Veterinary Research Communications, 31 (2007) 259–272
DOI: 10.1007/s11259-006-3432-z

Metabolism of Methenolone Acetate in a Veal Calf

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ABSTRACT

The use of anabolic steroids has been banned in the European Union since 1981. In this study, the metabolism of the anabolic steroid methenolone acetate, was investigated in a male veal calf. After daily oral administration of methenolone acetate, three main metabolites were detected in both urine and faeces samples. Among these metabolites, α-methenolone was apparently the main one, but 1-methyl-5α-androstan-3,17-diol and 3α-hydroxy-1-methyl-5α-androstan-17-one were also observed. The parent compound was still detectable in faeces. As a consequence, abuse of methenolone acetate as growth promoter can be monitored by analysing urine and faeces samples. A few days after the last treatment, however, no metabolites were observed. α-Methenolone was detectable in urine until 5 days after the last treatment, but in faeces no metabolites were detectable after 3 days.

Keywords: GC-MS, metabolism, methenolone acetate

Abbreviations: GC-MS, gas chromatography–mass spectrometry, MSTFA, *N*-methyl-N-(trimethylsilyl)-trifluoracetamide, HPLC, high-pressure liquid chromatography, TMS, trimethylsilyl

INTRODUCTION

The metabolism of methenolone acetate (Primobolan) has been thoroughly investigated in humans owing to its use as a doping agent among athletes. Like many other androgenic-anabolic steroids, it has been used in sports in the last two decades with the intention of increasing strength and improving performance. The use of methenolone acetate is prohibited in sports by the International Olympic Committee because of concerns over the potential incidence of adverse health effects and for ethical reasons (Goudreault and Massé, 1990). In residue analysis of veterinary medicinal products the use of anabolic steroids has been prohibited in the European Union since 1981 (EEC Directive 81/603, 1981, L222/32). Primobolan is used in the treatment of aplastic anaemia (Palva and Wasastjerna, 1972; Lockner, 1979) because of its therapeutic efficiency and lower hepatic toxicity compared to its 17α-alkylated analogues.

Owing to the substitution of the C-17 atom, methenolone acetate is protected from rapid hepatic metabolism and is therefore orally active. When administered orally, methenolone acetate is six times less androgenic than testosterone propionate and three times more

anabolic than orally active 17α-methyltestosterone (Goudreault and Massé, 1990). In early studies by Langecker (1962) and by Gerhards and colleagues (1965), it was shown that the major urinary metabolite of methenolone acetate in humans is the glucuronide of 3α-hydroxy-1-methylen-5α-androstan-17-one. More recently, Björkhem and Ek (1983) reported a method for the detection and quantification of 3α-hydroxy-1-methylen-5α-androstan-17-one and Goudreault and Massé (1990) and Massé and Goudreault (1992) investigated the urinary metabolites of methenolone acetate in humans.

Ho and colleagues (2005) studied the metabolism of methenolone acetate in horses. In urine, seven metabolites were observed, of which methenolone (17β-hydroxy-1-methyl-5α-androst-1-ene-3-one) was the main one. Among these metabolites, the major urinary metabolite in man, 3α-hydroxy-1-methylen-5α-androstan-17-one, was not detected.

The main objective of the present study was to look for target metabolites of methenolone acetate in urine and faeces after daily oral administration of methenolone acetate to a male veal calf.

MATERIALS AND METHODS

Reagents and chemicals

Methenolone was obtained from Steraloids (Wilton, NY, USA) and methenolone acetate from the patent holder. All other chemicals used were of analytical grade from Merck (Darmstadt, Germany).

Stock standard solutions of 1000 ng/µl were prepared in ethanol. Methanol was used for the preparation of working solutions. All standard and working solutions were stored at 4°C. The derivatization reagent $MSTFA^{2+}$ was prepared by dissolving 100 mg ammonium iodide (Sigma, St Louis, MO, USA) and 0.2 ml ethanethiol (Acros, Geel, Belgium) in 5 ml *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (Macherey-Nagel, Düren, Germany), followed by dilution of 1.5 ml of this solution with 10 ml MSTFA.

The derivatization reagent 2% methoxyamine hydrochloride in pyridine was obtained from Pierce (Rockford, IL, USA).

Instrumentation

Apparatus for extraction and clean-up. A series of devices was used for extraction and clean-up. The most important are the solid-phase extraction columns, Isolute Si-columns (3 cm^3/500 mg) and Isolute NH_2-colums (1 cm^3/100 mg) (IST International, Mid Glamorgan, UK) and the preparative HPLC apparatus (HPLC Intelligent pump, Merck, Darmstadt, Germany).

GC-MS apparatus. Chromatographic separation was achieved using a ThermoQuest CE Trace GC gas chromatograph (Thermo Finnigan, Austin, TX, USA) with split/splitless injector (250°C). Analysis of trimethylsilyl enol trimethylsilyl ether derivatives was performed using a non-polar 5% phenyl-polysilphenylene-siloxane SGE BPX-5 GC-column (25 m × 0.22 mm i.d., 0.25 µm); *O*-methyloxime trimethylsilyl ether derivatives were analysed using

TABLE I
Sampling plan

Day	Administration (time)	Urine sample (time)	Faeces sample (time)
0	08:00	21:00	21:00
1	08:00	09:20	09:10
2	09:00	08:45	08:30
3	08:00	09:00	–
4	08:00	17:30	16:30
5	08:00	10:55	–
6	08:00	10:00	09:30
7	08:00	11:00	10:45
8	08:00	17:30	16:45
9	08:00	09:00	08:45
10	08:00	11:00	10:30
11	08:00	08:30	08:15
12	08:00	08:15	08:00
13	08:00	08:15	08:00
14	–	09:00	08:45
15	–	08:45	08:30
16	–	10:45	10:30
17	–	08:45	08:30
18	–	16:00	15:45
21	–	15:15	15:15
23	–	14:30	14:15

a 35% phenyl-polysilphenylene-siloxane SGE BPX-35 GC-column (25 m × 0.22 mm i.d., 0.25 μm) (SGE Inc., Austin, TX, USA). Injection was performed at an oven temperature of 100°C, increased at a programmed rate of 17°C min up to until 250°C, then increased at 2°C/min up to 300°C, and finally maintained for 1.30 min. Helium gas was used as GC carrier gas at a flow rate of 1 ml/min. A Carlo Erba autosampler AS2000 (Thermo Finnigan) was used to inject the trimethylsilyl enol trimethylsilyl ether derivatives in splitless mode. Detection was done using a Polaris ion trap mass spectrometer (Thermo Finnigan) with electron impact ionization. Injection of the *O*-methyloxime trimethylsilyl ether derivatives was performed with an A200S autosampler (Thermo Finnigan) in splitless mode and detection was done using a Polaris Q ion trap mass spectrometer (Thermo Finnigan) with electron impact ionization. In both cases the ion source temperature was 200°C and the transfer line temperature was 275°C.

Animal experiment

For the animal experiment, a male veal calf (170 kg, piebald) was treated orally with daily doses of methenolone acetate for 2 weeks. The calf was fed with straw and hay *ad libitum* and 300 g concentrates each day. The dose given was 3 mg/kg body weight per day, administered

as tablets. During this period and during the week after treatment, urine and faeces samples were taken (Table I).

Extraction and clean-up

Extraction and clean-up of urine. Urine samples (25 ml) were first diluted with 0.2 mol/L acetate buffer (pH 5.2) and 100 µl glucuronidase was added to the samples. Hydrolysis was performed during 2 h at 62°C. Urine clean-up was performed by liquid–liquid extraction with diethyl ether. The ether phase was washed with sodium carbonate and ultrapure water and evaporated to dryness. To further reduce interfering matrix compounds, urine extracts were reconstituted in ethanol and fractionated into four fractions by preparative HPLC on a C_{18} reversed-phase column (Ultrasphere, 10 mm × 25 cm, 5 µm, Beckman Coulter, Fullerton, CA, USA). The mobile phase consisted of a mixture of methanol and water. A linear gradient was run (starting at 25% water and increasing to 100% methanol) at a flow rate of 3 ml/min (Smets and Vandewalle, 1984). Four fractions were collected, the first three of volume approximately 8.5 ml and the fourth fraction of approximately 27.5 ml, and each fraction was evaporated to dryness.

Extraction and clean-up of faeces. Faecal samples (10 g) were diluted with water and anabolic steroids were extracted using diethyl ether. The ether phase was evaporated to dryness and the samples were reconstituted in methanol and water was added. Defatting was performed with petroleum-benzine. The samples were evaporated to dryness and reconstituted in chloroform and hexane. Clean-up was carried out using solid-phase extraction with a silica column coupled to an NH_2-column. Elution was carried out with chloroform–acetone (40:10v/v) and the eluate was evaporated to dryness. To further reduce interfering matrix compounds, faecal extracts were reconstituted in ethanol and fractionated into four fractions by preparative HPLC on a C_{18} reversed-phase column (Hamoir *et al.*, 1998). Four fractions were collected and evaporated to dryness.

Derivatization. Trimethylsilyl enol trimethylsilyl ether derivatives (TMS): The final extracts were converted into trimethylsilyl enol trimethylsilyl ether derivatives with $MSTFA^{2+}$. A 25 µl aliquot of $MSTFA^{2+}$ was added to each vial and incubated for 60 min at 60 ± 2°C. One µl was injected into the gas chromatograph.

O-*methyloxime trimethylsilyl ether derivatives (MO-TMS):* The final extracts were dissolved in 50 µl of a 2% solution of methoxyamine hydrochloride in pyridine and heated at 70 ± 2°C for 30 min to produce *O*-methyloxime derivatives. Trimethylsilyl ether derivatives were then prepared by adding 25 µl MSTFA and heating the resulting mixture at 70 ± 2°C for 30 min. After evaporation, the derivatives were reconstituted in 25 µl hexane and 1 µl was injected into the gas chromatograph.

Data processing

First, possible metabolites were recovered by comparing the fractions of the urine/faecal samples before and after treatment by means of their GC-MS chromatograms. The metabolites were subsequently identified.

6-Dehydroprogesterone was added to both urine and faecal samples as internal standard before extraction and clean-up, and subsequently the ratio of the peak areas of the metabolite and the internal standard was calculated. The quantification of the metabolites was based on a comparison with the standard β-methenolone. β-Methenolone was added to blank urine and blank faecal samples together with the internal standard 6-dehydroprogesterone (spiked samples) to enable calculation of the ratio of the peak areas of β-methenolone and the internal standard. Thus, the metabolite/6-dehydroprogesterone area ratio was compared to the standard β-methenolone/6-dehydroprogesterone area ratio to estimate the concentration of the metabolite present in the urine/faecal sample.

RESULTS AND DISCUSSION

Phase I metabolites in urine

In order to identify possible metabolites, the four fractions of a urine sample before treatment were compared with those after treatment by means of their full-scan GC-MS chromatograms. Analysis of the ion chromatograms revealed three main metabolites (M1–M3) in fraction 4. Each metabolite had lost the acetate group present in the parent compound and two metabolites were further reduced (Figure 1).

Metabolite 1 (M1) had a TMS molecular ion of m/z 446. The fragment ions present in MS full scan (m/z 179, 195, 208, 341, 356 and 431; Figure 2), indicated the compound methenolone. However, the standard methenolone eluted at a later retention time than M1. The standard used was β-methenolone (17β-hydroxy-1-methyl-5α-androst-1-ene-3-one), since no standard of α-methenolone (17α-hydroxy-1-methyl-5α-androst-1-ene-3-one) was available. In urine samples the α-form can be expected (Leyssens *et al.*, 1994). Under the applied chromatographic conditions, α-forms will elute before their β-forms, which implies that M1 most probably is α-methenolone. Figure 3 shows the fragmentation of methenolone. Cleavage of the B-ring gives the fragment ions m/z179, 195 and 205. The main metabolite of methenolone acetate in humans, 3α-hydroxy-1-methylen-5α-androstan-17-one, also has a TMS molecular ion of m/z 446, but different fragment ions (Goudreault and Massé, 1990). To examine or exclude this possibility, another derivatization reaction was performed, using methoxyamine. Derivatization of the standard β-methenolone revealed two chromatographic peaks with identical mass spectra. These two chromatographic peaks were two possible isomers of the methoxyamine-derivatized methenolone. The MO-TMS molecular ion was m/z 403 and the fragment ions present in MS full scan were m/z 153, 166 (both by cleavage of the C-ring), 282 $(M-31-TMSOH)^+$ and 372 $(M-31)^+$. Goudreault and Massé (1990) described the MO-TMS derivatization of 3α-hydroxy-1-methylen-5α-androstan-17-one: a molecular ion of m/z 403 and fragment ions of m/z 142, 155 (both by cleavage of the A-ring) and 282. Figure 2 shows the MO-TMS mass spectrum of M1. This resembled the mass spectrum of the standard β-methenolone, but again the retention time of M1 is different from that of β-methenolone. These differences suggest that M1 is α-methenolone.

Metabolite 2 (M2) had a TMS molecular ion of m/z 450 (M). The fragment ions present in MS full scan were 435 $(M-Me)^+$, 360 $(M-TMSOH)^+$, 345 $(M-TMSOH-Me)^+$, 270 $(M-2TMSOH)^+$ and 255 $(M-2TMSOH-Me)^+$ which indicated an androstanediol

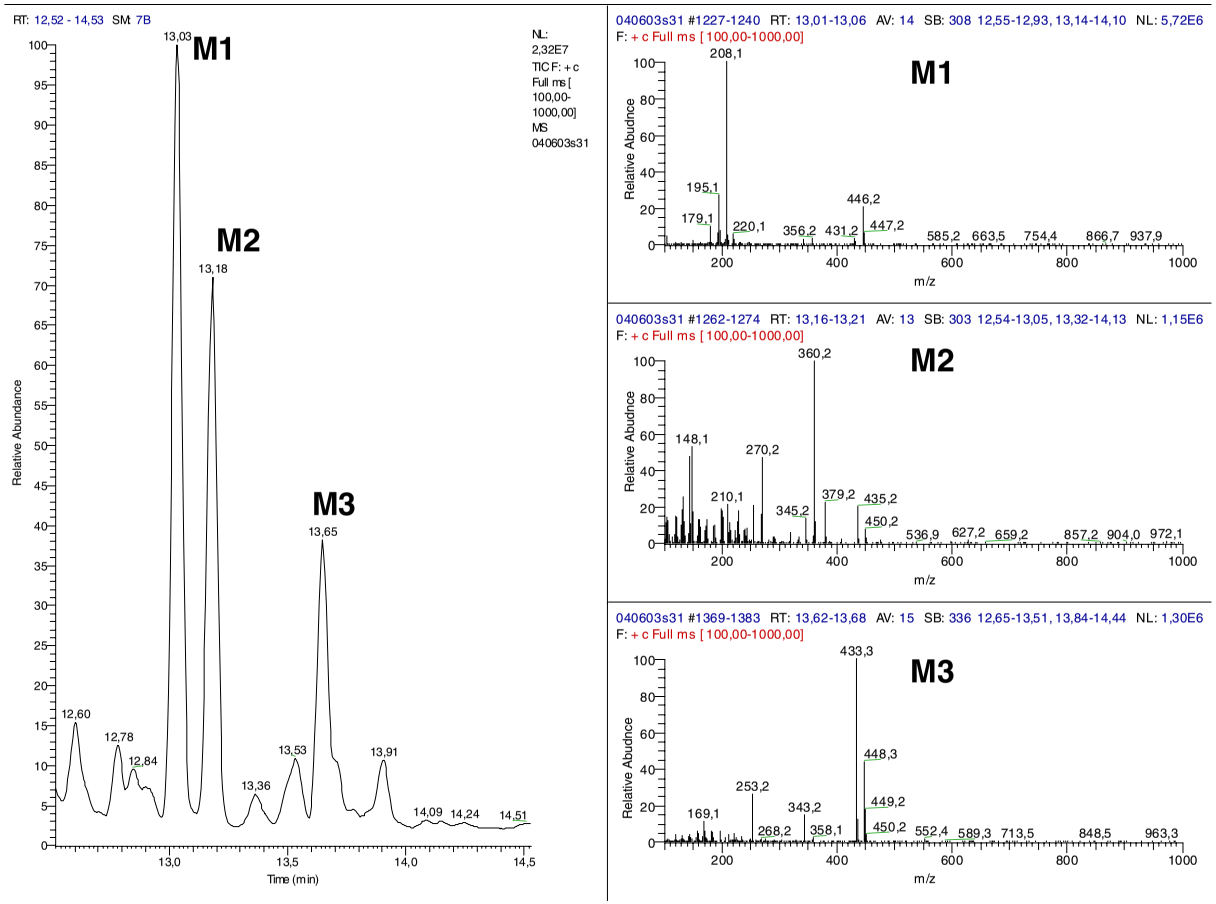


Figure 1. Chromatograms and mass spectra of the three main metabolites (M1, M2 and M3) of methenolone acetate as TMS derivatives in urine

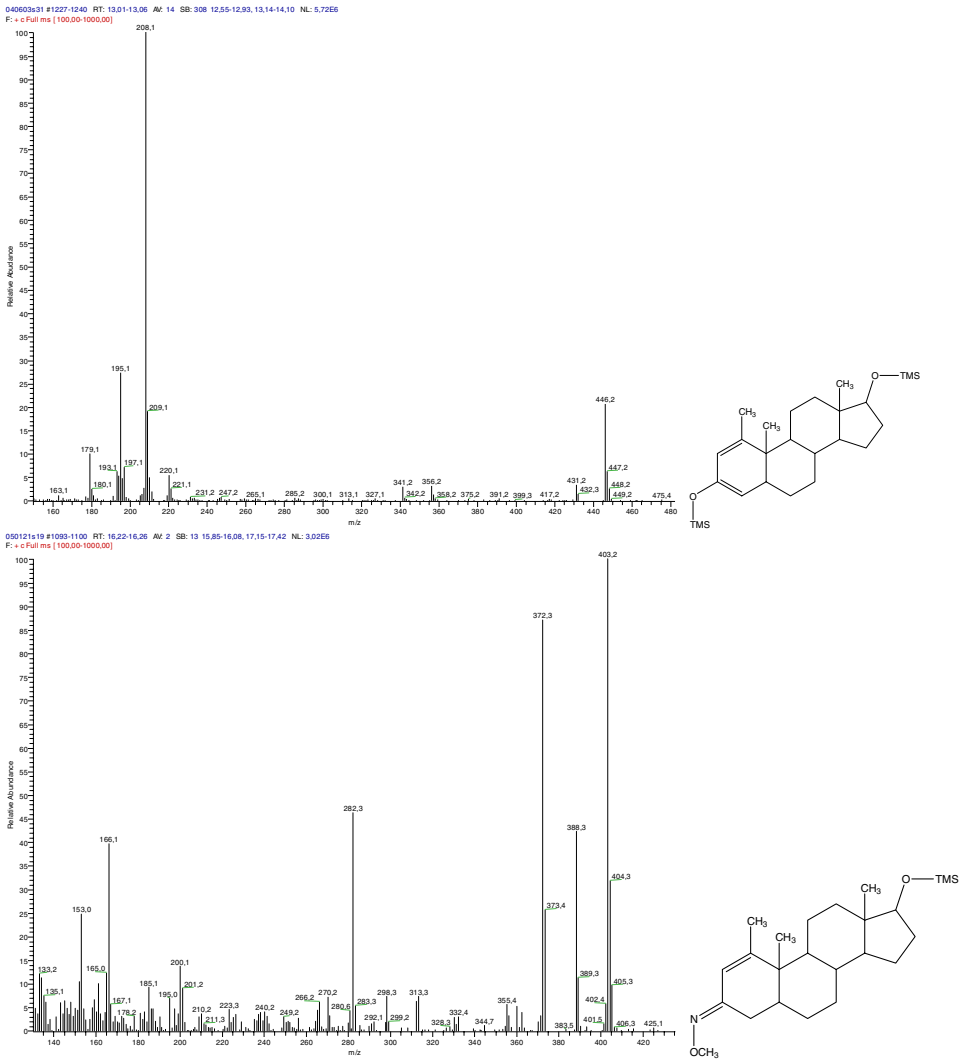


Figure 2. MS full scan of α-methenolone (M1) as TMS derivative (upper) and MO-TMS derivative (lower)

(Figure 4). As a consequence, to produce a molecular ion of m/z 450, the C1–C2 double bond is probably reduced. Therefore, M2 is 1-methyl-5α-androstan-3,17-diol. Since no keto groups are present in this metabolite, derivatization with methoxyamine gave the same mass spectrum as the TMS mass spectrum.

Metabolite 3 (M3) had a TMS molecular ion of m/z 448. In MS full scan the ion fragment at m/z 169 indicated the presence of a keto-group at position 17 ($C_9H_{17}OSi$) (Figure 5). As a consequence, the 3-keto and 1-ene group had to be reduced. Ungar and Dorfman (1952)

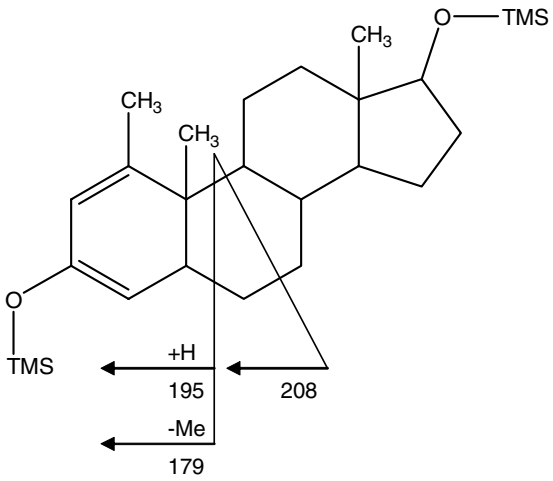


Figure 3. Fragmentation of the TMS derivative of methenolone

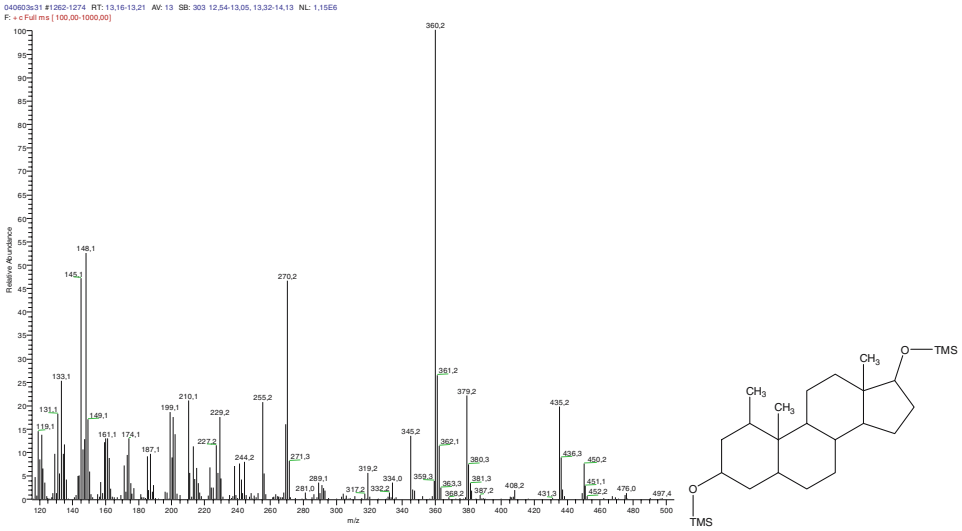


Figure 4. MS full scan of 1-methyl-5α-androstan-3,17-diol (M2) as TMS derivative

reported that 1-androstene-3,17-dione, a model compound of methenolone, was transformed into androsterone and isoandrosterone by reduction of the 1-ene and 3-keto groups. Therefore, M3 is probably 3α-hydroxy-1-methyl-5α-androstan-17-one. Another derivatization, using methoxyamine, was performed to examine this metabolite in more detail. Assuming that M3 is 3α-hydroxy-1-methyl-5α-androstan-17-one, the MO-TMS molecular ion should be m/z 405. In MS full scan the ions at m/z 284 $(M-31-TMSOH)^+$, 374 $(M-31)^+$ and 405 (M^+) confirmed the presence of one keto- and one hydroxyl-group and the reduction of

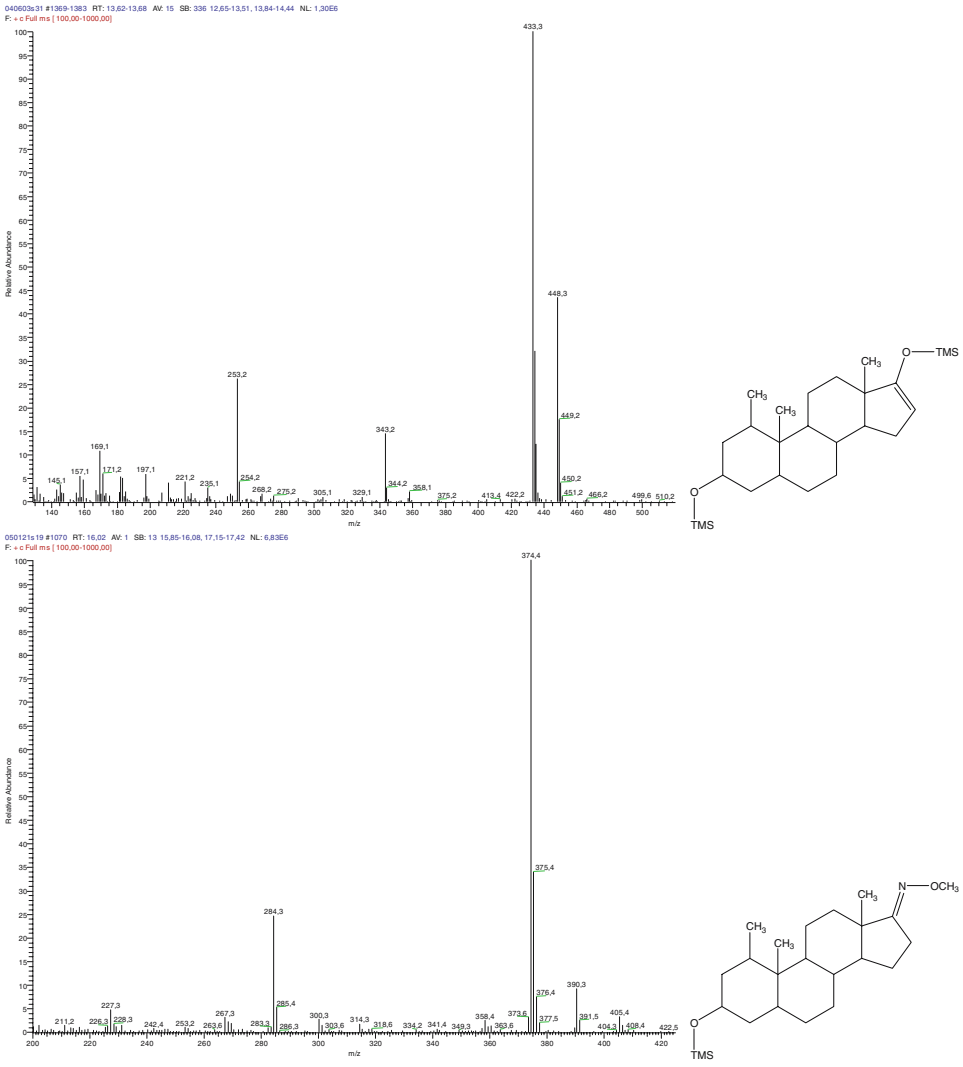


Figure 5. MS full scan of 3α-hydroxy-1-methyl-5α-androstan-17-one (M3) as TMS derivative (upper) and MO-TMS derivative (lower)

the C1–C2 double bond (Figure 5). These data suggest that M3 is 3α-hydroxy-1-methyl-5α-androstan-17-one, a metabolite that was also reported as a urinary metabolite of methenolone acetate in humans by Goudreault and Massé (1990).

Excretion profile in urine

A male veal calf (170 kg) was orally treated for 2 weeks with 3 mg/kg body weight per day. During this period and the week after treatment, urine and faeces samples were taken.

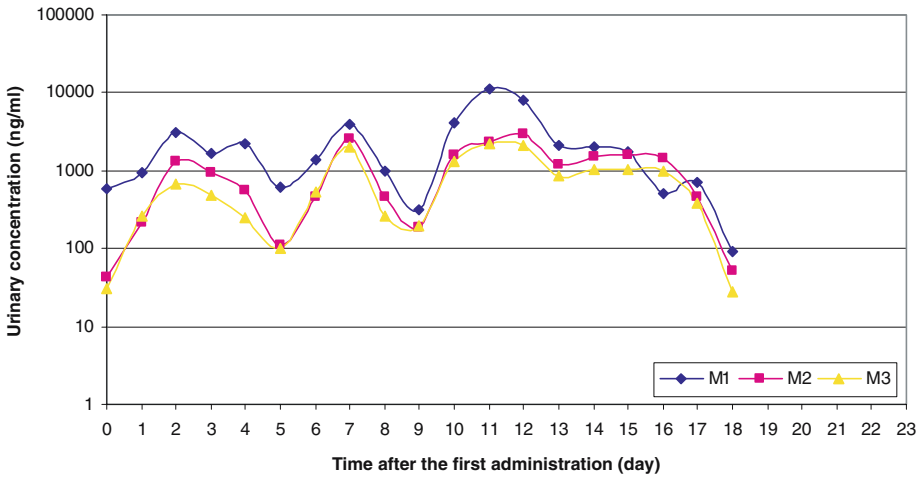


Figure 6. Excretion and elimination profile of metabolites M1, M2 and M3 in urine (last treatment during day 14)

Figure 6 shows the excretion profile of M1, M2 and M3 in urine. There is no correlation between the concentration of the metabolites and the time difference between the administration of methenolone acetate and the sampling of urine; the fluctuations in the excretion profile cannot be explained. M1 was apparently the main metabolite of methenolone acetate after oral administration. However, 5 days after the last treatment (day 14), no metabolites were detected in urine.

Phase I metabolites in faeces

In order to identify possible metabolites, the four fractions of a faeces sample before treatment were compared with those after treatment by means of their full-scan GC-MS chromatograms. Analysis of the ion chromatograms revealed the same metabolites recovered from urine (M1, M2, M3) and methenolone acetate itself in fraction 4.

Excretion profile in faeces

Figure 7 shows the excretion profile of M1, M2 and M3 in faeces. There is no correlation between the concentration of the metabolites and the time difference between the administration of methenolone acetate and the sampling of faeces; the fluctuations in the excretion profile cannot be explained. Again M1 was apparently the main metabolite of methenolone acetate after oral administration. In contrast to urine, already 3 days after the last treatment (day 14) metabolites were no longer detected in faeces. On the other hand, M1 was more dominant in faeces than in urine.

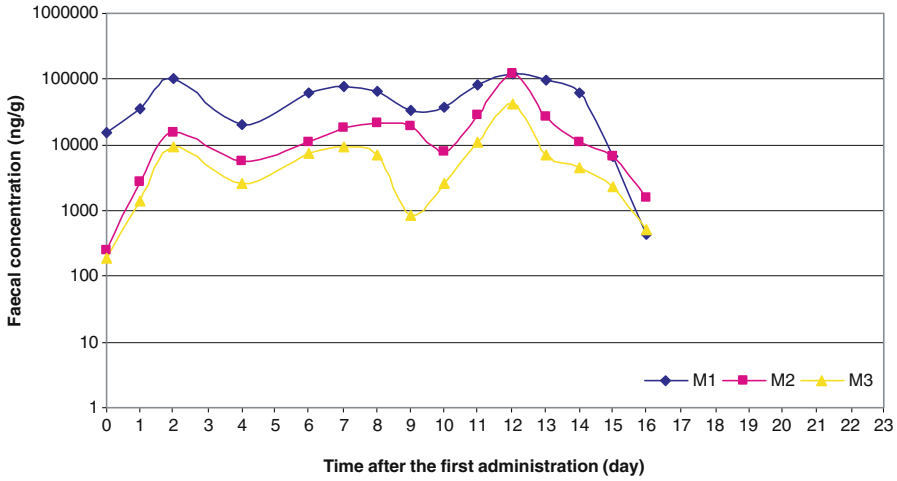


Figure 7. Excretion and elimination profile of metabolites M1, M2 and M3 in faeces (last treatment during day 14)

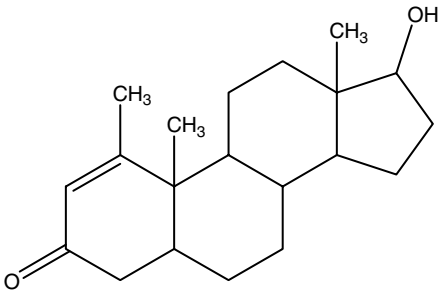
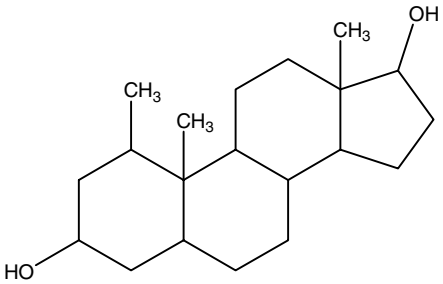
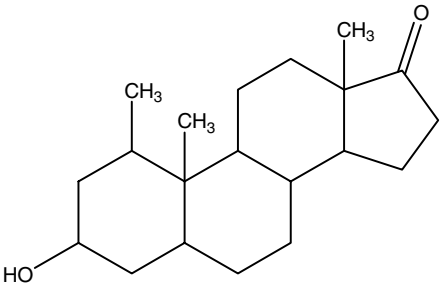
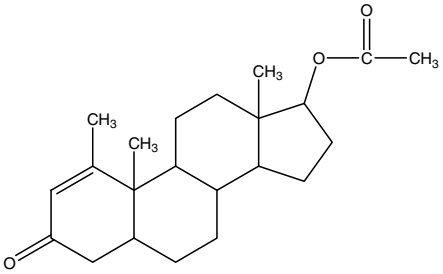
CONLUSIONS

Owing to the 1-position of the double bond of methenolone, the compound has some specific characteristics different from testosterone, which has a double bond in the 4-position. Methenolone is not able to aromatize to estrogens, which leads to a considerably higher anabolic activity than testosterone homologues. Therefore, methenolone has a possible use in breeding animals.

Abuse of methenolone acetate as growth promoter can be monitored by analysing urine or faeces. This study gives only an indication of the metabolites of methenolone acetate in urine and faeces since the metabolization study was performed using only one male veal calf. The differences in metabolites and the quantity of the metabolites between different sexes was not examined because it would be too expensive and too time-consuming to include many animals in the study. In both urine and faeces the recovered metabolites were α-methenolone (17α-hydroxy-1-methyl-5α-androst-1-ene-3-one) (M1), 1-methyl-5α-androstan-3,17-diol (M2) and 3α-hydroxy-1-methyl-5α-androstan-17-one (M3) (Table II). In faeces, even methenolone acetate itself was seen. α-Methenolone (M1) was apparently the main metabolite in both urine and faeces after oral administration of methenolone acetate. M1 was detectable in urine only until 5 days after the last treatment; in faeces these metabolites were no longer detectable after only 3 days. Abusive treatment of calves with methenolone acetate, can be detected by examining urine or faeces for metabolites M1, M2 and M3 up to a few days after the treatment. Thus, abuse can probably not be proven at the slaughterhouse. Therefore, in a control programme for anabolic steroids, it is important to take control samples at the farm as well as at the slaughterhouse.

Phase II metabolites have not yet been investigated. Only β-glucuronidase was used in the hydrolysis of urine and faeces, so, glucurono-conjugates were deconjugated and

TABLE II
Summary of the main metabolites of methenolone acetate in urine and faeces

	Metabolite	Chemical structure
M1	α-methenolone (17α-hydroxy-1-methyl-5α-androst-1-ene-3-one)	
M2	1-methyl-5α-androstan-3,17-diol	
M3	3α-hydroxy-1-methyl-5α-androstan-17-one	
	methenolone acetate	

detected, while possible sulpho-conjugates were not detected in this metabolism study. The metabolites M1, M2 and M3 can exist free or as glucuronide metabolites. To determine the profile of the phase II metabolites, an additional study is necessary in which specific separation of the metabolites is performed. The aim of this study was to look for target metabolites of methenolone acetate so as to be able to detect these metabolites in routine control urine samples in order to extend the range of anabolic steroids screened. Since conjugation is not necessary to facilitate the excretion of compounds in faeces, no deconjugation is necessary in analysing faecal samples. The abuse of methenolone acetate can be observed by detecting the parent compound and/or metabolites M1, M2 and M3 in faeces samples.

This study gives an indication of the metabolites of methenolone acetate in urine and faeces samples after oral administration to a male veal calf. Therefore, GC-MS was used as screen for the presence of metabolites. We show that abuse of methenolone acetate as growth promoter can be monitored by analysing urine and faeces samples with a routine GC-MS method for the detection of anabolic steroids in different matrices.

ACKOWLEDGEMENTS

The authors are grateful to Dirk Stockx and Ann Houvenaghel for assistance in experimental work and skilful operation of the GC-MS apparatus. The authors thank the Belgian Federal Ministry of Public Health and the Federal-Agency for the Safety of the Food Chain for the grant financing of this research (S6150).

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(Accepted: 27 October 2005; Published online: 23 December 2006)