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Detection of Stanozolol metabolites in bovine urine after intramuscular injection

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1. Introduction

In Europe the word „hormones" has a very bad reputation in the eyes of the consumer because of the possible danger for public health of some of these products. The European Union banned the use of anabolic steroids for cattle fattening in 1988 (Anonymous, 1988). The Veterinary Food Inspection of each member state has to follow this legislation. However, a law without a good functioning control system is not of much value. Analytical techniques, able to detect trace amounts of the parent drugs and their metabolites in several matrices are mandatory for the control mechanisms.

The list of steroids which could be abused in cattle fattening is long. Since the start of the control (in Belgium since 1973) a considerable number of substances were added to the list of steroids to be analysed for. Some examples of newly introduced products are: methandienone (methylboldenone), norethandrolone, norgestrel, fluoxymesterone etc. These steroids are not new on the pharmaceutical market but in order to escape from the increasing inspection the black market tries to replace the classical molecules by unexpected ones. In this regard, stanozolol is a relatively „old" molecule (U.S. patent in 1962 ; The Merck Index, 1996). Stanozolol has become an important product in veterinary inspection: it is often found in injection sites taken at the slaughterhouse (in 1995 it was detected 72 times in the 141 positive injection sites in Belgium (personal communication from Veterinary Inspection, 1997)). However, the same product was never detected in tissues (kidney fat, meat) and excreta (urine, faeces) taken at regulatory inspection.

In comparison with other anabolic steroids there is a difference in structure (fig 1).

Stanozolol (5α-androstane-17α-methyl-17β-ol (3,2-C) pyrazole)(Stan) resembles best methyltestosterone. Instead of the 3 ketogroup there is a pyrazole ring condensed to the androstane ring system. In the classical High Performance Thin Layer Chromatography (HPTLC) determination as described by Verbeke (1979) the detection limit of Stan is inferior to the detection limit of most other steroids (10–20 µg/kg instead of the normal action limit of 2 µg/kg). Also in Gas Chromatography Mass Spectrometry (GC-MS) the product has a different behaviour in comparison with related compounds.

In a related research area, sports doping, as well in man as in horseracing, Stan was considered as a problem too. The research group of the „Deutsche Sporthochschule Köln", GFR studied and synthetised urinary metabolites of anabolic steroids (a.o. Stan) in man (SCHÄNZER and others, 1990; SCHÄNZER and DONIKE, 1994)

The major urinary metabolites of Stanozolol in man are given in fig 2.

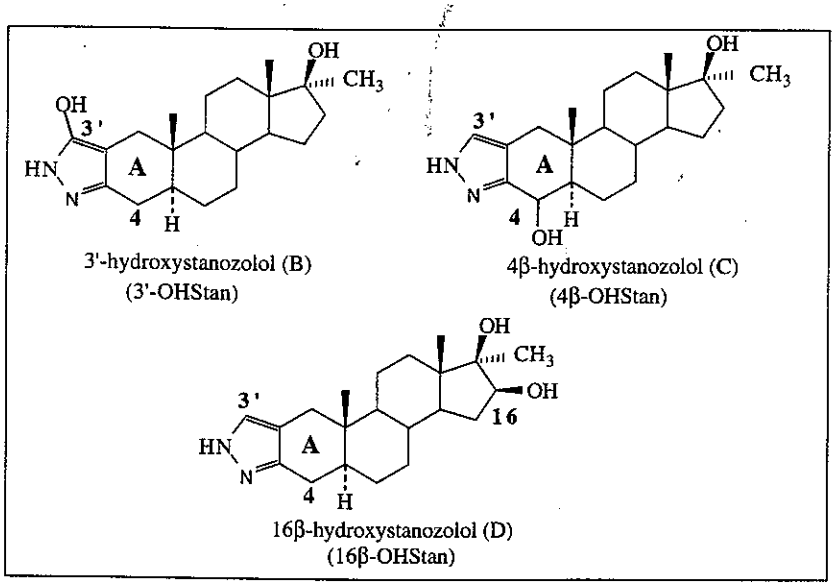


Fig 2: Main metabolites of stanozolol in man (SCHÄNZER and others, 1990)

Other authors used these metabolites in their effort to improve the GC-MS methodology for the determination of these compounds in doping control (MASSÉ and others, 1989) (CHOO and others, 1990). The use of Liquid Chromatography Tandem Mass Spectrometry (LC-MS-MS) for the identification of Stan and its major metabolites in human and equine urine was demonstrated by MUCK and HENION (1990). The long term detection and identification of Stan abuse in athletes was performed using High Resolution Mass Spectrometry (HRMS) in combination with Immunoaffinity Chromatography (IAC) clean-up. Using this technique the number of positives was increased considerably (SCHÄNZER and others, 1996).

In this investigation the metabolites of stanozolol in man, as obtained from the Doping laboratory of the „Deutsche Sporthochschule, Köln", GFR were tested out for their presence in cattle urine. In contrast to earlier experiments in sports doping, where Stan was administered orally, (MASSÉ and others, 1989 ; CHOO and others, 1990 ; MUCK and HENION, 1990 ; SCHÄNZER and others,

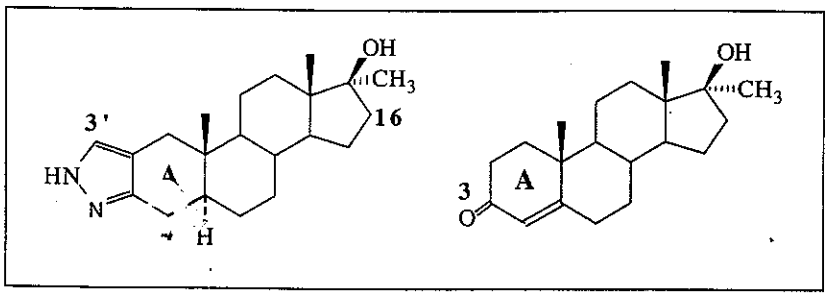


Fig 1: Formula of stanozolol in comparison with that of methyltestosterone

19[illegible]6), a Stan solution was injected intramuscularly to veal calves, urine samples were collected and analysed.

2. Experimental

2.1. Apparatus

The following apparatus were used: homogenisor (e.g. ultra-turrax T25), microwave oven (e.g. Zanker ZK1276), centrifuge (e.g. Sorvall RC-26), rotary vacuum evaporator (e.g. Bucchi R-114), waterbath (e.g. GFL), extraction flasks (250 and 500 ml), vacuum manifold (e.g. Vacubrand ME2), nitrogen evaporator (e.g. home made) and other types of evaporators (e.g. Speedvac SC210A), chromatographic columns and tanks, autosampler vials (e.g. Chromacol N8-07), Solid Phase Extraction (SPE) C18 columns (3 cc Baker 7020-03), standard laboratory equipment.

Platform mass spectrometer (Micromass UK Limited, Altrincham, UK) consisting of Hewlett Packard 6890 capillary gaschromatograph with 6890 autosampler and mass spectrometer with electron impact ionisation (EI+) : 70 eV

A model P4000 pump (TSP, San Jose, CA, USA) coupled to a LCQ Ion Trap Mass Analyser (Finnigan MAT, San Jose, CA, USA) with an electrospray interface and a Navigator 1.0, respectively. Symmetry C18 column (5 µm, 150 x 2.1 mm, Waters, Milford, USA).

2.2. Reagents and reference components

Most reference steroids were obtained from Steraloids (Wilton, N.Y., U.S.A.) or Sigma (St-Louis, MO, U.S.A.). Internal standards were d3-stanozolol (5α-androstane-17α-methyl-d_3-17β-ol (3,2-C) pyrazole) (Sigma 9271) (for LC-MSn). and 4α-hydroxystanozolol (4α-OHStan) (for GC-MS). The most important steroid used in this investigation is Stan (Sigma 7132). The metabolites of Stan were a generous gift of Dr. W. Schänzer of the „Deutsche Sporthochschule, Köln", GFR: 3'-hydroxystanozolol (3'-OHStan), 4ß-hydroxystanozolol (4ß-OHStan), 4α-hydroxystanozolol (4α-OHStan) and 16β-hydroxystanozolol (16ß-OHStan).

Helix Pomatia juice (Glucuronidase-sulfatase enzyme suspension) was obtained from Boehringer (Mannheim, Germany). Strombaject® (Stromba depot: an aqueous suspension of Stan; each dose contains 50 mg Stan in small pulverized particles) from Sanofi-Winthrop (Brussels, Belgium). MSTFA (N-Methyl N-trimethylsilyl-trifluoroacetamide) was from Macherey-Nagel (Düren, Germany) and TMSI (Iodotrimethylsilane) from Janssen Chimica (Geel, Belgium). DL-Dithiothreitol was purchased from Sigma-Chemie (Brussels, Belgium). MSTFA++ is prepared by dissolving 5‰ TMSI and a tip of a spatula point of dithiothreitol in MSTFA.

All solvents used were analytical grade from Merck (Darmstadt, Germany).

Stock solutions of anabolic steroids were prepared at 200 ng/µl in ethanol. Ten-fold dilutions of these stock solutions result in working solutions at a concentration of 20 ng/µl.

2.3. Clean-up procedures

Extraction

Ten ml urine was centrifuged at 2,700 g for 7 min and 3 ml acetate buffer 3 M (pH 4,6) was added. A SPE C18 column (3 cc Baker 7020-03) was conditioned with 5 ml methanol and 5 ml bidistilled water. The sample was brought onto a column and washed with 10 ml bidistilled water.

The column was eluted with 2 x 1 ml methanol and the eluate was evaporated to dryness under nitrogen. The sample was redisolved in 100 µl methanol, 5 ml acetate buffer 0.2 M (pH 4,6) and 50 µl Helix Pomatia juice was added. The sample was incubated overnight at 37 °C and afterwards centrifuged at 2,700 g for 10 min. The SPE columns were reconditioned with 5 ml methanol and 5 ml bidistilled water.

The sample was brought onto a column and washed with 5 ml bidistilled water, 5 ml methanol/water (20/80,V/V) and 2,5 ml hexane. The columns were dried for 1 min and eluted with 2 x 1,5 ml ethylacetate. The eluate was dried in a speedvac and dissolved in 500 µl ethanol followed by 5 ml water.

Purification on Immunoaffinity columns

The Immunoaffinity (IAC) columns (binding capacity to methyltestosterone 250 ng) (SCHÄNZER and others, 1996) was brought to ambient temperature and the conservation solution was removed. Afterwards 5 ml PBS buffer was added and the columns were rinsed with 10 ml water. The sample was brought onto the column and the column was rinsed with 10 ml water and 10 ml methanol/water (20/80,V/V). The column was eluted with 3 ml methanol/water (80/20,V/V).

2.4. Derivatisation for GC-MS

The final extract was derivatised to TMS enol-TMS ether (trimethylsilyl) derivatives with MSTFA++: the sample or 2,5 µl standard solution (50 ng) was transferred into an autosampler vial (700 µl) and dried under a nitrogen stream. MSTFA++ (40 µl) was added, the contents were mixed and incubated at 60°C during half an hour. Two µl (equivalent to 1,6 ng standard) was injected into the GC.

2.5. GC-MS conditions

GC-MS conditions: initial: 200 °C to 320 °C at 12 °C/min, Injector temperature : 300 °C (splitless injection 1,5 min), constant pressure of 12 kpa Column: HP5-MS (30 m x 0.25 mm ID., film thickness 0.25 µm).

Aquisition method: Selected Ion Monitoring on the ions given in table I.

The magnitude of the concentration of the metabolites was determined from a calibration curve of blank urine spiked with the metabolites and 4α-OHStan as internal standard (SCHÄNZER and others, 1996).

2.6. LC-MS conditions

The instrument was operated in MS/MS positive ion mode. The maximum injection time was set at 400ms with a total of 2 µscans per scan. An electrospray voltage of 4.5 kV and a capillary temperature of 220 °C were used provide a mobile phase. A methanol-1% aqueous acetic acid solution (70/30,V/V) was used as the mobile phase at a flow of 0.3 ml/min A standard mixture (ten nanogram each) of 3'-OHStan, 4β-OHStan, 16β-OHStan, Stan and Stan D3 (internal standard) was injected on column. Based on the retention times of an MS-full scan run the time axis was divided into two segments to continue in MS/MS-full scan. In the first segment the three metabolites eluted, in the second segment Stan and the internal standard coeluted. Therefore instead of further optimizing the LC-separation the second segment was subdivided into two scan events. Both compounds were separated mass spectrometrically.

segment 1: parent ion : 345; isolation width : 1; collision energy : 28%; full scan mass range : 100-350

segment 2: scan event 1: parent ion : 329.2; isolation width : 1; collision energy : 28%; full scan mass range : 100-350

scan event 2: parent ion : 332.2; isolation width : 1; collision energy : 29% full scan mass range : 100-350

2.7. Animal experiments

Two experiments were carried out:

A male calf (Holstein) of ca 40 kg (calf 1) was injected with a single intramuscular dose of 200 mg Stan (Sigma 7132) dissolved in 10 ml ethanol. Urine samples were collected for 3 days and frozen at - 24° C until analysis. Another animal (male, ca 40 kg, Holstein)(calf 2) was injected with 2 combined doses of Strombaject® (equivalent to 100 mg stanozolol). Urine samples were collected daily for 21 days and frozen at -24 °C until analysis

3. Results and discussion

3.1. Analysis of stanozolol: present situation

In Belgium anabolics are determined in different matrixes: injection sites, excreta as urine and faeces and tissue samples as kidney fat and meat. In injection sites stanozolol could be detected as well with HPTLC as with GC-MS (SMETS and others, 1991; BATJOENS and others, 1994). A survey of the hormones used in cattle fattening based on the analysis of Belgian injection sites demonstrated an increasing use of stanozolol from 1989 on to 1994 (VANOOSTHUYZE and others, 1994; HENDRIKS and others, 1994). In 1995 in 141 positive injection sites stanozolol was found 72 times (personal communication from Veterinary Inspection, 1997). However, the product was never detected in other matrices. Possible reasons for this phenomenon are: firstly that stanozolol is most probably metabolised very quickly so that the levels of the parent molecule

Table I : **Ions for Selected Ion monitoring of stanozolol metabolites by GC-MS**

Component	Ions
stanozolol	472 / 457 / 168 / 143
4β(α)-hydroxystanozolol	545 / 560 / 254 / 143
3'-hydroxystanozolol	560 / 545 / 520 / 254 /143
16β-hydroxystanozolol	560 / 381/ 231 / 218

Table II : **Ions for LC-MSn of stanozolol metabolites**

Component	Parent Ion (MH^+)	Daughter ions			
stanozolol	329	311		229	121
stanozolol-d3	332	314		232	124
4β-hydroxystanozolol	345	327	309		
3'-hydroxystanozolol	345	327		229	(159)
16β-hydroxystanozolol	345	327	309	227	159

in the different matrices examined are very low and secondly that the detection limit of the parent molecule with the HPTLC method is inferior to that of other anabolic steroids. Also with the clean-up of the HPTLC method (VERBEKE, 1979) a lower recovery for Stan (ca 10–20 %) in comparison with other steroids was obtained (De BRABANDER and others, 1997). Therefore a special clean-up based on immunoaffinity chromatography was used.

3.2. Immuno affinity chromatography

After SPE extraction the urine extracts were cleaned up by immunoaffinity chromatography (IAC) with an antibody raised against methyltestosterone which has a related structure to stanozolol (fig 1) (Van GINKEL, 1991). This immunoaffinity gel shows high cross reactivity to other 17β-hydroxy-17α-methyl steroids (SCHÄNZER and others, 1996) and can therefore be used for the isolation of stanozolol and its metabolites. The immunoaffinity column is very stable and can be used several times without any noticable loss in performance.

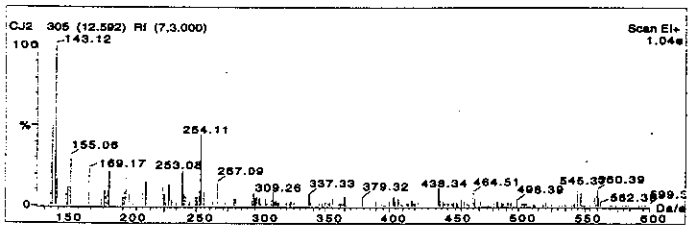
3.3. Detection of stanozolol metabolites

Both GC-MS and LC-MSn were used.

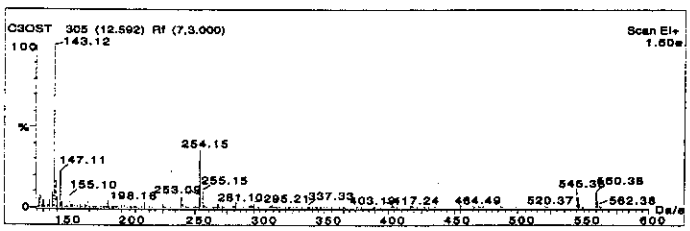
With GC-MS two of the urinary metabolites, obtained from Dr. W. SCHÄNZER were detected in the 4 urine samples taken from calf 1 after intramuscular injection but not in the blank. In fig 3 and 4 the full mass spectra of 3'-hydroxystanozolol and 16β-hydroxystanozolol are shown in comparison with those of the standards.

From these figures it can be seen that there is no doubt about the identity of both metabolites: for both more than 4 diagnostic ions are found at the correct retention time and in the correct ratios. However, it was remarked that the detection power of GC-MS for 16-OHStan is inferior to that of 3'-OHStan: for the injection of equal amounts of derivatised standards a much (ca 10 times) lower signal was obtained for 16-OHStan in comparison with 3'-OHStan. For 4-OHStan a response was found at the correct retention time but one of the diagnostic ions was lacking and therefore the data were considered as non relevant and not presented here.

The samples were also analysed by LC-MSn. With this technique the GC-MS results were confirmed. In fig 5 the MS2 spectra of stanozolol and the three metabolites were given. In the LC chromatogram 16-OH-

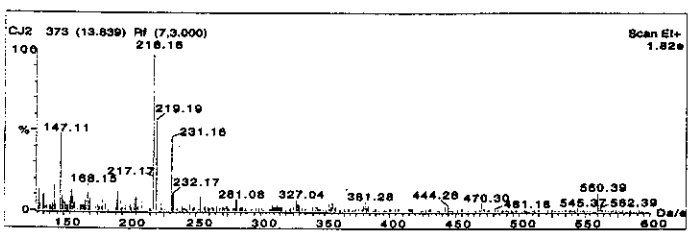


A: 3'-OHStan in urine

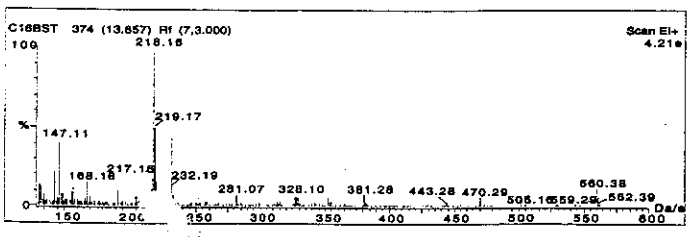


B : standard 3'-OHStan

Fig. 3: Mass spectrum of 3'-hydroxystanozolol in calve 1 urine (A) in comparison with that of the standard (B).

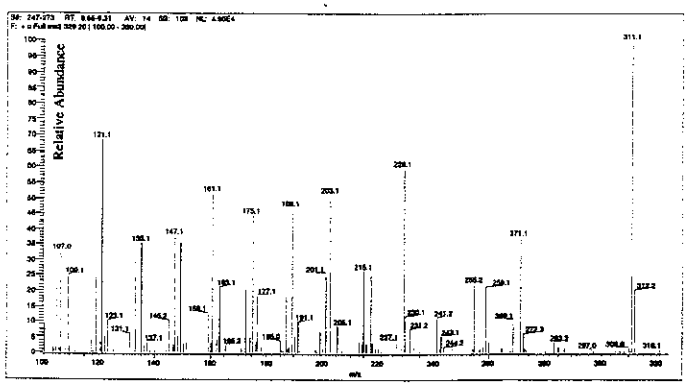


A : 16-βOHStan in urine

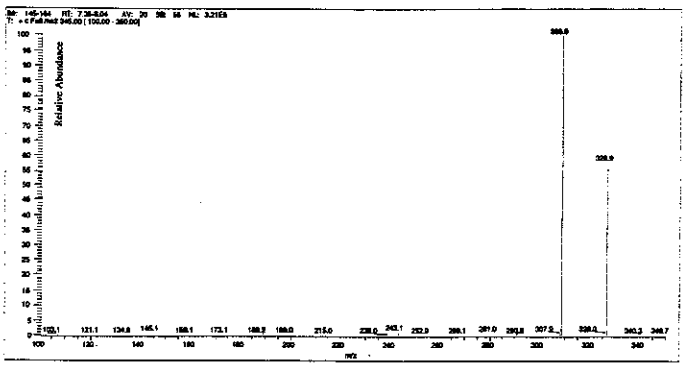


B: standard 16-βOHstan

Fig 4: Mass spectrum of 16ß-hydroxystanozolol in calve 1 urine (A) in comparison with that of the standard (B).



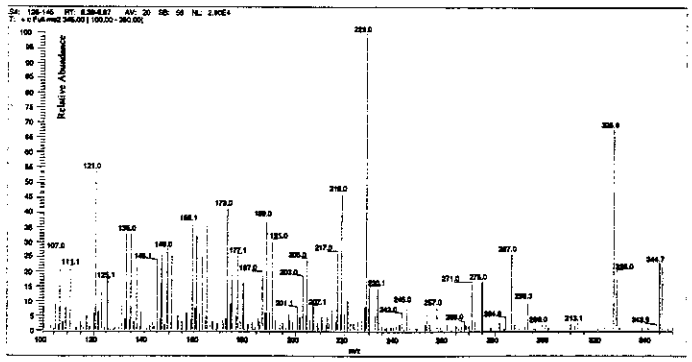
A: MS2 of Stanozolol



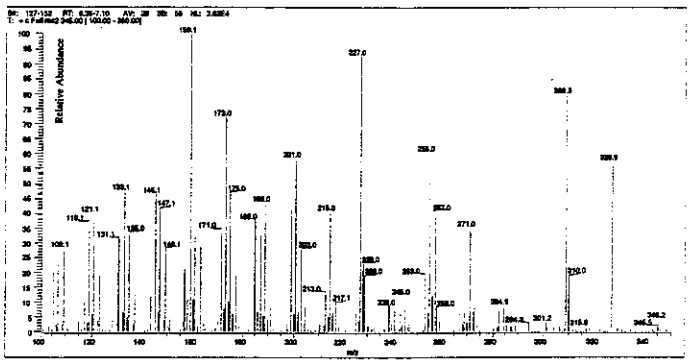
B: MS2 of 4ß-OHStan

Fig 5: MS2 spectra of stanozolol and the three metabolites.

Stan and 3'-OHStan coelute. In the urine of calf 1 a large peak was observed at the correct retention time (3.96 ; fig 6) showing the correct MS2 spectrum of 16-OHStan. The presence of a smaller amount of 3'-OHStan is masked by the larger amount of 16-OHStan because the typical ion 229 for 3'-OHStan is also present (although in a much lower abundance)

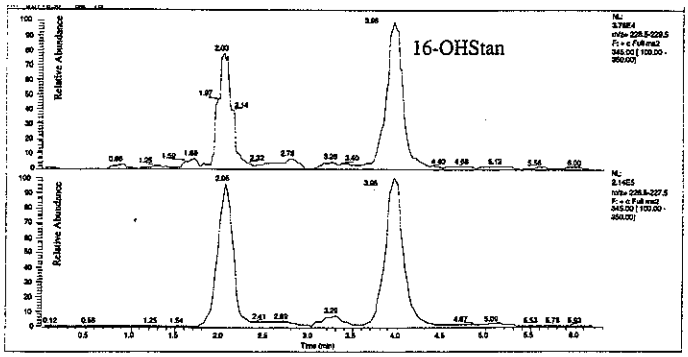


C: MS2 of 3'-OHStan



D: MS2 of 16β-OHStan

Fig 5: MS2 spectra of stanozolol and the three metabolites.



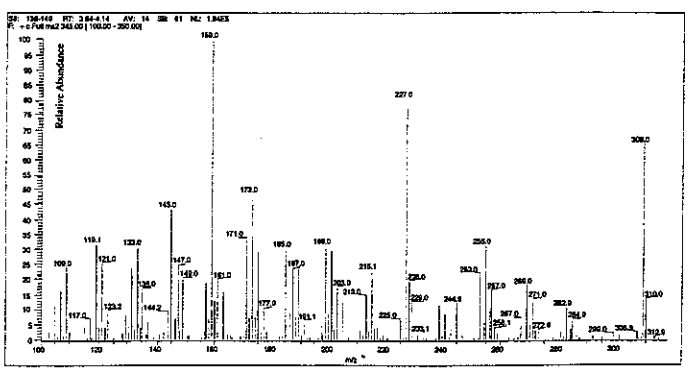


Fig 6: Chromatogram and MS2 spectrum taken at the retention time of 16OHStan (3'OHStan).

in the MS2 spectrum of 16-OHStan. In fig 6 the chromatogram and the MS2 spectrum taken at that retention time (3.96) are given. Also other peaks (e.g. 2.05) , possibly caused by non-identified metabolites were observed.

In LC-MSn the detection power of 16-OHStan is not inferior to that of 3'-OHStan: the LC-MSn signals obtained after injection of equal amounts of standards are of the same magnitude. The necessity for derivatisation of the sample for GC-MS may explain this phenomenon. Steric hindrance of the hydroxy groups on the 16 and 17 place may be the reason for an incomplete derivatisation. The detection of several metabolites is in concordance with the findings of MUCK and HENION (1990) and TEALE and others (1994) for horse urine.

In the urines taken from calf 2 (injected with 100 mg Strombaject®) the excretion of the two metabolites 16-OHStan and 3'-OHStan was followed in function of time using Selected Ion Monitoring (SIM) in GC-MS. The magnitude of the concentration of the metabolites was determined from a calibration curve of blank urine spiked with the metabolites and 4α-OHStan as internal standard (SCHÄNZER and others, 1996). These results were also confirmed qualitatively by LC-MS on four samples.

In fig 7 the evolution of the urinary excretion of the metabolites is given. It is shown clearly that the two metabolites appear in the urine after ca 3 days and are detectable with varying concentrations during ca 21 days after administration of Strombaject®.

4. Conclusion

In the urine of veal calves, treated with a single intramuscular (relatively high) doses of 200 mg stanozolol and 100 mg Strombaject® respectively, the stanozolol metabolites 3'-hydroxystanozolol and 16β-hydroxystanozolol were qualitatively detected by Gas Chromatography Mass Spectrometry (GC-MS) and Liquid Chromatography Tandem Mass Spectrometry (LC-MSn) using immunoaffinity chromatography (IAC) as a clean-up. These molecules are two of the metabolites of stanozolol in man, which were obtained from the doping laboratory of the „Deutsche Sporthochschule Köln", Cologne, GFR. It was found that after injection of Strombaject® the excretion of the metabolites was slow and could be used for the long term detection of the abuse of the anabolic agent.

Abstract

Stanozolol is an anabolic steroid which is often found in injection sites sampled from cattle at the slaughterhouse but has never been detected in tissues and excreta of animals during regulatory control. Most probably the difference in structure of stanazolol and the other steroids is the cause of this phenomenon. In sports doping (man and horseracing) stanozolol was subject for abuse too and became the subject of several research groups.

In this investigation veal calves were treated with intramuscular doses of stanozolol. In the urine of these calves the stanozolol metabolites 3'-hydroxystanozolol, and 16β-hydroxystanozolol were detected. These are two of the known urinary metabolites of stanozolol in man, which were obtained from the doping laboratory of the „Deutsche Sporthochschule Köln", Cologne, Germany.

Zusammenfassung

Stanazolol ist ein anabolisches Steroid und wird bei einer Kontrolle im Schlachthaus oft an der Spritzstelle bei Rindern bemerkt. Während gerichtlicher Kontrollen wurde dieses Produkt weder in Gewebe noch in Exkrementen nachgewiesen. Dies liegt höchstwahrscheinlich am großen Unterschied der chemischen Strukturen von Steroiden und Stanazolol.

Im Sport (bei Menschen wie auch bei Reitpferden) ist Stanazolol als Dopingmittel bekannt und wurde durch verschiedene Forschungsgruppen untersucht.

Bei unserem Versuch wurden Kälber mit Stanazolol intramuskulär gespritzt. In deren Urin wurden zwei Metaboliten nachgewiesen: 3'Hydroxystanazolol und 16β-Hydroxystanazolol. Im menschlichen Urin sind es zwei bekannte Metaboliten von Stanazolol, die uns vom Laboratorium für Doping-Kontrolle der Deutschen Sporthochschule Köln zur Verfügung gestellt wurden.

Acknowledgements

The authors are indebted to Dr. W. SCHÄNZER (Cologne) for the generous donation of the stanozolol metabolites and the scientific support.

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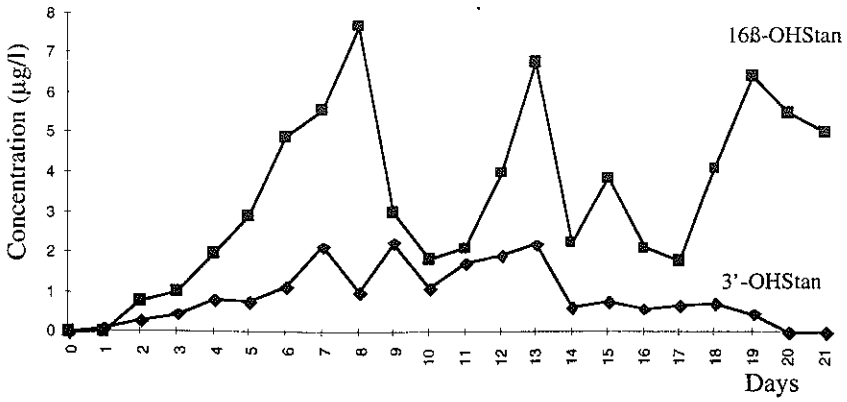

Fig 7: Evolution of the concentration of the metabolites of stanozolol in calve 2 urine in function of time (◊ : 3'OHStan ; • : 16βOHStan)

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