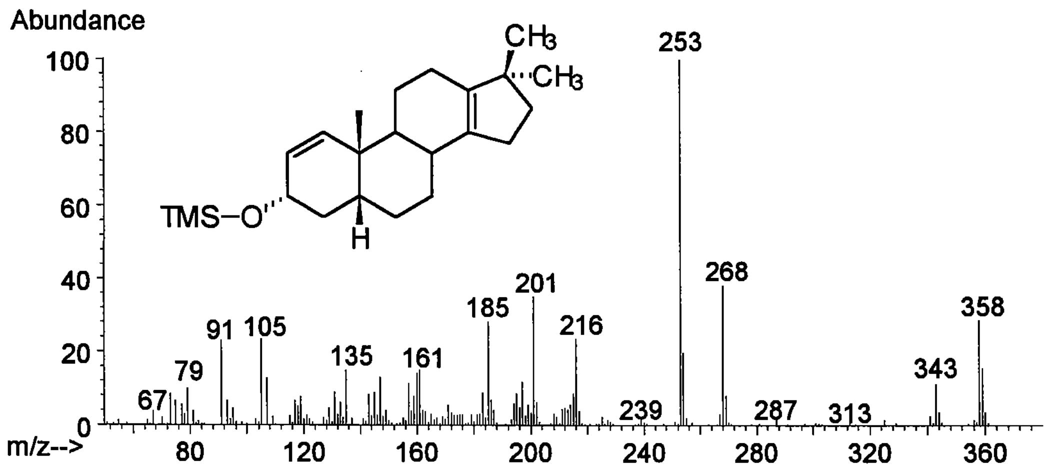

Fig.9 Ion Trap mass spectrum of 18-normetenol TMS (M^+ 358), 2ng on column

Daughter ion experiments show the best results with selected reaction monitoring of the fragment ion 253 using a collision energy of 1.0 eV (Fig.10). The fragment ion 253 is not present in the EI-fragmentation of stearic acid TMS and no interference occurs.

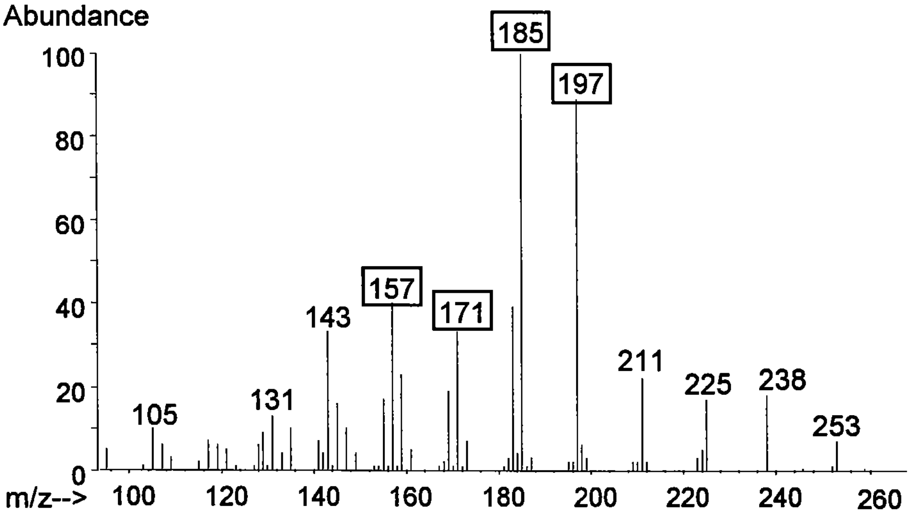

Fig.10 Daughter ion MS/MS spectrum of the selected fragment ion 253 of 18-normetenol TMS, collision energy 1.0 eV, 2 ng on column

For estimation of daughter ion intensities selected ion chromatograms of a reference standard of **I** using the ions 197, 185, 171 and 157 were plotted (Fig.11) and compared with a urine without HPLC isolation (Fig.12, spectra Fig.13) and a metandienone positive urine sample without (Fig.14, spectra Fig.15) and with HPLC isolation (Fig.16, Spectra Fig.17).

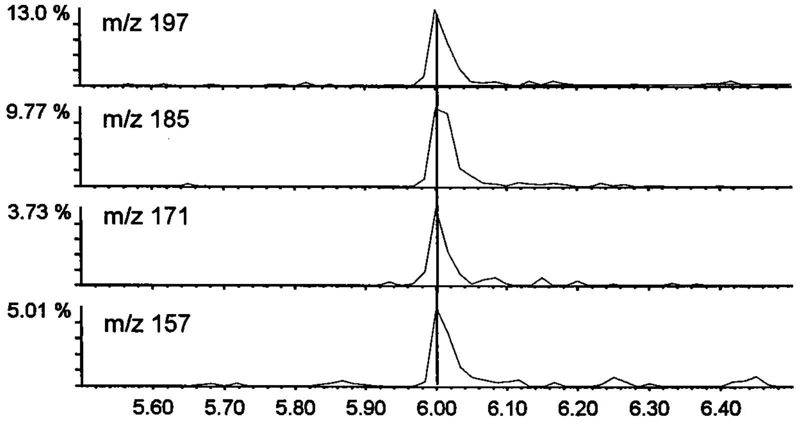

Fig. 11 MS/MS daughter ion chromatogram of the selected fragment ion 253 of 18-normetenol, reference standard 200 pg on column.

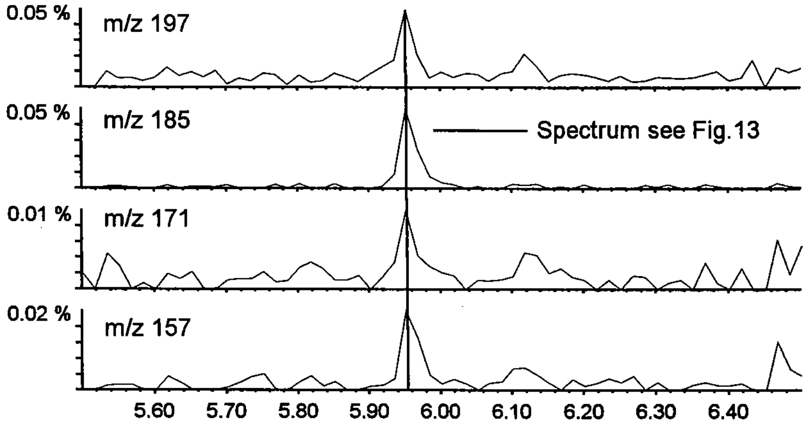

Fig. 12 MS/MS daughter ion chromatogram of the selected fragment ion 253 of a control urine with 2 ng 18-normetenol / ml of urine, approximately 200 pg on column, without HPLC clean-up.

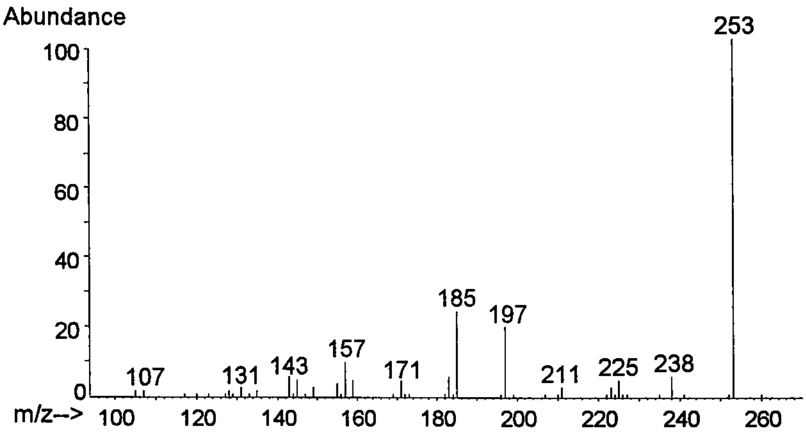

Fig. 13 Daughter ion MS/MS spectrum of the selected fragment ion 253 of a control urine with 2 ng 18-normetenol/ml urine of about 200 pg on column, without HPLC clean-up.

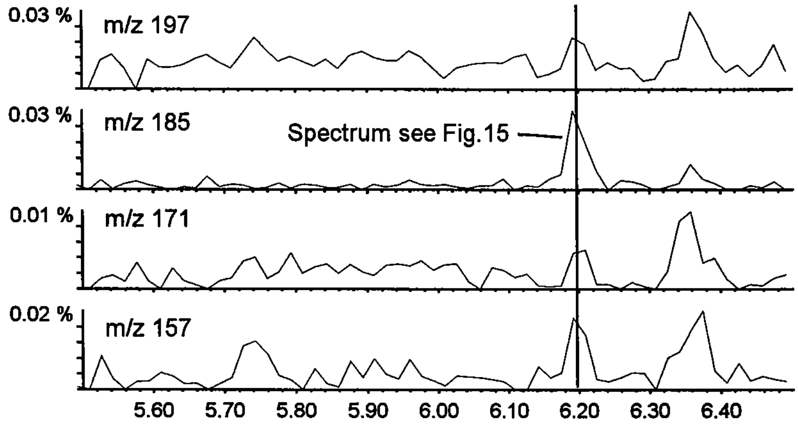

Fig. 14 MS/MS daughter ion chromatogram of the selected fragment ion 253 of a positive urine, without HPLC clean-up, retention time shift due to the high biological background.

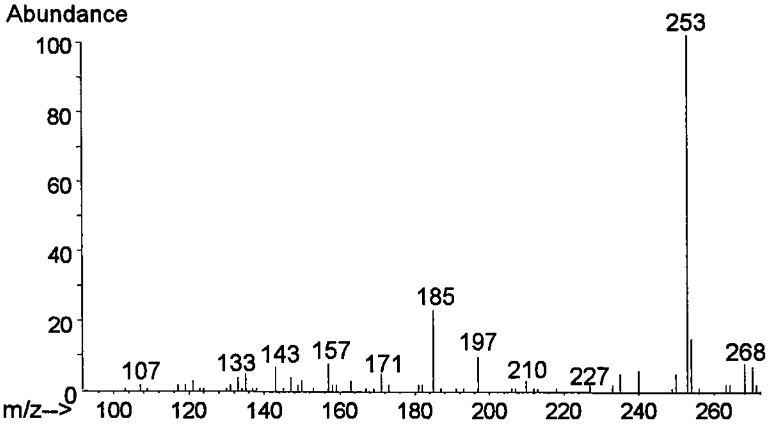

Fig. 15 Daughter ion MS/MS spectrum of the selected fragment ion 253 of a positive urine, without HPLC clean-up.

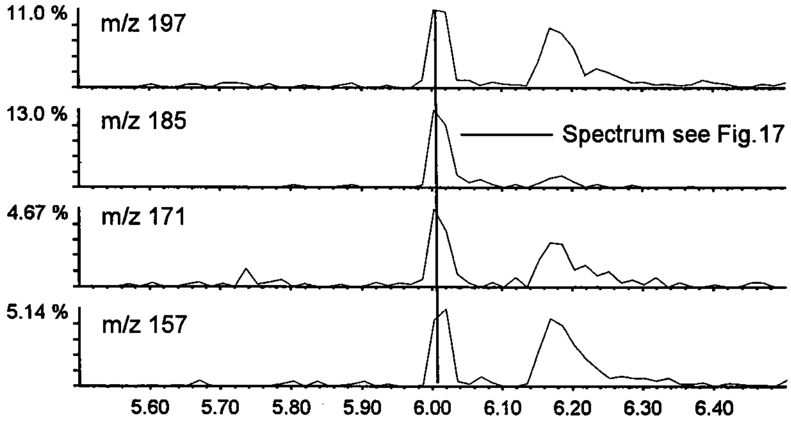

Fig. 16 MS/MS daughter ion chromatogram of the selected fragment ion 253 of a positive urine, with HPLC clean-up.

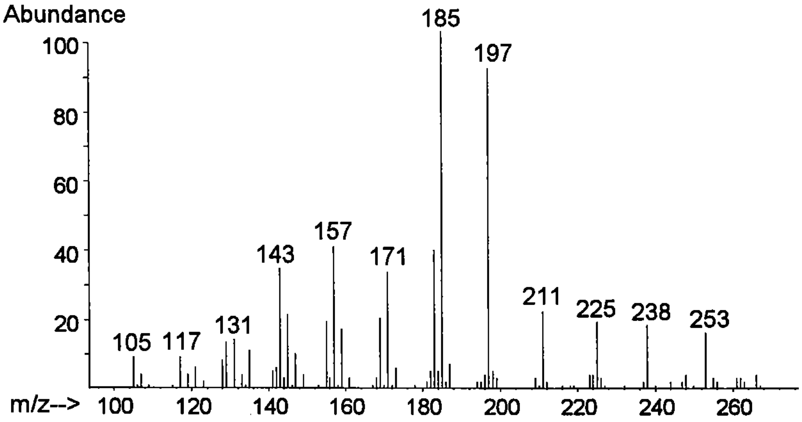

Fig.17 Daughter ion MS/MS spectrum of the fragment ion 253 of a positive urine, with HPLC clean-up.

Both urine samples, the 2 ng/ml control urine (Fig.12) and the positive urine (Fig.14) show suspicious signals in all four daughter ion traces 197, 185, 171 and 157 for 18-normetenol TMS. When ratios of intensities of the chromatographic signals and the full daughter ion spectra (Fig.13 and Fig.15) are compared to the reference standard (Fig.10 and Fig.11) MS criteria for identification are not fulfillef. This is mainly due to biological background which also yielded a shift in the retention time for the positve urine sample. Improved GC/MS chromatograms were obtained after HPLC clean-up was introduced. The result of the clean-up is presented in Fig.16 and Fig.17. A comparison of the daughter ion spectra of the positive sample (Fig.17) with the reference substance (Fig.11) shows high agreement for nearly all daughter ions with only slight variations in intensities.

Excretion studies

The unexpected finding that metabolites I and II are present with different amounts in positive urine samples was investigated. It was obvious that this difference was only observed in samples with low concentrations of metabolites I and II, which in fact is at the end of the elimination of metandienone metabolites.

After oral application of 5 mg of metandienone to a male volunteer metabolites I and II were estimated and the ratio of both metabolites was calculated (Fig18).

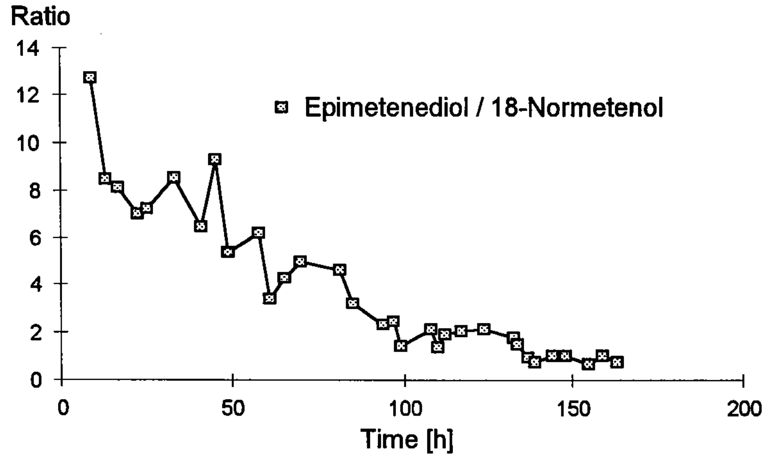

Fig.18 Ratio of urinary epimetenediol **II** to 18-normetenol **I** after a single oral application of 5 mg of metandienone.

The different rate of excretion of 18-normetenol and epimetenediol results in a high ratio in the beginning of the excretion curve caused by a higher excretion of epimetenediol. This ratio changed over time and at the end of elimination the 18-normetenol was the dominant metabolite.

This result cannot be explained by the proposed rearrangement of a sulphated precursor in urine. A possible explanation for this phenomenon is the assumption that the sulphated precursor partially decomposes in blood or in other intracellular compartments of the body. The rearrangment products 18-normetenol and epimetenediol are then excreted as conjugates with glucuronic acid into urine with different elimination half-times, yielding different ratios of both steroids in urine. To prove this assumption an excretion study was performed with oral administration of 0.1 mg of 18-normetenol and 0.1 mg of epimetenediol (solved in 10 ml of 40% ethanol) which were applied to a male volunteer at the same time. The result of the study shows a shift in the ratio of epimetenediol to 18-normetenol (Fig.19) which is dependent on time, comparable to the excretion results after application of 5 mg of metandienone (Fig.18). The recovery of epimetendiol was about 90% and approximately 30% for 18-normetenol.

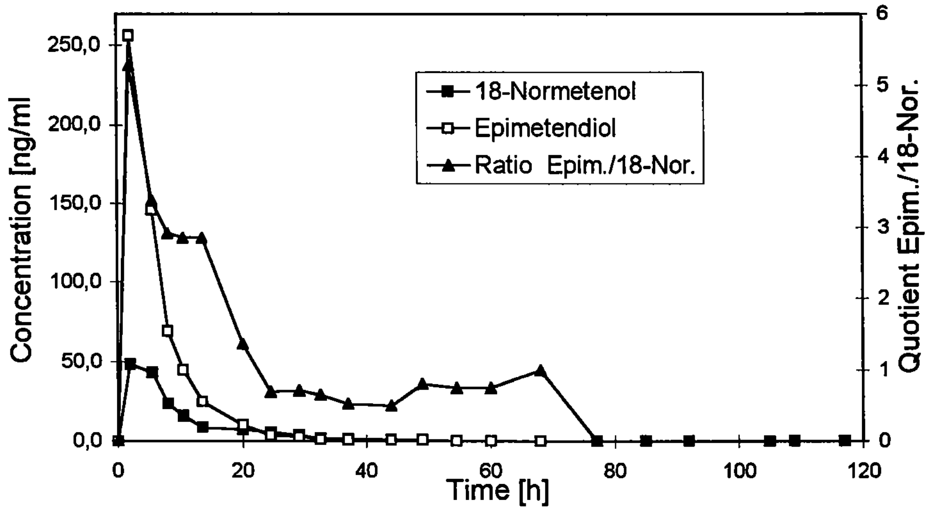

Fig.19 Excretion of 18-normetenol and epimetenediol after oral application of 0.1 mg each.

The difference can be caused by incomplete adsorption of 18-normetenol from the gastro-intestinal tract, enrichment in adipose cells, or other unknown metabolic influences. At the end of the excretion only 18-normetenol **I** was detected. This result confirmed that the difference in the intensities of metabolite **I** and **II** in positive urine samples depends on the extent of rearrangement of the sulphated precursor before both metabolites are excreted into urine. This ratio also depends on the time after the last adminstration, the amount of misused metandienone and the frequency of application. Individual variation in the enzymatically controlled metabolic pathways may also influence this pattern.

Conclusion

Both MS techniques, HRMS and ion trap MS/MS, can be used to confirm 18-normetenol at low concentrations (approximately 2 ng/ml urine) in positive metandienone urine samples. For both techniques it is mandatory to use an additional clean-up step, for example HPLC. Nevertheless, HRMS shows a better limit of detection, meaning that lower concentrations of 18-normetenol can be identified. For screening the HRMS showed improved results compared to the used ion trap instrument.

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