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Gas chromatographic–mass spectrometric identification of main metabolites of stanozolol in cattle after oral and subcutaneous administration

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Abstract

An analytical method has been developed in order to control the illegal use of stanozolol as growth promoter in livestock. The procedure was based on enzymatic hydrolysis, purification on a Clean Screen DAU column and derivatization with heptafluorobutyric anhydride prior to GC–MS analysis. This method allowed us to study the metabolism of stanozolol in cattle after oral and subcutaneous administrations. Urinary metabolites were identified by mass spectrometry. Stanozolol and 16-hydroxystanozolol were detected after oral administration, while 16-hydroxystanozolol and 4,16-dihydroxystanozolol were found after subcutaneous administration.

Keywords: Stanozolol

1. introduction

Stanozolol (17α-methyl-17β-hydroxy-5α-androstano-[3,2-C]-pyrazole) was first synthesised by Clinton and co-workers in 1959 [1,2] and used in cases of deficiency in protein synthesis and osteoporosis [3]. In spite of its prohibition by the International Olympic Committee (IOC) since 1974, this androgenic anabolic steroid was often abused in sport by athletes [4]. Stanozolol was also used in horseraces to enhance performances. Lately, the discovery of stanozolol in injection sites and anabolic preparations took in slaughterhouse revealed its illegal use as a growth promoter in breeding despite the ban in the European Union (EU) since 1988 (EC directive 81/602) [5].

The structure of this synthetic compound differs to a rather large extent from most anabolic steroids because of its pyrazole nucleus attached on the A-ring. This makes the extraction of stanozolol from complex matrices, as well as its GC analysis, eminently difficult. The analytical method used for stanozolol, as well as other steroids in urine is generally based on solid-phase extraction on a C_{18} cartridge, liquid–liquid extraction and purification by preparative HPLC [6,7].

GC coupled with mass spectrometry was used by several authors for the control of stanozolol in humans and horses.

Until now, no method is available for the simulta-

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neous detection and identification of stanozolol in cattle urine, and no metabolic study has been initiated on this compound. This lack of data does not permit to carry out an efficient control of stanozolol illegal use.

The aim of this paper was to focus on an analytical method that ensures a selective reproducible purification of stanozolol with good recovery. A derivatization technique was developed in order to avoid phenomenon of peak tailing. Furthermore, the main urinary metabolites of this anabolic steroid were studied after oral and subcutaneous administrations in two adult cows.

2. Experimental

2.1. Apparatus

A Hewlett-Packard (Palo Alto, CA, USA) 5972 mass selective detector coupled to a Model 5890 HP gas chromatograph was used. The magnetic MS, a reverse geometry double focusing instrument SX 102A (Jeol, Tokyo, Japan) was coupled to an HP-5890 GC. Two different capillary columns were used: OV-1 (30 m×0.25 mm I.D., film thickness 0.25 μm) (Interchrom, Montluçon, France) and OPTIMA 5 (15 m×0.25 mm I.D., film thickness 0.25 μm) (Macherey-Nagel, Habrot, France). The transfer line temperature was set at 280°C and the split/splitless injector was maintained at 250°C (1 min delay). The GC temperature programming rates were as follows: 120°C (2 min), 15°C min^{-1} until 300°C (6 min) and 120°C (2 min), 20°C min^{-1} until 250°C (0 min) then 10°C min^{-1} until 300°C (10 min). Helium (N55) was used as carrier gas at 1 ml min^{-1}. The electronic beam energy was set at 70 ev in the electronic impact (EI) mode.

2.2. Reagents and chemicals

Most of the reagents and solvents used were reagent-grade products from Merck (Darmstadt, Germany) and SDS (Peypin, France). *Helix pomatia* juice with enzymatic activities of β-glucuronidase 10^5 Fishman U/ml and sulfatase 10^6 Roy U/ml was purchased from Biosepra (Villeneuve-la-garenne, France). The SPE Clean Screen DAU 500 columns (500 mg of phase) were obtained from SDS. The N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) and the trimethyliodosilane (TMIS) were purchased from Fluka (Buchs, Switzerland) and dithiothreitol (DTE) was from Aldrich (Milwaukee, WI, USA). The reference steroids were obtained from Steraloids (Wilton, NH, USA), Research Plus (Bayonne, NJ, USA) and Sigma (St. Louis, MO, USA).

2.3. Animals

A 200 mg quantity of stanozolol dissolved in ethanol solution and pure water was injected subcutaneously to a Holstein cow (C1). Urine was collected daily for 8 days and stored frozen (−16°C).

A 500 mg quantity of stanozolol diluted in glacial acetic acid solution and pure water was administered orally to a second Holstein cow (C2). Urine was collected daily for 8 days and stored frozen (−16°C).

2.4. Method

2.4.1. Extraction and purification procedure

Urine (10 ml) was hydrolysed at least during 15 h at 52°C with 50 μl of *Helix pomatia* juice after addition of 1 ml of 2 *M* acetate buffer (pH 5.2). pH was adjusted to 6.0 with 4 ml of 0.1 *M* phosphate buffer (pH 6.0). The sample was slowly applied to a Clean Screen DAU column previously conditioned with 2 ml of methanol, 2 ml of ultra pure water (>14 MΩ cm) and 2 ml of 0.1 *M* phosphate buffer (pH 6.0). The column was washed with 1 ml of 1 *M* acetic acid, immediately followed by 6 ml of methanol. The analytes were finally eluted with 6 ml of ethyl acetate–ammonium hydroxide at 32% (97:3, v/v) solution, which was evaporated to dryness under a stream of nitrogen. The dry residues were dissolved in 200 μl of methanol and transferred into a reaction vial. Any remaining residue was dissolved with a further 100 μl of methanol. The extract was evaporated to dryness under a stream of nitrogen (40°C).

2.5. Derivatization prior to GC–MS analysis

The dry residue was derivatized according to the two following reactions: reaction A: 40 min at 60°C with 25 μl of MSTFA–TMIS–DTE (1000:5:5, v/v/

w). Reaction B: 30 min at 70°C with 25 μl of HFBA. 2 μl was injected into the GC–MS system.

3. Results and discussion

3.1. Extraction/purification

The published articles about the detection of stanozolol do mention extraction on C_{18} cartridge [6] or on Amberlite XAD-2 resin [8–10], which are both adapted for steroids with hydrophobic and neutral characters. The low recoveries obtained for the extraction of this compound on C_{18} cartridge led us to consider its properties.

The presence of the pyrazole nucleus condensed to the androstane ring confers its ionizing properties to the stanozolol. Its purification can be achieved on a Clean Screen DAU column, which contains both a C_8 and a benzene sulfonic acid functionality [11].

The stanozolol extraction yields of 20 ppb spiked urines were estimated using methylchlorotestosterone (20 ppb) as external standard. The mean recovery obtained was 90±6% (n=10) (Fig. 1).

3.2. Derivatization

Stanozolol has two ionizable hydrogen atoms (Fig. 2). Possible hydrogen binding may exist between labile hydrogens of stanozolol and the active sites of the column or glassware. This phenomenon is responsible for peak tailing.

In order to stabilise stanozolol and avoid the peak tailing, a derivatization step using a MSTFA–TMIS–DTE mixture was carried out. The derivative behaviour was studied under electronic impact ionisation (Fig. 3). Good sensitivity was obtained, but fragmentation did not give enough informative data because the N-TMS bond was not sufficiently stable.

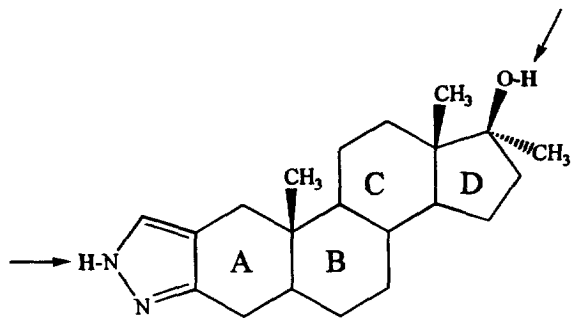

Fig. 2. Labile hydrogen position of stanozolol.

Using reaction B the bis-HFB derivative was obtained. However, a compound with 214 u less than the expected one was detected. A high resolution study (HR) allowed us to determine the elemental composition of the corresponding structure $C_{25}H_{29}ON_2F_7$ (506.2168) (Fig. 4). Ions detected at m/z 147.1174 ($C_{11}H_{15}$) and m/z 161.1330 ($C_{12}H_{17}$) would appear to indicate B-ring fragmentation (Fig. 5) and a loss of water at the 17β position. These observations led us to suppose a Wagner-Meervein rearrangement [12]. The properties based on the use of this HFB derivative were characterised by a high sensitivity as well as a good specificity (Fig. 6); the

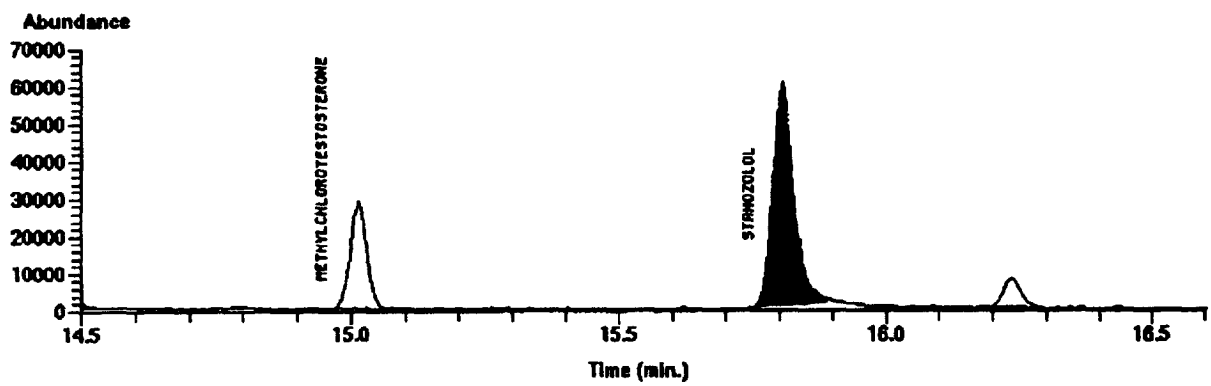

Fig. 1. Total ion current profile (TIC) of stanozolol (20 ppb) after purification on a Clean Screen Dau column.

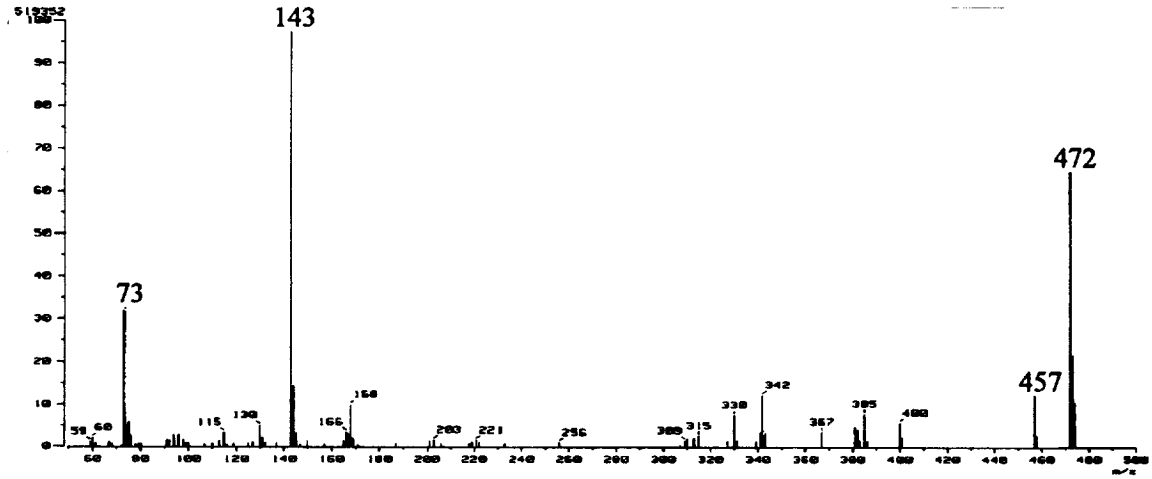

Fig. 3. Mass spectrum of the N,O-diTMS derivative of stanozolol in EI ionization and full SCAN mode (pure substance).

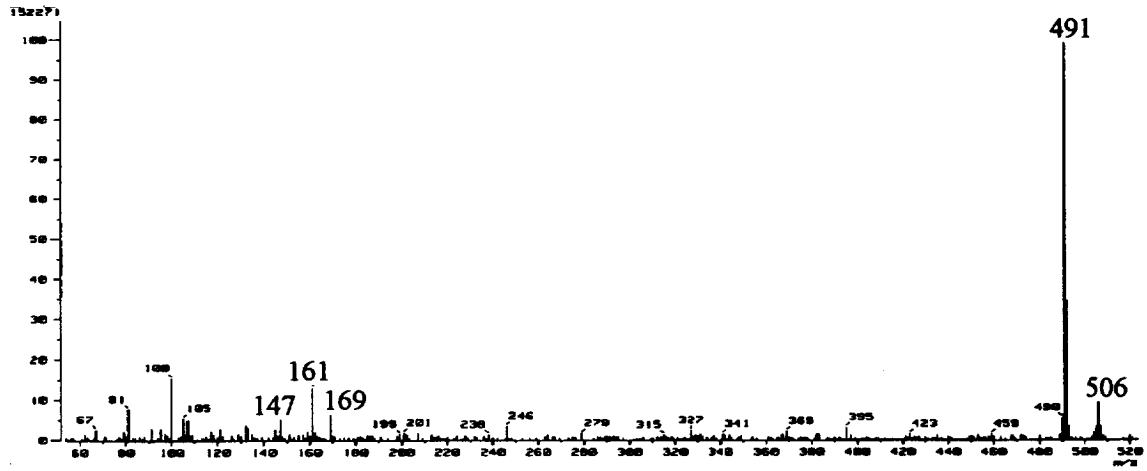

Fig. 4. Mass spectrum of the N-HFB derivative of stanozolol in EI ionization and full SCAN mode (pure substance).

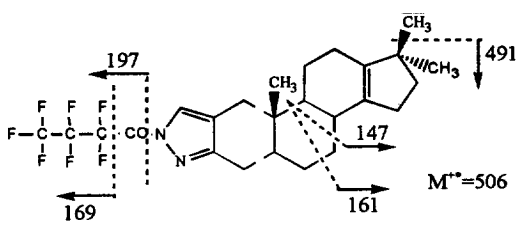

Fig. 5. Proposition of main fragmentations of the N-HFB derivative of stanozolol confirmed in high resolution (HR).

N-HFB bond which formed seemed to be stable in time.

3.3. Detection of metabolites in cow urine

3.3.1. Subcutaneous injection

After the (A) derivatization, the urinary extract of C1 was injected into GC–MS (EI ionization, quadrupole mass spectrometer).

The comparison between the urines collected

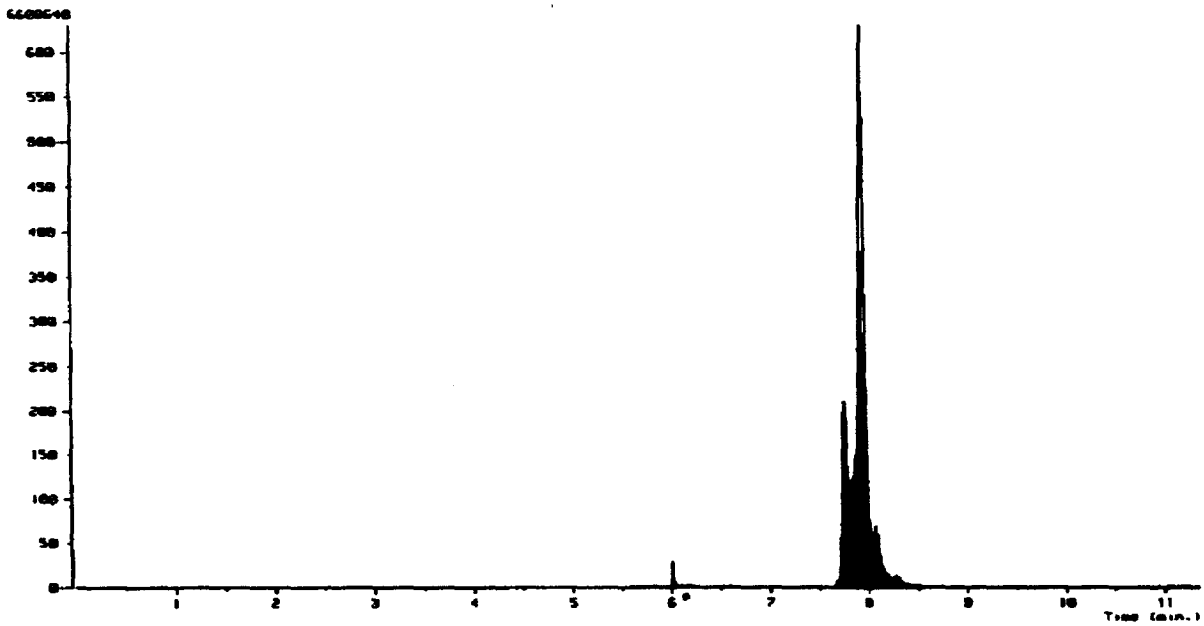

Fig. 6. Total ion current profile (TIC) of the N-HFB derivative of stanozolol (pure sample).

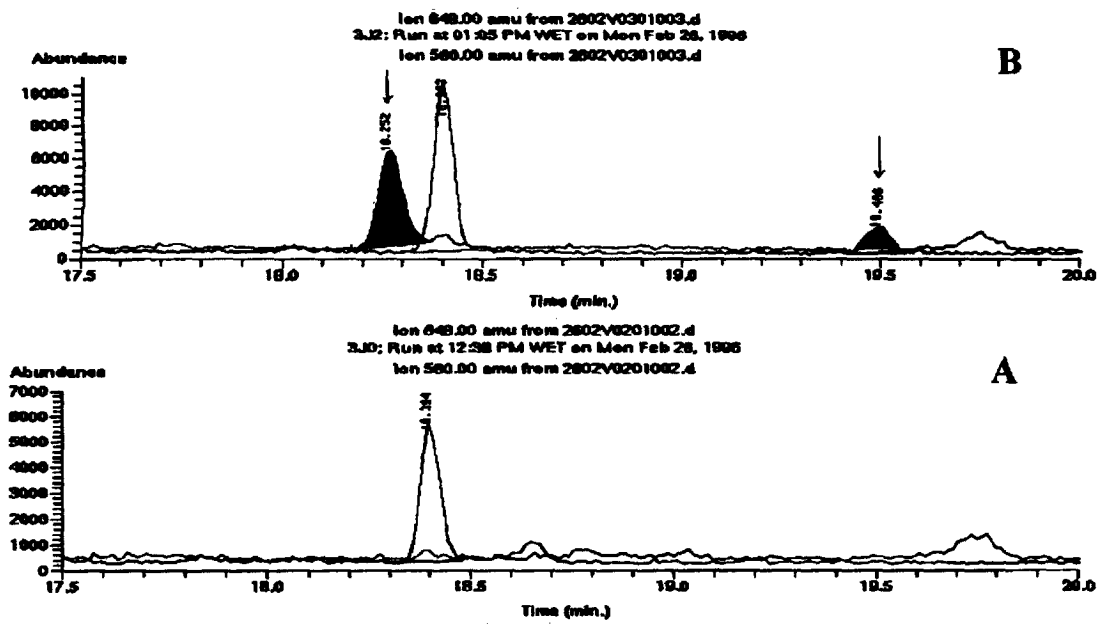

Fig. 7. Comparison of a total ion chromatogram (TIC) of a urinary extract J_0 (A) with TIC obtained with a urinary extract J_2 (B) (collected two days after subcutaneous administration). Two potential metabolites of stanozolol in urine J_2 were identified during this experiment.

before injection (J_0) and two days (J_2) after administration of stanozolol permitted the detection of metabolites. Conversely, stanozolol ($M^{+\cdot}$=472) and its isomers were not detected in these samples. Two chromatographic peaks were observed in J_2 (Fig. 7). They correspond to the molecular ions of potential metabolites, respectively at *m/z* 560 and *m/z* 648.

The ion at *m/z* 560 has been assigned to be a monohydroxylated metabolite. The fragmentation study in the EI mode enabled the correlation of the structure with specific fragments (Fig. 8). The ion *m/z* 168 originating from the cleavage of the A-ring with migration of two hydrogen atoms indicated that no hydroxy function was present on the pyrazole and the A-ring (Fig. 9). The ion fragments at *m/z* 231 and 218 indicate that the 16 position of the nucleus was hydroxylated (Fig. 9). No information was available to determine the α or β position of the alcohol function.

The ion at *m/z* 648 has been assigned to a dihydroxylated metabolite (Fig. 10). The ion fragments *m/z* 231 and 218 confirmed the presence of a hydroxy group in the 16 position (Fig. 11). The second hydroxy group characterised by the ion *m/z* 254 should be located either in the 3′ position of the pyrazole ring or in the 4 position (Fig. 11). This ion corresponds to a retro-Diels-Alder fragmentation. In order to identify this metabolite, we referred to the work of Schänzer and Donike [8] relative to the identification of two possible compounds in human urine. Comparison between 3′,16-dihydroxy-

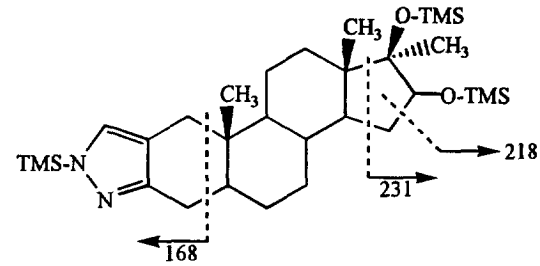

Fig. 9. Suggestion of main fragmentations of the trimethylsilylated 16-hydroxystanozolol ($M^{+\cdot}$=560) observed in the EI mode.

stanozolol and 4,16-dihydroxystanozolol mass spectra in the human indicated an ion 558 $[M-OTMS]^{+\cdot}$ which was only present in the mass spectrum of 4,16-dihydroxystanozolol. This ion was also observed in cattle and may confirm the identify of 4,16-dihydroxystanozolol. This TMSOH loss in 4 position rather than 3′ position could be explained by the benzylic cleavage in α, β position of the aromatic ring. Nevertheless, we were not able to conclude concerning the α or β position of the 4-hydroxy. Finally, two hydroxystanozolol metabolites were identified in urine after subcutaneous administration: 16-hydroxystanozolol and 4,16-dihydroxystanozolol.

3.3.2. Oral administration

The urinary extract of C2 was analysed by GC–MS (EI, magnetic mass spectrometer) in the full

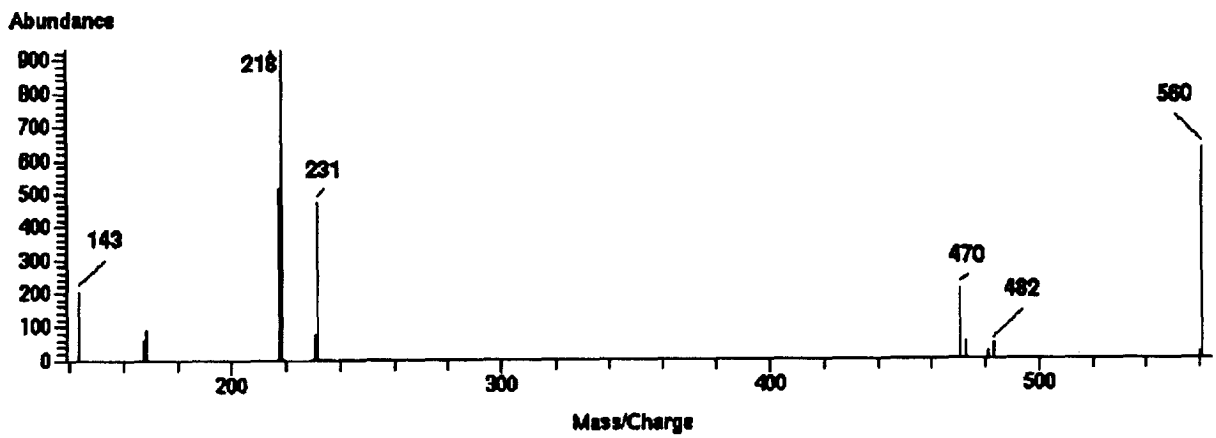

Fig. 8. Mass spectrum of the monohydroxylated metabolite ($M^{+\cdot}$=560) obtained by EI ionization and detected by comparison between a standard urine (J_0) and a urine (J_2) collected two days after subcutaneous administration of stanozolol.

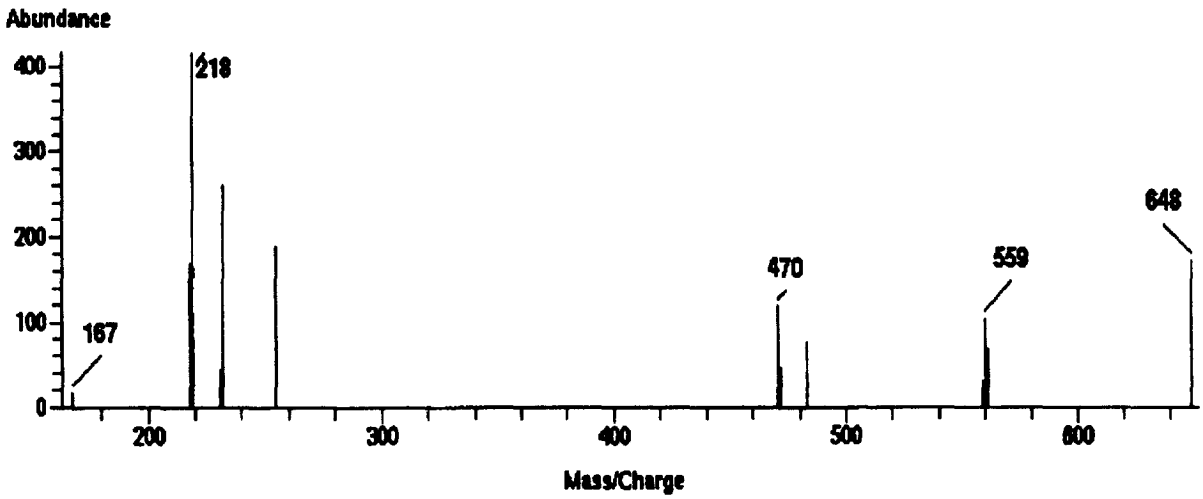

Fig. 10. Mass spectrum of the dihydroxylated metabolite ($M^{+\cdot}$=648) observed in EI mode and detected by comparison between a standard urine (J_0) and a urine (J_2) collected two days after subcutaneous administration of stanozolol.

SCAN mode after B derivatization. At the first time, the comparison of ion chromatograms between J_0 and J_2 urines allowed us to detect the presence of stanozolol (Fig. 12A). The diagnostic ions have been observed at the same retention time as the stanozolol standard and in the same relative ratio when compared to internal standard. Secondly, the comparison between J_0 and J_2 urines showed the appearance of a chromatographic peak in J_2 (Fig. 12B) corresponding to the molecular ion at *m/z* 932. A study of the main fragments (Fig. 13) led us to the following hypothesis: the ion at *m/z* 292 is characteristic of the cleavage of bonds C_1–C_{10} and C_4–C_5 with migration of two hydrogen atoms and with a charge retention located on the A-ring (Fig. 14); this would indicate the absence of hydroxy group in 3′ or 4 position. The cleavage of bonds C_{14}–C_{15} and C_{13}–C_{17} on the D-ring (ion *m/z* 451) (Fig. 14) with a charge retention located on the A,B,C-ring seems to prove the presence of an alcohol function on the D-ring. The hydroxy-position on the D-ring was determined by the study of TMS derivatives: the stanozolol metabolite afforded characteristic fragment ions at *m/z* 218 and 231 indicating a 16-hydroxy structure.

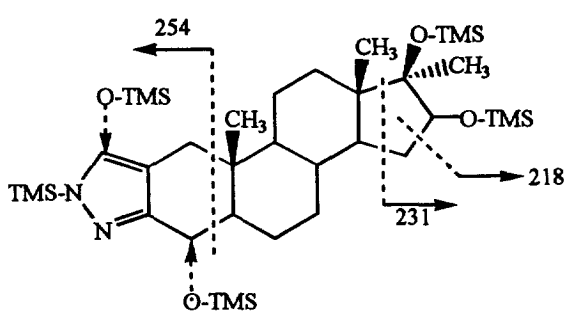

Fig. 11. Suggestion of main fragmentations of the dihydroxylated metabolite derivated TMS of stanozolol ($M^{+\cdot}$=648) observed in the EI mode.

The study of stanozolol metabolism in cattle led us to identify stanozolol and 16-hydroxystanozolol after oral administration and 16-hydroxystanozolol and 4,16-dihydroxystanozolol after subcutaneous injection. The HFB derivatization allowed us to detect low concentrations of stanozolol (around 1 ppt) in cow urine after oral administration.

The analytical method developed for stanozolol still needs to be validated. An urinary excretion profile of the suspected metabolites should be studied as well as the determination of the metabolites stereoisomerism. These investigations may be the starting point of further studies, which could confirm unambiguously the structure of these first metabolites.

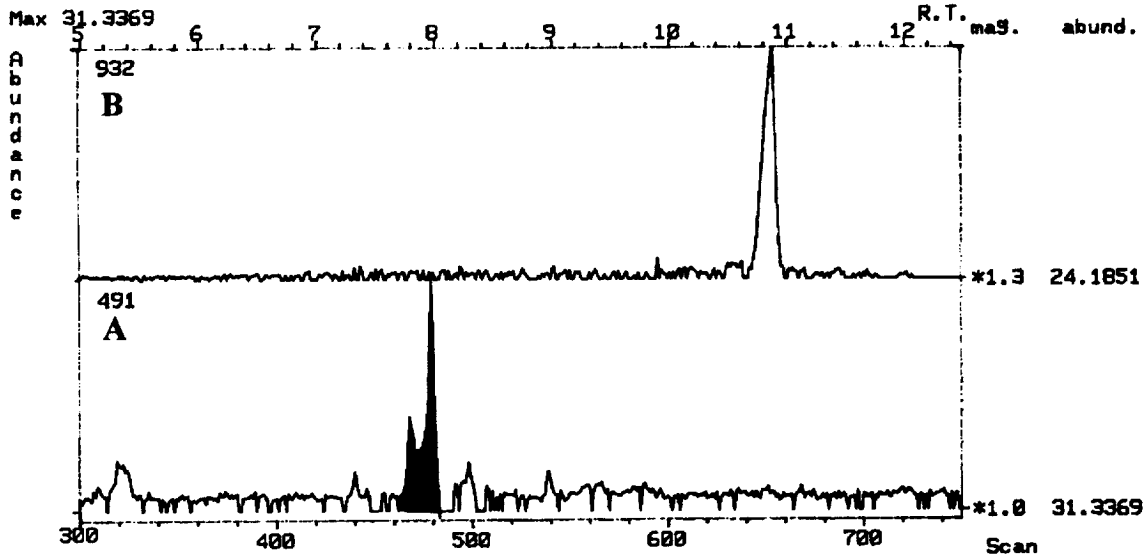

Fig. 12. Stanozolol ($M^{+\cdot}-15=491$) (A) identified in urine J_2 (collected two days after oral administration of stanozolol) and a potential metabolite of stanozolol ($M^{+\cdot}=932$) (B) detected in urine J_2 after oral administration.

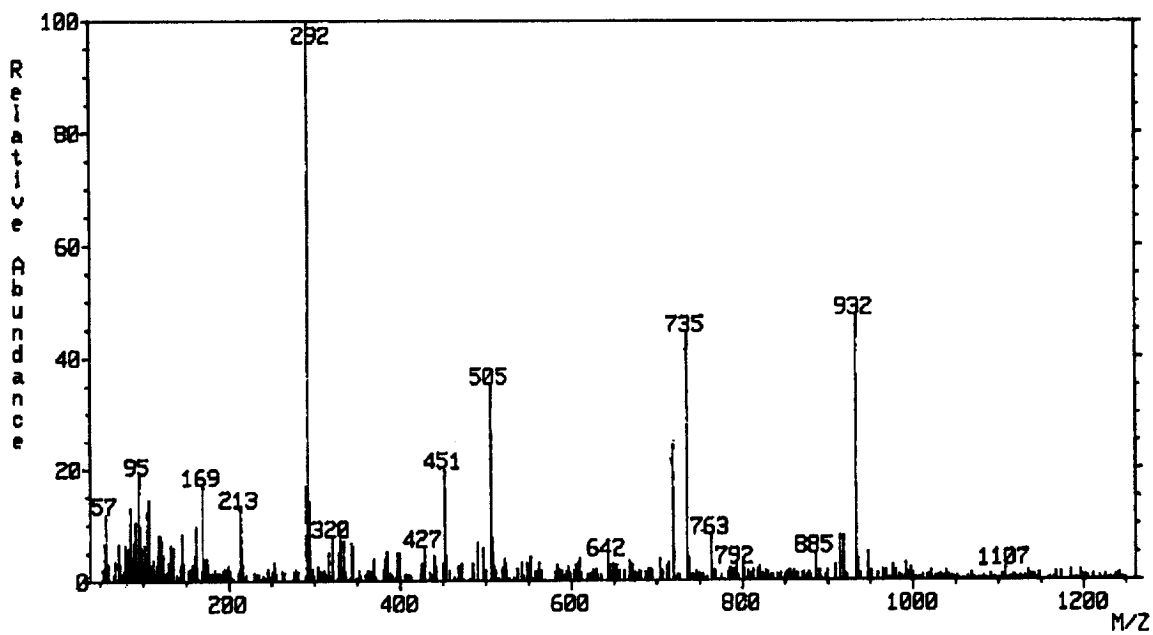

Fig. 13. Mass spectrum of monohydroxystanozolol ($M^{+\cdot}=932$) observed in EI mode and detected by comparison of a standard urine J_0 and an urine J_2 collected two days after oral administration of stanozolol.

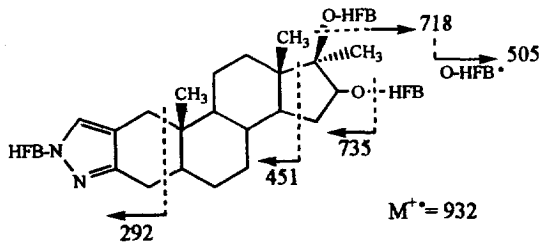

Fig. 14. Suggestion of main fragmentations of the 16-hydroxystanozolol derivated HFB observed in the EI mode: $[M-CF_3(CF_2)_2CO]^+$ at *m/z* 735; $[M-CF_3(CF_2)_2COOH]^{+\cdot}$ at *m/z* 718 and $[M-CF_3(CF_2)_2COOH-CF_3(CF_2)_2CO]^+$ at *m/z* 505.

References

[1] R.O. Clinton, A.J. Manson, F.W. Stanner, A.L. Beyler, G.O. Potts, A. Arnold, J. Am. Chem. Soc. 81 (1959) 1513–1514.

[2] R.O. Clinton, A.J. Manson, F.W. Stanner, H.C. Newmann, R.G. Christansen, R.L. Clarke, J.H. Akermann, D.F. Page, J.W. Dean, W.B. Dickinson, C. Carabateas, J. Am. Chem. Soc. 83 (1961) 1478–1491.

[3] C.H. Chesnut, J.L. Ivery, H.E. Guber, M. Mattews, W.B. Nelp, Metabolism 32 (1983) 571–580.

[4] W.M. Mück, J.D. Henion, Biomed. Environ. Mass Spectrom. 19 (1990) 37–51.

[5] EC directive 81/602, Off. J. Eur. Commun., No. L 222 (1981) 32.

[6] R. Massé, C. Ayotte, H. Bi, R. Dugal, J. Chromatogr. 497 (1989) 17–37.

[7] B. Le Bizec, M.P. Montrade, F. Monteau, F. André, Anal. Chim. Acta 2751-2 (1993) 123–133.

[8] W. Schänzer, M. Donike, J. Steroid. Biochem. 36 (1990) 153–174.

[9] W. Schänzer, M. Donike, Anal. Chim. Acta 275 (1993) 23–48.

[10] H.Y.P. Choo, O.S. Kwon, J. Park, J. Anal. Toxicol. 14 (1990) 109–112.

[11] M.C. Dumasia, E. Houghton, J. Chromatogr. 564 (1991) 504–513.

[12] D. Stöckl, R. De Sagher, L.M. Thienpont, G. Debruyckere, C.H. Van Peteghem, Analyst 119 (1994) 2587–2589.