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Introduction

Anabolic androgenic steroids (AAS) are the most frequently abused doping substances. Following the IOC statistic of reported A-sample results more than 60% of detected doping agents are AAS. The misuse of AAS in sports is controlled since their first ban in 1974. The detection of AAS in urine of athletes was limited until 1990 only to competition testing. This was insufficient as AAS are misused in the time period "out of competition" to enhance performance. It was a common practice for athletes to stop AAS intake at a distinct period before competition to prevent detection in by the drug testing laboratory. Even a high number of positives were found at competition testing. To improve the testing of AAS misuse 1. the testing of athletes was extended to the time period "out of competition" and

2. the sensitivity and retrospectivity of the used analytical techniques was increased. The latter was achieved using high resolution mass spectrometry (HRMS) in routine doping control [1]. HRMS is not a brand new technique, even in the eighties HRMS instruments were applied in dope analysis, not on a routine basis for screening purposes but in confirmation analysis. A routine application of HRMS with a high number of sample analysis per day was not possible as instrumental operation and data evaluation was work extensive and not automated. This disadvantage changed when modern software for HRMS got available and enabled dope testing laboratories to measure a high number of samples per day.

Besides instrumental improvement research was focused on the metabolism of AAS with the aim to identify metabolites detectable for the longest time after administration. Here we present an overview of long-term excreted metabolites of metandienone and mestanolone.

![Chemical structures of metandienone and mestanolone](image)

Fig. 1 Structure formula of metandienone and mestanolone
Metandienone

Metandienone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-on) has been marketed since 1959. It is one of the most misused AAS. Metabolism studies of metandienone have been reported in 1963 by Segaloff and Rongone [2], who identified 6β-hydroxy-metandienone as the main excreted metabolite. They also detected an isomer of metandienone but could not confirm its chemical structure. Identification of this metabolite as 17-epimetandienone was achieved in 1971 by Macdonald et al. [3].

![Metandienone and its metabolites](image)

**Fig. 2** Metabolism of metandienone (reported in 1963 and 1971)

Further metabolism studies were published by Dürbeck et al. [4] in 1979 using GC/MS analysis of trimethylsilylated products for structure elucidation. They investigated only unconjugated excreted metabolites and confirmed three metabolites (17-epimetandienone, 6β-hydroxymetandienone, 6β-hydroxy-17-epimetandienone) and two 'artifacts' (17β-hydroxy-17α-methylandrosta-1,4,6-trien-3-one and 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one). Both 'artifacts' are nowadays discussed as metabolites. The formation process of the first 'artifact' is still unexplained whereas the generation of the second 'artifact' will be discussed as follows.

In 1991 Schänzer et al. [5] reported conjugated excreted metabolites of metandienone which were detectable for a longer period after application than the known unconjugated metabolites. Main excreted conjugated metabolites are 17α-methyl-5β-androstane-3α,17β-diol, 17α-methyl-5β-androst-1-ene-3α,17β-diol III, and 17β-methyl-5β-androst-1-ene-3α,17α-diol I. The latter is considered as a long-term excreted metabolite and is together with 17,17-dimethyl-18-nor-5β-androsta-1,13-dien-3-one II the metabolite of choice for the long-term detection of metandienone misuse [1].
The actual known and identified metabolites of metandienone in human are listed in Fig. 3.

The mechanism of 17-epimerisation of metandienone and other 17α-methyl-17β-hydroxy AAS has been discussed for several years. In 1989 Edlund et al. [6] showed that 17β-sulphated metandienone spontaneously hydrolyzed in water to several dehydration products and to 17-epimetandienone. The obtained reaction products of metandienone 17β-sulphate when solved in water are shown in Fig. 4. Edlund et al. proposed and proved that in metandienone metabolism of horses a 17β-sulphate is formed. After excretion of the 17β-sulphate into urine it decomposes to an intermediate carbenium ion which rearranges mainly to 17-epimetandienone and 17,17-dimethyl-18-norandrosta-1,4,13-trien-3-one and to several dehydration products.
with a 16-ene, 17-ene and 12-ene structure and finally, to a low extent, a rearrangement product with a 13-hydroxy-17,17-dimethyl structure is formed (Fig.5). The same metabolism and rearrangement process occurs in human metabolism of metandienone and other 17α-methyl-17β-hydroxy steroids [7, 8].

2 17,17-Dimethyl-18-norandrosta-1,4-dien-3-one
3 17-Methylandrosta-1,4,12-trien-3-one
4 17-Methylandrosta-1,4,16-trien-3-one
5 17-Methylengandrosta-1,4-dien-3-one
6 13-Hydroxy-17,17-dimethylandrosta-1,4-dien-3-one
7 17-Epimetandienone

Fig.4 GC/FID-chromatogram of 17-epimerization reaction products of metandienone (steroids are derivatized with MSTFA/lmi)

Fig.5 Assumed reaction scheme for 17-epimerization and dehydration and of 17α-methyl-17β-hydroxy 17β-sulphated steroids
Based on these data the metabolic pathway to obtain the long-term metabolites I and II can be described as a rearrangement of a 17β-sulphate of 17α-methyl-5β-androst-1-ene 3α,17β-diol III after excretion into urine (Fig.6). As the rearrangement products are not bonded to a sulphate group they should be extractable from urine prior to deconjugation, but both metabolites are not simply extractable with an organic non-water miscible solvent. They are still conjugated, not with sulphate but with glucuronic acid. This conjugate can be hydrolyzed by treatment with a specific enzyme preparation, β-glucuronidase from E.coli. The assumption of a β-glucuronic acid conjugate is allowed as β-glucuronidase is highly specific to steroidal β-glucuronic acid conjugates. Nevertheless more evidence for a β-glucuronide should be presented. Research for structure elucidation is under investigation.

![Chemical Structures](image)

**Fig.6** Proposed rearrangement process for the origin of metabolite I and II

An interesting question in the metabolism of metandienone is at what stage of the metabolism sulphatation and glucuronidation occurs and which metabolite is the target compound for conjugation to generate 17α-methyl-5β-androst-1-ene-3α,17β-diol III 17β-sulphate 3α-(β)-glucuronide. A possible metabolic pathway is proposed in Fig.7.
17β-Sulphation is assumed to react directly with metandienone or the 5β-reduced metandienone. This assumption is based on the results of an excretion study with 17α-methyl-5β-androst-1-ene-3α,17β-diol III [5] confirming the excretion of III itself and the reduced metabolite 17α-methyl-5β-androstan-3α,17β-diol. Thus no 17-epimer of III was detected, 17β-sulphation of III can be excluded and the sulphation as a prerequisite for the urinary formation of I and II results from a precursor of III (Fig. 7). Glucuronidation is only possible after reduction of the 3-keto group of metandienone to a 3-hydroxy function.

Fig. 7 Proposed metabolic pathway for formation of 17α-methyl-5β-androst-1-ene-3α,17β-diol 17β-sulphate 3α-(β)-glucuronide

The improvement in sensitivity and selectivity by HRMS yielded a prolonged detection time of metandienone after its last ingestion. This is demonstrated in Fig. 8, showing the excretion curve of 17β-methyl-5β-androst-1-ene-3α,17α-diol I after oral administration of a single dose of 5 mg of metandienone to a male volunteer. The diagram shows that HRMS can double the detection time and the additional
improvement in sample preparation by HPLC results in a distinct prolonged detection time. Certainly the HPLC clean-up step is only applied in the confirmation of positive samples. The data points from 200 - 400h after application represent urinary concentrations of I much lower than 0,1 ng/ml. In the common practice of dope analysis these values are too low to fulfill and to guarantee all criteria for a positive identification of metabolite I.

![Graph showing Epimetenediol concentrations over time](image-url)

**Fig. 8** The detection of 17β-methyl-5β-androst-1-ene-3α,17α-diol I with different MS-techniques and improved sample clean-up after oral administration of 5 mg of metandienone

The detection of metandienone metabolite I and II by GC/MS and GC/HRMS is performed after derivatization of both compounds yielding trimethylsilyl (TMS) ethers [1]. This can be obtained via the enolization reaction mixture MSTFA / ammonium iodide / ethanethiol S-TMS (1000/2/6, v/w/v) or the reagent MSTFA/imidazol (100/2, v/w). The obtained EI spectra are presented in Fig. 9 and 10 and show the characteristic fragmentation pattern of trimethylsilylated 17-methyl-17-hydroxy metabolites with a high abundant and characteristic D-ring fragment of m/z 143. It should also be mentioned that the EI-spectra of both 17-epimeric bis-TMS ethers I and III are nearly identical and differ only slightly in the intensity of a few ion fragments.
The identification of long-term excreted metabolites and the improvement in mass spectrometric detection [1], as the introduction of HRMS in routine screening analysis, have increased the number of positive samples in the Cologne laboratory in 1995 and 1996. Table 1 summarises the results of reported positive A-samples based on low resolution mass spectrometry (LRMS) in comparison to the HRMS. The results of metandienone positive samples, especially 63 findings in 1995 only with HRMS, show the effectiveness of this analytical technique. Up today, based on a limited number of positive findings, the advantage of HRMS screening for AAS and
β2-agonists could only be proved for metandienone, stanozolol, clenbuterol and 4-chlorodehydromethyltestosterone in the Cologne laboratory.

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

<table>
<thead>
<tr>
<th>Positive A-samples</th>
<th>1995</th>
<th>1996</th>
</tr>
</thead>
<tbody>
<tr>
<td>All banned substances</td>
<td>155</td>
<td>100</td>
</tr>
<tr>
<td>Anabolic androgenic steroids</td>
<td>117</td>
<td>69</td>
</tr>
<tr>
<td>Metandienone total</td>
<td>78</td>
<td>28</td>
</tr>
<tr>
<td>LRMS</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>HRMS*</td>
<td>63</td>
<td>21</td>
</tr>
<tr>
<td>Stanozolol total</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>LRMS</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>HRMS*</td>
<td>9</td>
<td>2</td>
</tr>
</tbody>
</table>

* Metabolites were only detected in screening by HRMS

Mestanolone

Mestanolone (17β-hydroxy-17α-methyl-5α-androstan-3-one) is the corresponding 17α-methyl steroid of dihydrotestosterone (DHT). It was first synthesized in 1935 by Rudzicka et al. [9]. The bioavailability of mestanolone for therapeutic use was not very sufficient because the fast metabolism of the 3-keto group yielding a 3α-hydroxy metabolite. This metabolite has less pharmacological effects and is rapidly conjugated and excreted. Our attention was attracted to mestanolone when information about AAS misuse in the former german democratic republic (GDR) became public in the beginning of the nineties. The main misused AAS in the GDR was 4-chlorodehydro-methyltestosterone. But in the last decade mestanolone became more attractive as investigations with this classic androgen showed positive results and athletic performance seemed to be improved, especially in women.

Our strategy in investigation of the mestanolone metabolism was to identify long-term excreted metabolites to follow the misuse of this drug for a longer period after the last application as in the past. The main excreted metabolite of mestanolone is 17α-methyl-5α-androstan-3α,17β-diol 3 which is excreted into urine after conjugation (Fig.11). This conjugate can be specifically hydrolyzed with β-glucuronidase from E.coli and therefore a conjugation with glucuronic acid is
indicated. Investigations to characterize more precisely the conjugate structure of this metabolite are in preparation.

Fig. 11  Metabolism of mestanolone, R = proposed conjugate: β-glucuronide, 17β-methyl-5α-androstan-3α, 17α-diol 1, 17, 17-dimethyl-18-nor-5α-androst-13-en-3α-ol 2 and 17α-methyl-5α-androstan-3α, 17β-diol 3

Excretion studies with 20 mg of mestanolone orally applied showed that in the beginning metabolite 3 is the most abundant metabolite [7]. As proved in the metabolism of metandienone (see above) the possibility of a sulphated metabolite, which should be converted to a 17-epimeric product and to a corresponding rearrangement product with a 17, 17-dimethyl-18-nor-13-ene structure after excretion into urine was investigated (Fig. 12). Therefore the possible 17-epimers of mestanolone itself and the main metabolite 3 were synthesised [7] and the excreted metabolites of mestanolone after administration of 20 mg of mestanolone were quantified. The 17-epimer of mestanolone itself was not detected. This finding is in agreement with the result that also mestanolone itself is not excreted neither unconjugated nor conjugated. But the result is different to the metabolism of dihydrotestosterone (the mestanolone homologous steroid without a 17α-methyl group), which is excreted after oral application to a high extent conjugated with glucuronic acid at the 17β-hydroxy group. This conjugation is sterically hindered in mestanolone by the 17α-methyl group. The 3-keto function of mestanolone is rapidly reduced to a 3α-hydroxy structure and the metabolite is
excreted after conjugation. The corresponding 17-epimer (17β-methyl-5α-androstane-3α,17α-diol 1) of the reduced mestanolone 3 was identified [7]. This metabolite was produced in comparison to the main metabolite 3 with approximately 1% in the first 72 hours after application. Additionally it was possible to identify in low amount 17,17-dimethyl-18-nor-5α-androst-13-en-3α-ol 2, the proposed rearrangement product of 17β-sulphated 3. As both metabolites 1 and 2 were obtained only after hydrolysis with β-glucuronidase from E.coli the steroid conjugation with glucuronic acid and the proposed rearrangement process in Fig.12 is most probably.

![Chemical Structure](image)

Fig.12 Proposed urinary rearrangement process of 17α-methyl-5α-androstane-3α,17β-diol 3 17β-sulphate 3α-glucuronide.

The isolated metabolites 1 and 2 were confirmed as long-term excreted metabolites. Whereas the concentration of the main metabolite 3 in urine decreased rapidly in the course of time both rearrangement products 1 and 2 are excreted more slowly (Fig.13). This different excretion profile caused a change in the ratio of substances 1 and 2 to compound 3.
Fig. 13  Excretion of mestanolone metabolites after oral administration of 20 mg of mestanolone (semilogarithmic scaling).

Fig. 14 presents the GC/HRMS-chromatograms for the three metabolites 1, 2 and 3 11 hours and 10 days after administration of 20 mg of mestanolone. After 10 days only the long-term detected metabolites 1 and 2 could be identified and metabolite 3, which was a hundredfold more abundant in the first day, was no longer detectable. Therefore it is clearly evident that only the long-term metabolites 1 and 2 are suitable for the long-term determination of mestanolone misuse.

Conclusion

The use of HRMS in screening of synthetic anabolic androgenic steroids has increased the retrospecticity in detection of steroid misuse. Compared to the high number of metandienone positives in Cologne by HRMS there was no positive finding for mestanolone in the same time period, even long-term excreted metabolites were controlled.
Fig. 14  GC/HRMS analysis of mestanolone metabolites 1, 2 and 3 and the internal standard 17α-methyl-5α-androstan-3β,17β-diol (ISTD) A) 11 hours and B) 10 days after administration of 20 mg of mestanolone. Sample preparation: To 2 ml of urine are added 0.5 ml 1M phosphate buffer pH 7.0 and 50 μl of β-glucuronidase from E. Coli (Boeringer Mannheim). The urine sample is incubated for 1 h at 50°C and after addition of sodium carbonate to adjust the pH 11-12 extracted with 5 mL of n-pentane. The organic layer is concentrated to dryness and derivatized with 100 μl MSTFA/ammonium iodide/ethanethiol TMS (1000/2/6, v/w/v) and heated for 15 min at 60°C.
References


