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Detection of 19-nortestosterone and its urinary metabolites in miniature pigs by gas chromatography–mass spectrometry

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ABSTRACT

The metabolism of 19-nortestosterone was investigated in a miniature non-castrated male pig (boar), in a castrated pig (barrow) and in a female pig (sow). Urine samples were taken before and at regular intervals after the injection of 100 mg of Laurabolin (nortestosterone laurate). The sample clean-up consists in preliminary solid-phase extraction, followed by high-performance liquid chromatographic purification and fractionation. Finally, gas chromatography–mass spectrometry is used to identify the 19-nortestosterone metabolites.

INTRODUCTION

Nandrolone (17β-19-nortestosterone; 17β-19-NT) is a popular anabolic steroid in animal breeding, frequently found in illegal hormone "cocktails". Recently, however, some problems arose with the detection of 17β-19-NT in the urine of slaughter animals. A strikingly high percentage of 19-nortestosterone-positive cases in pigs led to the conclusion that 17β-19-nortestosterone is an endogenous compound in male pigs (boars) [1,2]. In this study, the effect of the injection of Laurabolin (nortestosterone laurate; Intervet, Brussels, Belgium) on the urinary steroid excretion pattern was investigated in three miniature pigs: a non-castrated male pig (boar), a castrated pig (barrow) and a female pig (sow). Urine samples were collected before and at regular intervals after the injection of Laurabolin.

Based on a preliminary literature study of nandrolone metabolism in different animal species, urine samples from the miniature pigs were analysed for 17β-19-NT and its epimer 17α-19-NT and for further possible metabolites, particularly isomers of 3-hydroxyestran-17-one and isomers of estrane-3,17-diol.

19-Nortestosterone has already been detected as a natural steroid in stallion urine [3,4], it has been identified in the follicular fluid of the mare [5,6] and its

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secretion by the testis of the stallion [7,8] has been reported. 19-Nortestosterone has also been identified in human follicular fluid [5,9]. Only some of the above references specified the 17β-epimeric form of 19-nortestosterone. In bovine species, C-17 epimerization is a major pathway of metabolism and 17α-19-NT is encountered in bovine urine after the administration of 17β-19-NT-containing veterinary preparations [10–12].

Isomers of 3-hydroxyestran-17-one have been detected as nortestosterone metabolites in the urine of crossbred and thoroughbred horses [13–15] and in the urine of equine male castrates and fillies [16]. In the horse, 5α-estrane-3β,17α-diol is endogenous in non-castrated stallions [3] and after administration of 19-nortestosterone-containing veterinary preparations, estrane-3,17-diols have been traced [13–17]. The stereochemistry of the most prominent metabolite is 5α-estrane-3β,17α-diol; the minor metabolite has a 5α,3β,17β configuration [18].

EXPERIMENTAL

Animals

The miniature pigs, two years old and weighing about 50 kg, were 75% Göttinger and 25% Vietnamese breed.

Standards

19-Noretiocholanolone (5β-estran-3α-ol-17-one), 19-norepiandrosterone (5α-estran-3β-ol-17-one) and 19-norepietiocholanolone (5β-estran-3β-ol-17-one) were kindly donated by Schering (Berlin/Bergkamen, Germany). 19-Norandrosterone (5α-estran-3α-ol-17-one), 5α-estrane-3α,17β-diol and 5α-estrane-3β,17α-diol were purchased from Steraloids (Wilton, NH, U.S.A.).

Solvents and glassware

Methanol and water were of high-performance liquid chromatographic (HPLC) grade from Alltech (Deerfield, IL, U.S.A.). Ethyl acetate was obtained from Janssen Chimica (Geel, Belgium).

Derivatization vials were silanized with a solution of 10% dichlorodimethylsilane (Merck-Schuchardt, Hohenbrunn bei München, Germany) in toluene before use.

Instrumentation

Octadecyl (C_{18}) and amino (NH_2) disposable columns for solid-phase extraction were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). For HPLC, the following equipment was used: a Model 6000A pump and WISP 710B automatic injector (Waters Assoc., Milford, MA, U.S.A.), a Model SP 8400 variable-wavelength detector (Spectra Physics, Santa Clara, CA, U.S.A.) operated at 254 nm

and a Helirac 2212 fraction collector (Pharmacia–LKB, Uppsala, Sweden). The HPLC analytical column was a LiChrospher 100 RP-18 (5 μm) column (12.5 mm × 4 mm I.D.)(Merck, Darmstadt, Germany) and was protected by a pellicular reversed-phase (30–50 μm) guard column (75 mm × 2.1 mm I.D.) (Chrompack, Middelburg, The Netherlands).

Gas chromatographic–mass spectrometric (GC–MS) analyses were carried out on a Model HP-5890 gas chromatograph, equipped with an HP Ultra 2 fused-silica (cross-linked 5% phenyl methyl silicone) capillary column (25 m × 0.2 mm I.D.), film thickness 0.33 μm (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Derivatization agents

Heptafluorobutyric anhydride (HFBA) was purchased from Macherey-Nagel (Düren, Germany), methoxyamine hydrochloride and N-trimethylsilylimidazole (TSIM) from Pierce (Rockford, IL, U.S.A.) and Lipidex-5000 from Packard Instruments (Brussels, Belgium).

Sample preparation

Steroid extraction was based on the procedure of Schmidt *et al.* [19]. Free and conjugated steroids, contained in 10 ml of urine, were extracted on a C_{18} column. The conjugated steroids were hydrolysed by means of *Helix pomatia* digestive juice (Boehringer, Mannheim, Germany) at 37°C for 16 h. The steroids were subsequently extracted on a C_{18} column. Additional purification was done by placing an amino column (NH_2) in series with the C_{18} column. An aliquot of the extract was finally purified and fractionated by HPLC [mobile phase methanol–water (65:35) at a flow-rate of 1 ml/min]. Because of the lack of chromophoric groups in some of the specified steroids (see *Standards*), fractions were analysed by GC–MS of a standard mixture of 17β-19-nortestosterone and its possible metabolites. The first fraction (fraction I, 5–7.5 min) contained the two 19-nortestosterone epimers and the second fraction (fraction II, 7.5–11 min) the four 3-hydroxyestran-17-one isomers and the two available estrane-3,17-diol isomers (see *Standards*).

Derivatization

Heptafluorobutyrates were prepared by adding 50 μl of heptafluorobutyric anhydride and 200 μl of benzene to the dried extract. After 1 h at 60°C, the derivatization mixture was removed under nitrogen and the derivative was dissolved in 20 μl of hexane.

Methoxime-trimethylsilyl (MO-TMS) ethers were prepared following the procedure of Thenot and Horning [20] and purified using Lipidex 5000 gel chromatography [21]. The purified derivative was dissolved in 20 μl of hexane.

GC–MS analysis

A 2-μl volume of the derivative was injected into the GC–MS instrument. The splitting ratio was 1:10. The carrier gas was high-purity helium (N60) (L'Air Liquide, Liège, Belgium) at a column head pressure of 80 kPa. The injection temperature and transfer line temperature were 290°C. For the heptafluorobutyrates, the oven temperature was programmed from 200 to 270°C at 5°C/min and maintained at 270°C for 14 min. For the MO-TMS derivatives, the oven temperature was programmed from 230 to 280°C at 5°C/min and maintained at 280°C for 15 min.

The mass spectrometer was operated in the electron-impact mode. The electron beam voltage was 70 eV. For data acquired by selected ion monitoring, dwell times of 100 ms were used.

RESULTS

The recovery from solid-phase extraction for 17β-19-NT was calculated by means of radioactive tracers and liquid scintillation counting. The mean of five determinations was 78.4 ± 5.4%. The recovery from HPLC processing was 76.4 ± 4.2% (n = 4).

Table I shows identification and confirmation data for 17β-19-nortestosterone and its possible metabolites, as obtained by processing the standard steroids. Identification data were obtained by HFB derivatization and confirmation was carried out by formation of MO-TMS derivatives.

TABLE I

IDENTIFICATION AND CONFIRMATION DATA FOR 17β-19-NORTESTOSTERONE AND ITS POSSIBLE METABOLITES

Compound[a]	Identification data (HFB derivatization)		Confirmation data (MO-TMS derivatization)	
	Characteristic ions (m/z)	MU[b]	Characteristic ions (m/z)	MU[b]
17α-19-NT	666,453,306	23.63	375,344,254	26.59
17β-19-NT	24.38			27.18
5α-E-3α-ol-17-one	472,454,428	23.52	377,346,256	25.28
5β-E-3α-ol-17-one	24.06			25.85
5β-E-3β-ol-17-one	24.18			25.81
5α-E-3β-ol-17-one	24.55			26.07
5α-E-3α,17β-diol	456,441,415	23.06	407,332,242	25.18
5α-E-3β,17α-diol	23.22			25.47

[a] -E- = -estran-.

[b] Methylene unit value.

TABLE II

DETERMINATION OF 17β-19-NT AND 17α-19-NT

Compound	Boar		Barrow		Sow	
	Before[a]	After[a]	Before	After	Before	After
17β-19-NT	+	+	N.D.[b]	+	N.D.	+
17α-19-NT	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

[a] Before and after injection of Laurabolin (100 mg).
[b] Not detected.

Both GC retention data, expressed as methylene unit values, and characteristic ions were used for identification. For a positive identification, all characteristic ions must appear simultaneously at the correct retention time.

Determination of 17β-19-NT and 17α-19-NT

Fraction I of the HPLC eluate was analysed for the presence of 17β-19-NT and 17α-19-NT. The results for the determination of the two 19-NT epimers are summarized in Table II.

Only the boar urine contained 17β-19-NT before injection of Laurabolin, which proves its secretion by the testis [1,2,22]. After injection, 17β-19-NT was detected in all samples. Fig. 1 shows the mass spectrum of 17β-19-NT-diHFB in the untreated boar (top), compared with that of the reference 17β-19-NT-diHFB (bottom). Fig. 2 shows ion chromatograms (ions of *m/z* 666, 453 and 306) for the sow, before (top) and after (bottom) the injection of Laurabolin.

No 17α-19-NT could be detected in either the pre- or post-administration samples. This proves that, in contrast to what is observed in bovine species [10–12], C-17 epimerization is not a major pathway of metabolism in miniature pigs.

Determination of isomers of 3-hydroxyestran-17-one

Fraction II of the HPLC eluate was analysed for the presence of 5α-estran-3α-ol-17-one, 5β-estran-3α-ol-17-one, 5α-estran-3β-ol-17-one and 5β-estran-3β-ol-17-one. The results for the determination of the 3-hydroxyestran-17-one isomers are summarized in Table III. None of the above 17-oxo-19-nor steroids could be detected in the pre-administration samples. However, in the post-administration samples, 5β-estran-3α-ol-17-one and 5α-estran-3β-ol-17-one were found in the samples from the boar, barrow and sow, and 5α-estran-3α-ol-17-one could additionally be demonstrated in the post-administration samples from the barrow. Fig. 3 shows ion chromatograms (ions of *m/z* 472, 454 and 428) for the barrow, before (top) and two weeks after (bottom) Laurabolin injection.

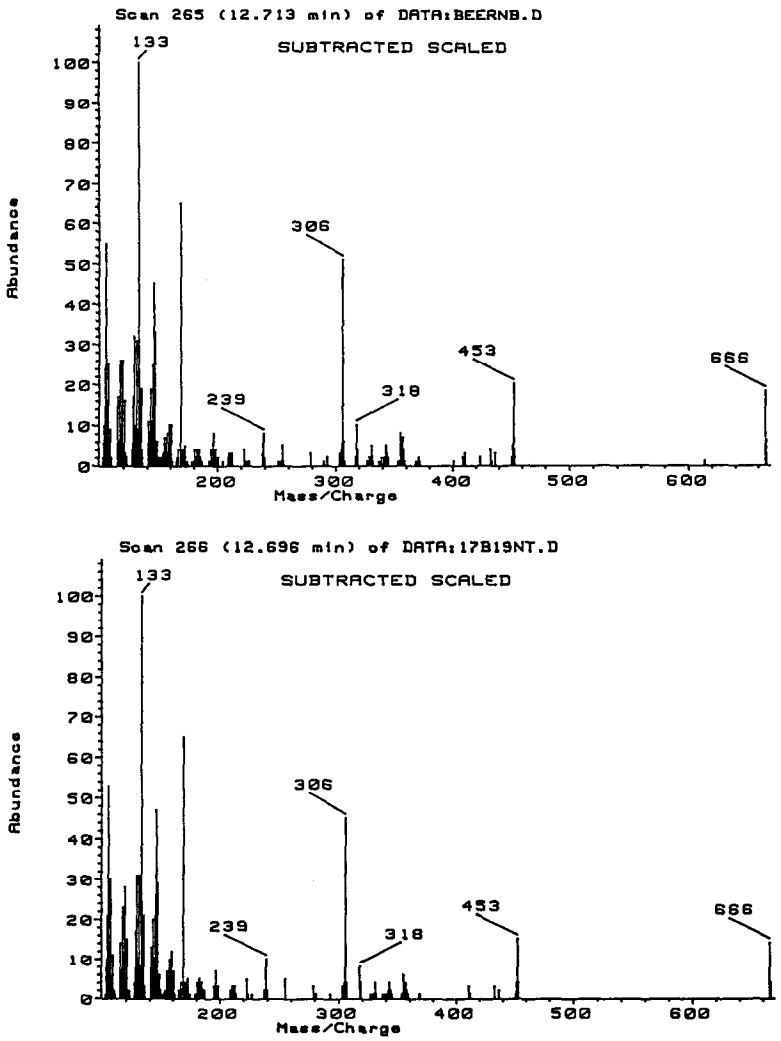

Fig. 1. Mass spectra of 17β-19-NT-diHFB in the untreated boar (top) and of the reference 17β-19-NT-diHFB (bottom).

Determination of isomers of estrane-3,17-diol

Concerning the detection of estrane-3,17-diols (data summarized in Tables IV and V), neither of the two isomers available as reference compounds were detected, in either the pre- or post-administration samples. However, two other isomers (E-diol I and E-diol II) were found in the urine samples from the injected boar

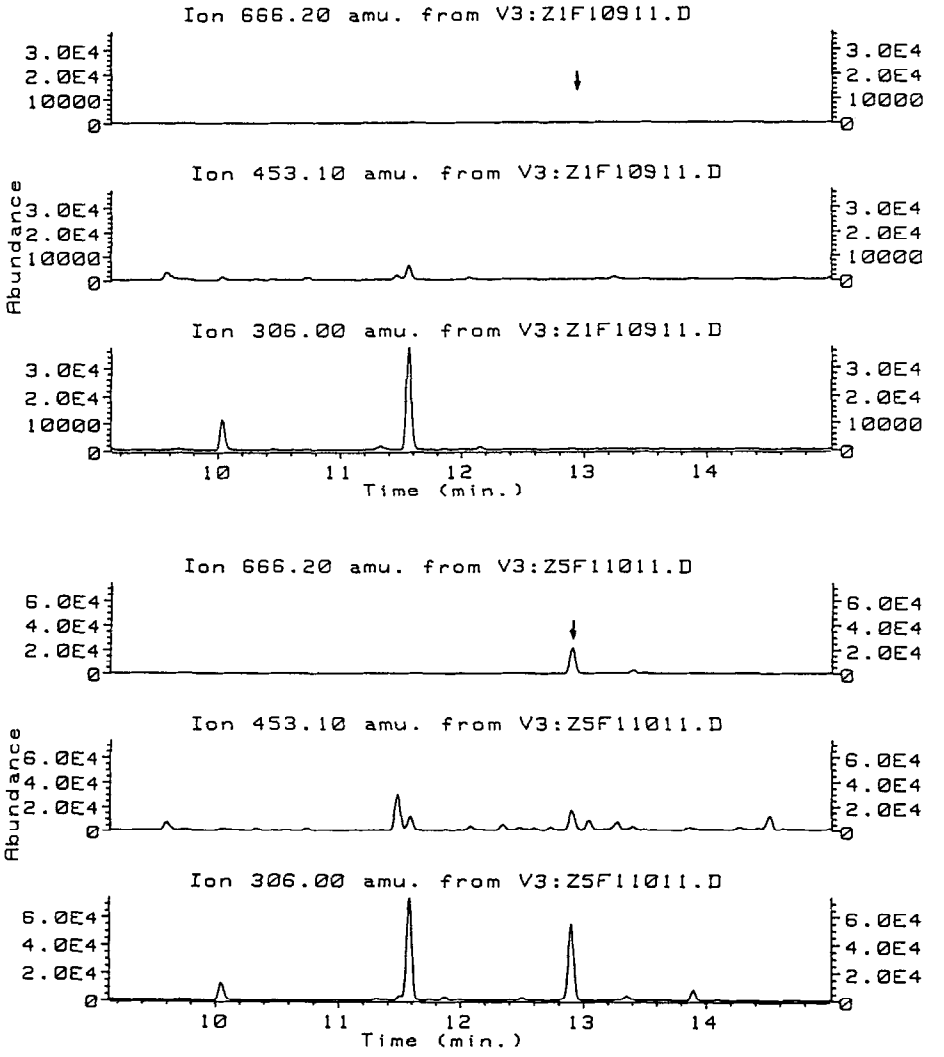

Fig. 2. Selected ion monitoring of ions of *m/z* 666, 453 and 306. Top, non-injected sow; bottom, sow two weeks after injection of Laurabolin (100 mg) (arrow indicates 17β-19-nortestosterone).

and barrow and one in the injected sow (E-diol I). MO-TMS derivatization of the extracts was employed to confirm the structure of the estrane-3,17-diols. Based on a recent report [18], the presumed stereochemistry of the first-eluting compound (E-diol I) is the 5β-estrane-3α,17β-diol and that of the second-eluting compound (E-diol II) is the 5α,3β,17β-isomer.

TABLE III

DETERMINATION OF 3-HYDROXYESTRAN-17-ONES

Compound[a]	Boar		Barrow		Sow	
	Before[b]	After[b]	Before	After	Before	After
5α-E-3α-ol-17-one	N.D.[c]	N.D.	N.D.	+	N.D.	N.D.
5β-E-3α-ol-17-one	N.D.	+	N.D.	+	N.D.	+
5β-E-3β-ol-17-one	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
5α-E-3β-ol-17-one	N.D.	+	N.D.	+	N.D.	+

[a] -E- = -estran-.
[b] Before and after injection of Laurabolin (100 mg).
[c] Not detected.

TABLE IV

DETERMINATION OF ISOMERS OF ESTRANE-3,17-DIOL

Compound[a]	Boar		Barrow		Sow	
	Before[b]	After[b]	Before	After	Before	After
5α-E-3α,17β-diol	N.D.[c]	N.D.	N.D.	N.D.	N.D.	N.D.
5α-E-3β,17α-diol	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
E-diol I	N.D.	+	N.D.	+	N.D.	+
E-diol II	N.D.	+	N.D.	+	N.D.	N.D.

[a–c] See Table III.

Figs. 4 and 5 show the mass spectra of E-diol I-diTMS in the barrow two weeks after injection and of E-diol II-diHFB in the boar 3 weeks after injection, respectively.

CONCLUSIONS

The presence of some 19-nortestosterone metabolites in miniature pigs has been demonstrated. Further investigations will focus on the eventual concordance between results obtained on miniature pigs and those on feeding pigs (Belgian landrace). In this respect, GC–MS could be used as an alternative to radioimmunoassay screening and the high-performance thin-layer chromatographic confirmation tests, mostly employed for the detection of the

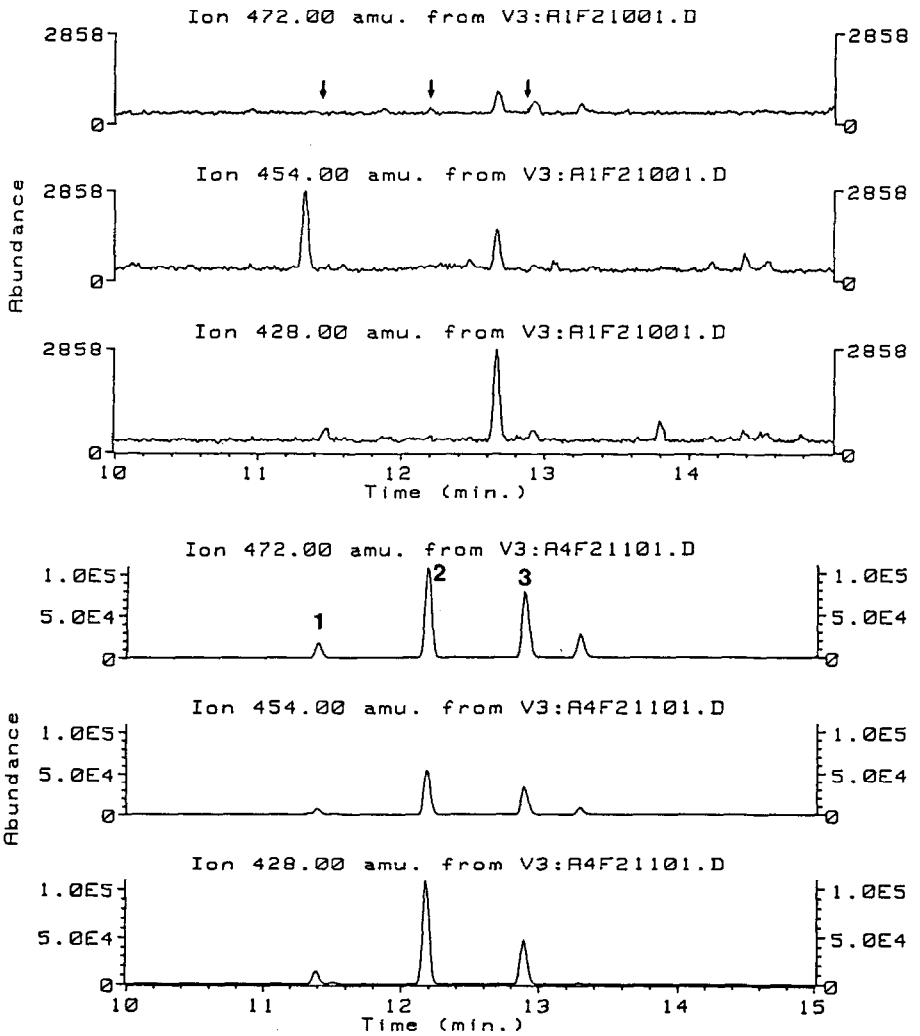

Fig. 3. Selected ion monitoring of ions of *m/z* 472, 454 and 428. Top, non-injected barrow; bottom, barrow two weeks after injection of Laurabolin (100 mg). Peaks: 1 = 5α-Estran-3α-ol-17-one; 2 = 5β-estran-3α-ol-17-one; 3 = 5α-estran-3β-ol-17-one.

TABLE V

METHYLENE UNIT VALUES AND PRESUMED STEREOCHEMISTRY OF THE TWO ISOMERS OF ESTRANE-3,17-DIOL

Compound	Methylene unit value		Presumed[a] stereochemistry
	HFB	MO-TMS	
E-diol I	23.60	25.60	5β-Estrane-3α,17β-diol
E-diol II	24.06	25.96	5α-Estrane-3β,17β-diol

[a] Ref. 18.

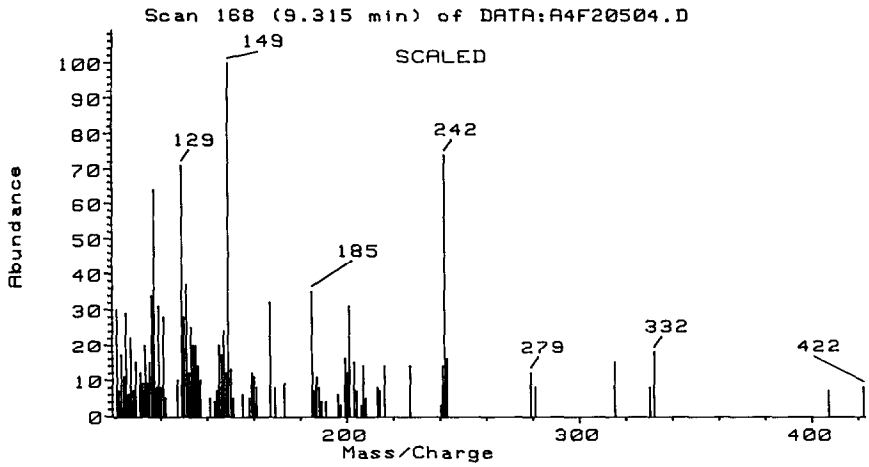

Fig. 4. Mass spectrum of E-diol I-diTMS in the barrow two weeks after injection of Laurabolin (100 mg).

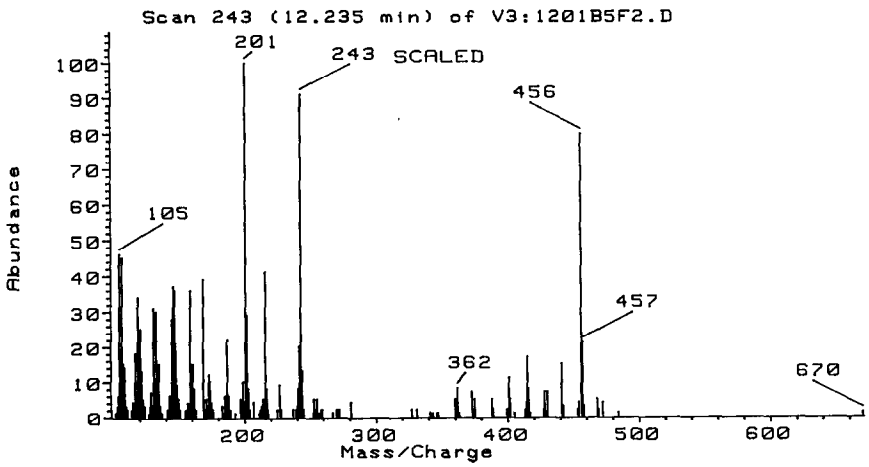

Fig. 5. Mass spectrum of E-diol II-diHFB in the boar three weeks after injection of Laurabolin (100 mg).

illegal use of 19-nortestosterone. Moreover, not only the parent molecule but also the metabolites can be screened.

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REFERENCES

1 G. Maghuin-Rogister, A. Bosseloir, P. Gaspar, C. Dasnois and G. Pelzer, *Ann. Méd. Vét.*, 132 (1988) 437.
2 G. Debruyckere, C. Van Peteghem, H. F. De Brabander and M. Debackere, *Vet. Q.*, 12 (1990) 246.
3 E. Houghton, J. Copsey, M. C. Dumasia, P. E. Haywood, M. S. Moss and P. Teale, *Biomed. Mass Spectrom.*, 11 (1984) 96.
4 E. Houghton, M. C. Dumasia, M. S. Moss and P. Teale, in G. J. Johnston (Editor), *Proceedings of the 37th and 38th Meetings of the AORC, Toronto and Denver, June 12–17, 1983 and 1984*, AORC, Denver, CO, pp. 61-71.
5 L. Dehennin, P. Silberzahn, A. Reiffsteck and I. Zwain, *Pathol. Biol.*, 32 (1984) 828.
6 P. Silberzahn, L. Dehennin, I. Zwain and A. Reiffsteck, *Endocrinology*, 117 (1985) 2176.
7 E. Benoît, F. Garnier, D. Courtot and P. Delatour, *Ann. Rech. Vét.*, 16 (1985) 379.
8 T. Dintinger, J.-L. Gaillard, I. Zwain, R. Bouhamidi and P. Silberzahn, *J. Steroid Biochem.*, 32 (1989) 537.
9 L Dehennin, M. Jondet and R. Scholler, *J. Steroid Biochem.*, 26 (1987) 399.
10 E. Benoît, J. L. Guyot, D. Courtot and P. Delatour, *Ann. Rech. Vét.*, 20 (1989) 485.
11 W. Haasnoot, R. Schilt, A. R. M. Hamers and F. A. Huf, *J. Chromatogr.*, 489 (1989) 157.
12 L. A. van Ginkel, R. W. Stephany, H. J. van Rossum, H. van Blitterswijk, P. W. Zoontjes, R. C. M. Hooijschuur and J. Zuydendorp, *J. Chromatogr.*, 489 (1989) 95.
13 E. Houghton, *Xenobiotica*, 7 (1977) 683.
14 E. Houghton, G. A. Oxley, M. S. Moss and S. Evans, *Biomed. Mass Spectrom.*, 5 (1978) 170.
15 E. Houghton and M. C. Dumasia, *Xenobiotica*, 10 (1980) 381.
16 E. Houghton, M. C. Dumasia, P. Teale, M. S. Moss and S. Sinkins, *J. Chromatogr.*, 383 (1986) 1.
17 M. C. Dumasia and E. Houghton, *Xenobiotica*, 14 (1984) 647.
18 E. Houghton, A. Ginn, P. Teale, M. C. Dumasia and J. Copsey, *J. Chromatogr.*, 479 (1989) 73.
19 N. A. Schmidt, H. J. Borburgh, T. J. Penders and C. W. Weykamp, *J. Steroid Biochem.*, 31 (1985) 637.
20 J. P. Thenot and E. C. Horning, *Anal. Lett.*, 5 (1972) 21.
21 M. Axelson and J. Sjövall, *J. Steroid Biochem.*, 5 (1974) 733.
22 A. Ruokonen and R. Vihko, *J. Steroid. Biochem.*, 5 (1974) 33.