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17α-Ethyl-5β-estrane-3α,17β-diol, a biological marker for the abuse of norethandrolone and ethylestrenol in slaughter cattle

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Abstract

The metabolism of the illegal growth promoter ethylestrenol (EES) was evaluated in bovine liver cells and subcellular fractions of bovine liver preparations. Incubations with bovine microsomal preparations revealed that EES is extensively biotransformed into norethandrolone (NE), another illegal growth promoter. Furthermore, incubations of monolayer cultures of hepatocytes with NE indicated that NE itself is rapidly reduced to 17α-ethyl-5β-estrane-3α,17β-diol (EED). In vivo tests confirmed that, after administration of either EES or NE, EED is excreted as a major metabolite. Therefore, it was concluded that, both in urine and faeces samples, EED can be used as a biological marker for the illegal use of EES and/or NE. Moreover, by monitoring EED in urine or faeces samples, the detection period after NE administration is significantly prolonged. These findings were further confirmed by three cases of norethandrolone abuse in a routine screening program for forbidden growth promoters.

Keywords: Steroids; 17α-Ethyl-5β-estrane-3α,17β-diol; Norethandrolone; Ethylestrenol

1. Introduction

At present, the abuse of anabolic steroids in slaughter cattle is mainly demonstrated by the detection of the parent compounds, excreted in urine and faeces or by their presence in kidneyfat. However, many anabolic steroids are extensively metabolised and the parent compounds are often not detectable, or only shortly after administration [1–4]. This is clearly shown by the screening method for the illegal use of anabolics such as 4-chlorotestosterone acetate (CLTA), nortestosterone and trenbolone, where the forbidden products can be demonstrated by the presence of unique metabolites [5–12]. Unfortunately, the available information on the relative importance of individual metabolites in the overall biotransformation and subsequent elimination of

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growth promoters in cattle, is still scarce. It is therefore probable that the applicability of the analysis of metabolites for the regulatory control of anabolic steroids is not yet fully exploited.

Liver tissue plays a major role in the metabolism of xenobiotics. This is due to the presence of several metabolising enzyme systems, including a group of membrane-bound mixed-function oxidative enzymes, the cytochrome P450 system (CYP450). Oxidations by these microsomal mixed-function oxidase systems are major pathways in the metabolism of numerous endogenous and exogenous compounds [13–15]. The hepatic oxidative metabolism reflects the activity of several cytochrome P450 isoenzymes [16]. In vitro biotransformation studies using isolated liver tissue preparations have therefore become an important alternative for in vivo metabolism studies [13].

Microsomal fractions of liver or other organs, containing vesicular isolates of the endoplasmic reticulum are preferably used to study specific biotransformation steps or to detect and/or produce intermediates of metabolic pathways [13,14]. The main advantages of microsomal preparations are simplicity, relatively low cost and prolonged stability [13,17].

Whole liver cells can also be used to study the formation of metabolites. They have an important advantage as compared to subcellular fractions because more accurate quantitative studies of the different metabolic routes and the formation of cytosolic metabolites can be monitored directly within the system. A serious limitation however is the rapid loss of differentiation of isolated liver cells [13].

Due to its highly hydrophobic character, ethylestrenol (EES) is strongly retained by reversed-stationary phases, which implicates a different behaviour during solid-phase extraction and high-performance liquid chromatography (HPLC) clean-up of steroids for residue analysis. To elute EES from the C_{18} stationary phases used both in the clean-up of urine samples and the HPLC fractionation of urine and faeces samples 100% MeOH or ethyl acetate was necessary as solvent. So, the inclusion of EES in the multi-residue screening for anabolic steroids using strong hydrophobic stationary phases in the clean-up is impossible. Together with EES many matrix compounds are eluted. This matrix does not allow a reproducible derivatisation nor a sensitive detection of EES by gas chromatography–mass spectrometry (GC–MS). Therefore, the majority of current multi-residue methods fail to detect EES. Additionally, from a pharmacokinetic point of view, it is most likely, that EES uptake in the blood stream after intramuscular or subcutaneous administration is very slow. Due to its hydrophobicity EES most likely will accumulate in fat tissue. Last year, several cases of norethandrolone (NE) abuse were detected along the national screening program. In excreta, always low amounts of NE were found. Many samples did not meet the identification criteria and had to be reported as negatives. As a consequence, the additional identification of metabolites of NE would add to the reliability of the multi-residue screening. It is even possible that the detection of a specific metabolite clearly related with the mother substance can prolong the detection of the abuse. The availability of a valid screening method for EES and a longer detection period for NE are of importance for regulatory controls.

In the present study, both monolayer cultures of intact liver cells and microsomal preparations were used to examine the metabolic pathways of EES and NE. CLTA, of which the in vivo biotransformation has been studied in our laboratory, was used as a reference substance to evaluate the in vitro systems. Since the identification of unique biological markers for the illegal use of EES and NE was the main purpose of the study, the conclusions of the in vitro experiments were verified by in vivo experiments and by case studies in routine control procedures.

2. Experimental

2.1. Experimental animals and samples

Microsomes were prepared from livers of four animals, of which two adult females (Friesian–Holstein and Meuse–Rhine–Yssel) of approximately 600 kg and a cow and a bull, both of mixed breed and 36-weeks old. Hepatocytes were isolated from livers from two Friesian–Holstein bulls of about one year old.

Thirty six mg EES was administered intramuscularly to a 12-month-old heifer ($\pm$100 kg) and 250 mg NE to a 15-month-old heifer ($\pm$300 kg). Fifty mg EES were dissolved in 10 ml and 250 mg NE in

12 ml of pharmaceutical grade Miglyol–*N*-methyl-2-pyrrolidone (1:1, v/v) (Sigma, St. Louis, MO, USA). Blank samples (=before treatment) of urine and faeces were collected for two days. Samples of urine and faeces were collected at 13, 37, 62, 93, 109, 134 h and 7, 14 and 21 days after the injection of EES and at 12, 36, 60, 84, 110, 160 h and 14 and 21 days after injection of NE. These samples were kept frozen at −20°C until analysis.

In a previous in vivo study on the metabolism of CLTA all samples of urine were stored at −20°C. Under these conditions the metabolites formed were found to be stable [5].

Thirteen urine and faeces samples of untreated animals were available at different laboratories: the Belgian reference laboratory (Wetenschappelijk Instituut voor Volksgezondheid–Louis Pasteur, Brussels, Belgium) and TNO Nutrition and Food Research Institute (Zeist, The Netherlands).

2.2. Chemicals and standards

All reagents and solvents were of analytical grade. Ultrapure water was derived from a Waters Milli-RO water purification system (Millipore, Bedford, MA, USA). HPLC-grade methanol was obtained from Labscan (Dublin, Ireland), diethyl ether and *tert*.-butylmethyl ether from Merck (Darmstadt, Germany) and chloroform and dichloromethane from UCB (Brussels, Belgium). Tri-Sil-TBT (Pierce, Rockford, IL, USA), which consists of trimethylsilylimidazole–*N*, *O* - bis - (trimethylsilyl)-acetamide–trimethylsilylchlorosilane (3:3:2, v/v/v) was used for trimethylsilylation. Ethoxyamine-hydrochloride (Fluka, Buchs, Switzerland) was used as a 2% solution in pyridine (Sigma, St. Louis, MO, USA). Succus *Helix pomatia* containing 105 Fishman units/ml glucuronidase and 106 Roy units/ml sulphatase was obtained from Sepracor (Villeneuve la Garenne, France). Bond-Elut C_{18} 3 ml, Bond-Elut silica 3 ml, Bond-Elut amino 1 ml and Chem-Elut 20 ml columns were from Varian (Harbor City, CA, USA). All anabolic steroids were obtained from the Belgian reference laboratory, Wetenschappelijk Instituut voor Volksgezondheid–Louis Pasteur (Brussels, Belgium). A pure standard of a NE metabolite described as 17α-ethyl-5β-estrane-3α,17β-diol (EED) was provided by the Institut für Biochemie, Deutsche Sporthochschule Köln, Germany. All reagents and chemicals for the preparation and incubation of microsomes were from Sigma (St. Louis, MO, USA), except glucose-6-phosphate-dehydrogenase (grade II, from yeast) (Boehringer Mannheim, Mannheim, Germany). Powdered William's Medium E, phenol red (10.7 mg/l), BSA (fraction V), glutamine, hydrocortisone-21-hemisuccinate sodium, bovine insulin, gentamicin sulphate and collagenase (type IV) used for the isolation and incubation of hepatocytes were from Sigma and newborn calf serum from Flow Labs. (Irvine, UK). All reagents used for the chemical synthesis of the metabolites were from Aldrich (St. Louis, MO, USA).

2.3. Small scale synthesis of 17α-ethyl-5β-estrane-3α,17β-diol

The synthesis of 17α-ethyl-5β-estrane-3α,17β-diol (EED) was performed on a mg scale, following the method described by Schänzer and Donike [18]. NE was reduced in methanol–6 mol/l sodium hydroxide (20:1, v/v) using in situ generated hydrogen, by slowly adding concentrated hydrochloric acid 12 mol/l to an aqueous solution of 12% (m/v) sodium borohydride. To stabilise the sodium borohydride solution, sodium hydroxide 0.2 mol/l was added. Palladium (10%) on charcoal was used as a catalyst. The reduction under hydrogen atmosphere was performed in a diazomethane generator. After 15 min of vigorous stirring at room temperature, the reaction mixture was diluted with water and extracted with *tert*.-butylmethyl ether. The organic layers were evaporated to dryness. 17α-Ethyl-5β-estrane-17β-ol-3-one, the major reaction product, was further reduced to EED with lithium aluminium hydride. The reaction yield of 17α-ethyl-5β-estrane-3α,17β-diol was approximately 73%. Three mg was isolated by HPLC and identified by ^{1}H nuclear magnetic resonance (NMR) and MS and by comparing the GC–MS spectrum with a reference standard.

2.4. In vitro experiments

2.4.1. Preparation of microsomes

Fifty-gram aliquots of liver were frozen in liquid nitrogen within 15 min after the slaughter of the animal. Homogenisation was performed in 1.15% (w/v) KCl–0.1 mmol/l EDTA using a Potter–Elve-

jehem PFTE glass homogeniser. The homogenate was centrifuged at 10 000 *g* for 25 min at 4°C and the supernatant was centrifuged at 100 000 *g* for 75 min at 4°C. The resulting pellet was suspended in homogenising buffer and centrifuged at 100 000 *g* for 75 min. The pellets were suspended in 0.1 *M* phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 0.1 mmol/l EDTA, and were stored at −80°C. Protein concentrations were assessed by the method of Lowry et al. using bovine serum albumin (BSA) as a standard [19].

2.4.2. Isolation of hepatocytes

The caudate liver lobe (ca. 100 g of tissue) of a bull was obtained within 10 min after bleeding. To remove the blood, the tissue was rinsed with ice-cold Euro-Collins buffer (15 mmol/l KH_2PO_4, 42 mmol/l K_2HPO_4, 15 mmol/l KCl, 1 mmol/l $NaHCO_3$, 0.2 mol/l glucose) supplemented with 1 mmol/l EGTA. Hepatocytes were isolated according to Van 't Klooster et al. [13], based on the method of Seglen [20].

2.4.3. Incubation of steroids with microsomal preparations

Steroids (50 μg) were incubated for 30 min at 37°C in 1 ml phosphate buffer (50 mmol/l, pH 7.4) containing $MgCl_2$ (30 mmol/l), NADP (1 mmol/l), glucose-6-phosphate (10 mmol/l), glucose-6-phosphate dehydrogenase (2 U/ml) and a microsomal preparation (±600 μg protein).

2.4.4. Incubation of steroids with isolated hepatocytes

Cells were cultured at a density of $4 \cdot 10^6$ cells/60 mm culture dish (Greiner, Alphen a/d Rijn, The Netherlands) in 4 ml Williams' E supplemented with 4% newborn calf serum, 1.67 mmol/l glutamine, 50 μg/ml gentamicin-sulphate, 1 μmol/l hydrocortisone, 1 μmol/l insulin, 0.5 mmol/l $CaCl_2$ and 0.5 mmol/l $MgCl_2$. Cells were incubated for 4 h in a humidified atmosphere of air (95%) and CO_2 (5%) at 37°C. Then the medium was replaced by medium without serum, $CaCl_2$ and $MgCl_2$. After an incubation of 20 h, the hepatocytes were incubated with the steroid under study for 6 and 24 h at final concentrations of 100 μmol/l and 10 μmol/l of the steroid. Stock solutions of 100 mmol/l an 10 mmol/l of the steroids were prepared in MeOH (final concentration of methanol in the incubation medium 0.1%). The incubated media were kept at −20°C until GC–MS analysis.

2.5. Sample preparations

2.5.1. Isolation of steroids from microsomal incubation mixtures

Liquid–liquid extraction of the metabolites was performed by adding 6 ml of diethyl ether and 6 ml of *tert*.-butylmethyl ether to 1 ml of the incubation mixture. The tubes were capped and shaken by hand. After freezing the aqueous layer, the organic solvent was removed, and evaporated under a gentle stream of nitrogen. The extracts were analysed by HPLC and GC–MS as described below.

2.5.2. Isolation of steroids from liver cell incubation mixtures

Two ml of the incubation mixture of hepatocytes was adjusted to pH 5.2 with acetate buffer (0.2 mol/l, pH 4.8). Fifty μl of Succus *Helix Pomatia* were added and the mixture was subsequently incubated overnight at 37°C. After incubation the mixture was applied on a Chem-Elut column and steroids were eluted by subsequent addition of 5 ml of *tert*.-butylmethyl ether and 5 ml of chloroform. All extracts were analysed by GC–MS, as described below.

2.5.3. Clean-up of urine and faeces samples

The details of the extraction procedure for the aliquots of 50 ml of urine were described earlier [5,21,22]. The extraction of the faeces samples was performed based on a method described previously [23]. Briefly, to 20 g of faeces equilenin (500 ng) was added as internal standard. To homogenise the faecal substance for extraction with diethyl ether, 13 ml Ultrapure water was added. This suspension was extracted subsequently for 2 h with 50 ml of diethyl ether and for 1 h with 50 ml diethyl ether. To separate the aqueous suspension from the diethyl ether layer, the samples were centrifuged for 10 min at 1500 *g*. The ether layers were always decanted over a cotton-wool plug in a round-bottom flask to remove solids. The diethyl ether was removed under vacuum at 60°C and the remainder was dissolved in 8 ml methanol. Four ml of a 0.33 mol/l phosphoric

acid solution were added to remove the fat. This solution was filtered before pouring it on a Chem-Elut column. The column was washed with hexane and eluted with dichloromethane. Subsequently the dichloromethane was removed under vacuum and the residue dissolved in 600 μl chloroform. Six ml of hexane was added to the solution for a further clean-up of the extracts on a Bond-Elut silica column on top of an amino column, as described for urine samples [5,21,22].

2.5.4. HPLC fractionation of urine and faeces extracts

The modular HPLC system was equipped with a high-pressure pump (Spectra Physics, San Jose, CA, USA; Model 231 with Rheodyne 7010 injection valve), a variable-wavelength monitor (LKB, Bromma, Sweden; Model 2151 operating at 244 nm), a fraction collector (Gilson Model 202, Villiers-le-Bel, France) and a recorder (Shimadzu, Kyoto, Japan; Chromatopack CR-1B). The HPLC column was a 250×10 mm I.D. stainless steel tube packed with 5 μm silica (ODS Ultrasphere, Beckman, Fullerton, CA, USA). Methanol–water (73:23, v/v) was used as mobile phase for isocratic elution at a rate of 2.5 ml/min. The collector was operated in the time mode. To redissolve the dried extracts, 250 μl of a methanolic solution of naphthalene (0.20 mg/ml) and 100 μl of Ultrapure water were used. This sample was fractionated in eight fractions and collected with time windows, relative to naphthalene as internal standard, ranging from 0.46 to 0.61 (fraction I), 0.61 to 0.73 (fraction II), 0.73 to 0.86 (fraction III), 0.86 to 1.04 (fraction IV), 1.04 to 1.24 (fraction V), 1.24 to 1.44 (fraction VI), 1.44 to 1.65 (fraction VII), 1.65 to 1.85 (fraction VIII) [5,24,25]. All fractions were dried separately under a stream of nitrogen at 60°C and dissolved in 500 μl methanol. Two hundred fifty μl was dried in a GC–MS vial and derivatised.

2.6. HPLC analysis

For the analysis of microsomal incubation mixtures of NE and 4-chlorotestosterone (CLT) a Hewlett-Packard 1050 system equipped with a HP 61306 diode array detector was used. CLT, NE and their hydroxylated and oxidised metabolites were detected at 244 and 254 nm. The mobile phase for isocratic elution was methanol–water (67:33, v/v) at a flow-rate of 1 ml/min. The HPLC column was a 250×4.6 mm I.D. stainless steel tube packed with 5 μm silica (Chromspher C_{18}, Chrompack, Middelburg, The Netherlands).

The extraction recovery was tested by comparing the peak-area ratios of the studied steroids with the peak-area of progesterone, used as internal standard, obtained from respectively aqueous standards and fortified incubation mixtures. All microsomal preparations were heat inactivated at 80°C for 5 min. Calibration curves were obtained by spiking heat inactivated microsomal preparations with standards at concentrations ranging from 10 ng/μl to 1 μg/μl.

2.7. Gas chromatography–mass spectrometry

2.7.1. GC–MS conditions

The GC–MS analyses were performed on a GCQ Finnigan system (San Jose, CA, USA) or on a Varian Saturn I system (Walnut Creek, CA, USA). The GC column used was a DB-5 MS 30 m×0.32 mm I.D. fused-silica column with 0.25 μm film thickness (J&W, Folsom, CA, USA). Temperature settings were as follows: injector at 260°C, transfer line 275°C, ion source 200°C, oven program: initial temperature: 50°C for 20 s, 50°C to 190°C at 50°C/min, 190 to 320°C at 4.6°C/min, 320°C for 4 min. The carrier gas was helium at a gas velocity of 40 cm/s for the GCQ and 1 ml/min for the Saturn I. Aliquots (1 μl) were injected on a split–splitless injector in the split mode. The preparation of the ethoxime-trimethylsilyl (EOTMS) derivatives was described earlier in detail [5].

2.7.2. Data analysis

To identify the metabolites, a reversed library search method was used. For a positive identification of NE and EED, the same criteria were used as described earlier [5].

Semi-quantitative results for NE and EED were obtained by measuring the peak area ratio of a selected fragment ion to the area of one particular fragment ion of the internal standard (5 ng/μl): m/z 360 of 5α-androstan-3α-ol-17-one and m/z 242 of 5α-estrane-3β,17α-diol. The latter was used as inter-

nal standard to analyse the EED fraction V. 5α-Androstan-3α-ol-17-one was used as internal standard for all other fractions. Each analyte was quantified in triplicate using three different fragment ions. The masses *m/z* 417, 327 and 298 of the EOTMS derivative of NE and the masses *m/z* 421, 331 and 241 of the trimethylsilyl (TMS) derivative of EED were used to quantify. To calibrate the instrument direct control samples of 10, 5, 2, 1 and 0.5 ng/μl were injected.

2.7.3. Quality control of the overall methods

The GC–MS and high-performance thin-layer chromatography (HPTLC) methods and the extraction recoveries for each batch of 10 samples were controlled by the simultaneous processing of a blank sample spiked at 2 ng/ml for urine and 10 ng/g for faeces. Equilenin was added at 10 ng/ml to urine samples and 25 ng/g to faeces samples as internal standard to control for every sample of the animal experiment the general extraction recovery [5].

2.8. High-performance thin layer chromatography

For the HPTLC screening of routine samples the 4×4 mode, according to De Brabander et al. [26], was used. Briefly, 8 μl of the HPLC fractions IV and V were applied to a 10×10 cm silica gel 60 plate (Merck). Chromatography was carried out in two dimensions using chloroform–acetone (27:3, v/v) as solvent I and cyclohexane–ethyl acetate–ethanol (18:12:0.75, v/v/v) as solvent II. For the staining and detection of NE a 5% sulphuric acid solution in acetic anhydride [27] was used. This allows the detection of NE at concentration levels of 2 ng/ml in urine and 5 ng/g in faeces.

2.9. ^{1}H NMR spectroscopy

The ^{1}H-NMR spectrum was recorded on a Varian system at 400 MHz. The spectrum was recorded at ambient temperature as a 12 mmol/l solution in $C^{2}HCl_{3}$. Chemical shifts were expressed in parts per million (ppm) relative to tetramethylsilane.

3. Results and discussion

3.1. In vitro experiments

Since the in vivo biotransformation of EES and NE in cattle is poorly understood, the metabolic conversion was studied in vitro, using liver microsomes and isolated hepatocytes. Previously the urinary excretion products of CLT in cattle have been characterised in our laboratory [5]. Therefore, CLT was used as a reference compound. This made it possible to compare, on the one hand, the in vitro results obtained by different techniques, and on the other hand, to compare the in vitro metabolism with the in vivo metabolism. Moreover, the isotope ratios of the chlorine group, present in the CLT molecule facilitated the location of formerly unknown chlorinated metabolites.

The three major in vivo biodegradation products of CLT, i.e., 4-chloroandrost-4-ene-3,17-dione (CLAD), 4-chloroandrost-4-ene-3α,17β-diol and 4-chloroandrost-4-ene-3-ol-17-one, were also present in microsomal liver preparations and incubations with isolated hepatocytes. Although epimerisation is a common in vivo biotransformation route, no 17α-CLT was found [5,28]. Only a trace of an important hydroxylated urine metabolite, 4-chloroandrost-4-ene-3ξ,17ξ,x-triol, was detected in vitro. In addition, new compounds, not yet described in the urine of CLTA-treated animals, were present in the microsomal preparations yielding mass spectra with similar electron impact (EI) fragmentation patterns as some of the hydroxylated metabolites found in cattle urine, but eluting at different retention times.

Incubation of microsomal liver preparations from four different animals treated with EES, revealed NE as the major biotransformation product of EES, as shown in Fig. 1A. Several minor peaks were tentatively assigned to hydroxylated analogues. Considering its strong hydrophobic nature, oxidation is of major importance to eliminate EES from the body [29,30].

However, in comparison with CLT, the conversion of EES by microsomes was extremely low. This is illustrated in Table 1, which compares the conversion of CLT into CLAD, one of the four major in vitro metabolites of CLT, with the conversion of

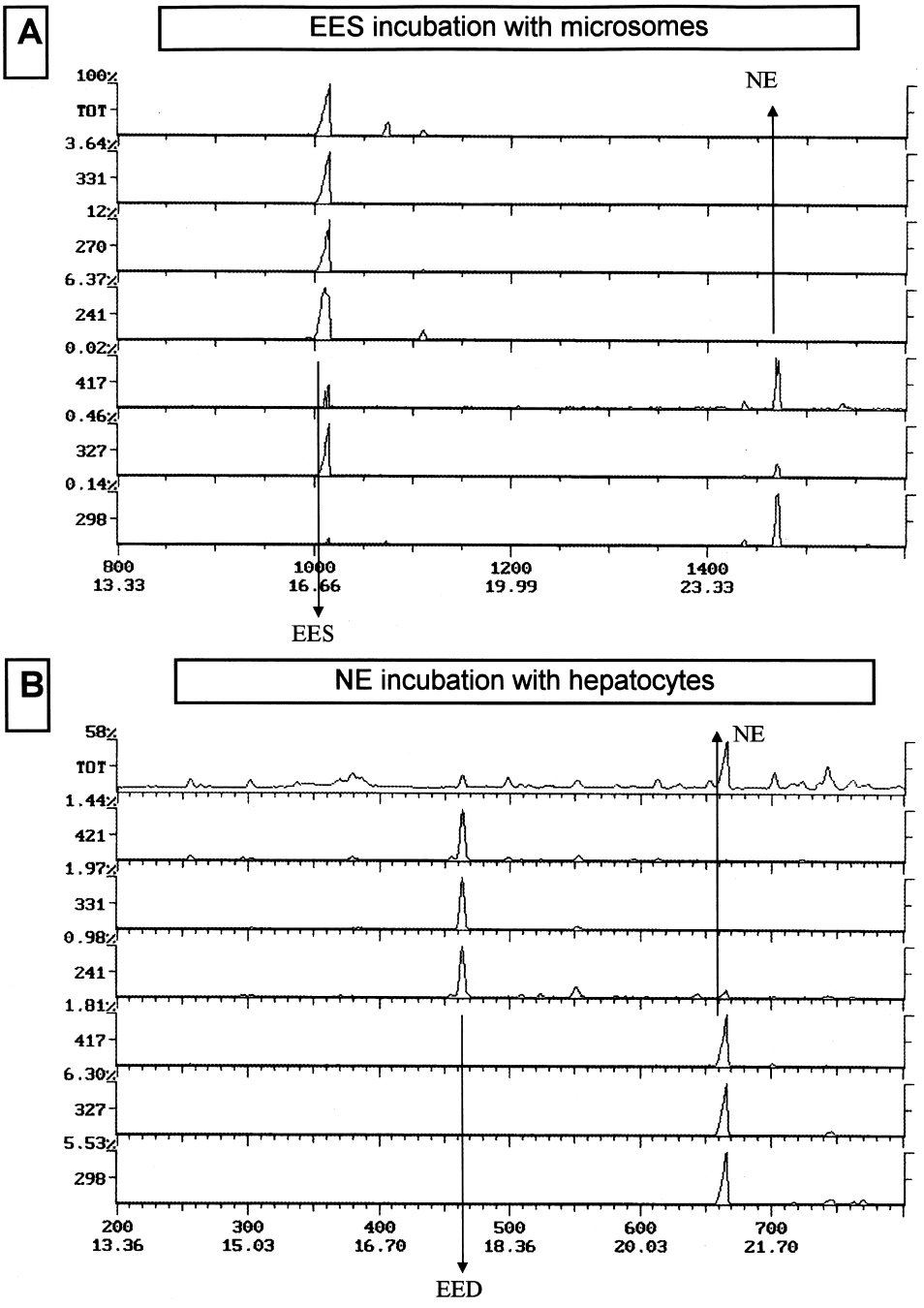

Fig. 1. (A) Total ion current and single ion chromatograms are shown for the three most important diagnostic ions of EES and NE, pointing towards NE as most important metabolite of EES after incubation with liver microsomal preparations (recorded on a Varian Saturn I system). (B) EED as the major in vitro metabolite after a 6 h incubation of NE with isolated hepatocytes. Total ion current and ion chromatograms for the three most important diagnostic ions of EED and NE (recorded on a Finigan GCQ system).

Table 1
Comparison of the metabolic activities of four microsomal preparations

Batch code	Conversion CLT (%)	Yield	
		CLAD[a] (%)	NE (%)
951101	55	5.8	1.8
951102	33	3.4	1.2
960305	45	5.1	1.6
960306	14.3	1.75	0.8

[a] CLAD used as a reference for the activity of the cytochrome P450.

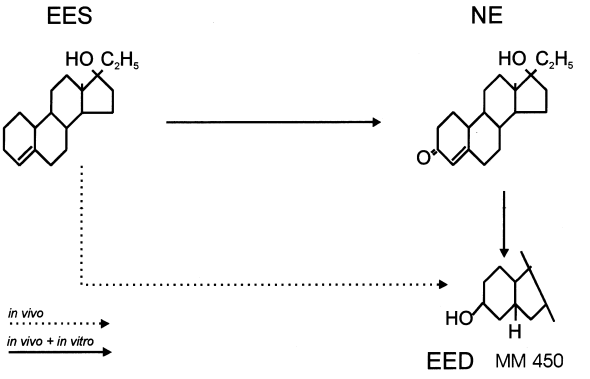

Fig. 3. The in vitro metabolic pathway of EES and NE to EED.

EES into NE, the only major in vitro metabolite of EES. The percentage of CLT consumption and its recovery as CLAD, determined by means of HPLC–UV, were considered to reflect the metabolic activity of the microsomal preparations for the steroid.

Accurate measurements of the EES consumption by HPLC–UV was hampered by its strong hydrophobic nature and its low UV absorbance, even at 210 nm. Therefore, the metabolic conversion rate of EES into NE by the four separate batches of microsomes was estimated by GC–MS analysis of NE. A calibration curve was established by analysing heat-inactivated microsomal preparations (60°C for 10 min), spiked with increasing amounts of NE.

To study the biodegradation of NE, NE itself was used as substrate in an incubation experiment. Similarly to EES, only hydroxylated products were tentatively identified while no further reduction products were detected. Additional experiments on the metabolism of NE were performed with isolated liver cells. To identify possible intermediates, NE was incubated for 24 h at 100 μmol/l and 10 μmol/l, and for 6 h at 100 μmol/l. The major compound, as revealed by GC–MS, was 17α-ethyl-5ξ-estrane-3ξ,17β-diol, which was not observed in the microsomal preparations incubated with NE. A typical chromatogram and the electron impact spectrum of this compound are shown in Figs. 1B and 2A. The metabolite was further characterised as EED as described below.

In summary, studies with microsomal preparations provided evidence that EES is mainly transformed into NE, with only minor oxidation products. Incubations with whole liver cells revealed that EED is the most prominent metabolite of NE. This allowed us to outline an hypothetical metabolic pathway of EES and to assign a central role to NE, which is easily converted into EED (Fig. 3).

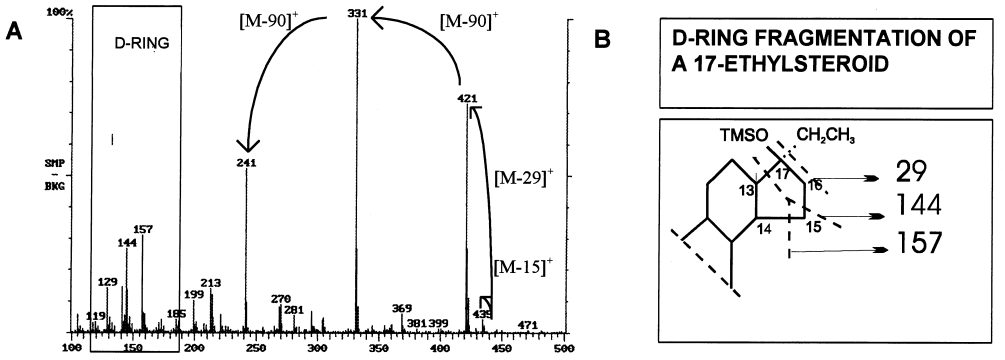

Fig. 2. (A) The full scan electron impact (EI) mass spectrum of EED recorded on a Finnigan GCQ system. (B) D-ring fragmentation in the trimethylsilyl derivative of EED gives rise to the fragment ions m/z 157 and m/z 144 present in the spectrum of EED. Immediate loss of the ethyl group at position 17 causes a loss of the molecular ion m/z 450.

Despite differences in race, sex and feeding habits between the animals studied, all experiments pointed towards the same metabolic pathway, as described in Fig. 3. As a consequence, these experiments indicate that EED is a reliable biological marker for the detection of NE and EES abuse in cattle, independent of biological variations. This hypothesis was confirmed by in vivo experiments.

3.2. In vivo experiments

Doses of 36 mg of EES and 250 mg NE, respectively, were administered to a heifer of approx. 100 kg and to a heifer of approx. 300 kg. Urine and faeces samples, collected at regular time intervals, were analysed by GC–MS.

During the first three days after treatment with EES, NE nor any other metabolites of EES were detected in urine and faeces samples. NE became detectable only in urine samples during a period of three days after the lag period at concentrations lower than 2 ng/ml, whereas faeces samples remained negative for NE. However, EED could be detected in both urine and faeces. Semi-quantitative results for NE and EED (Fig. 4), based on a five-point calibration curve for both compounds, showed that in faeces, EED is the only conversion product that points to the administration of EES. As for the urinary excretion, EED levels by far exceeded the NE levels. As previously described, EES itself was not detectable in urine nor in faeces.

After administration of NE, large quantities of the parent compound were excreted in urine during the first week. The presence of NE in faeces was limited to 36 h after NE administration (Fig. 4). In the HPLC fraction V, EED and one of its isomers, not detected in the in vitro experiments, were detected (Fig. 5). This isomer disappeared along with NE. EED levels exceeded those of NE in both urine and faeces (Fig. 4). The high excretion of EED in faeces prolonged the detection of NE treatment for almost a week.

The high amounts of EED, excreted in urine, and its longer detectability in faeces as compared with NE, can be explained by the fast conversion of NE into EED in the liver. Therefore, it was concluded

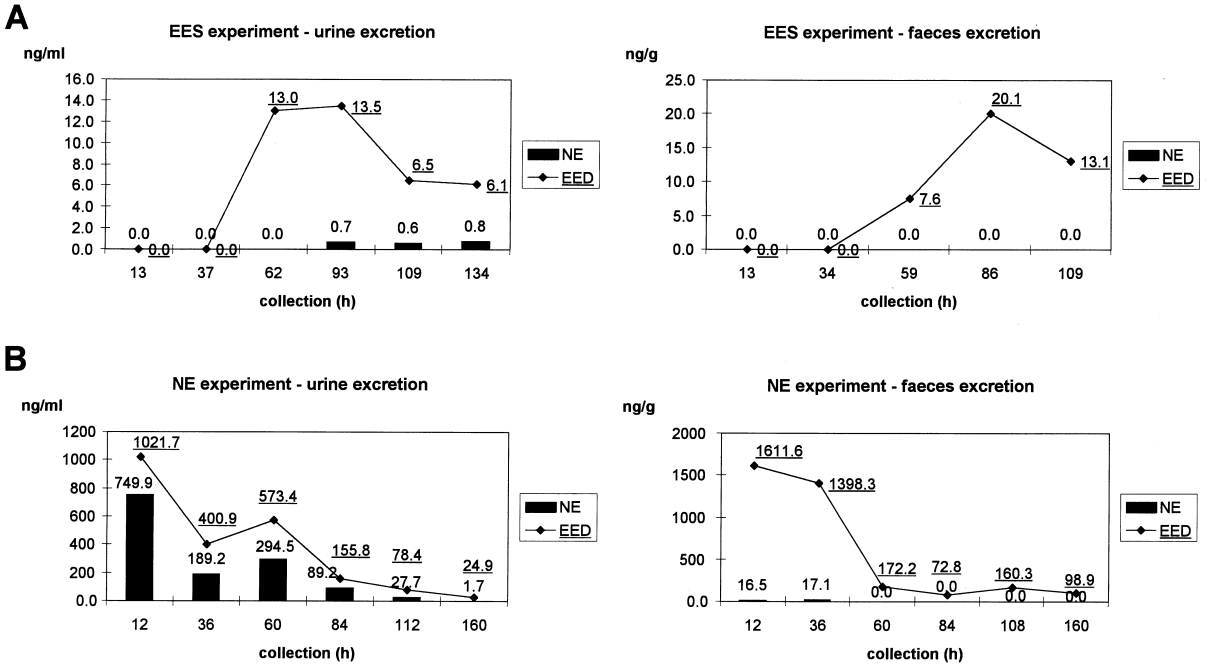

Fig. 4. (A) Excretion profile of EED and NE in urine and faeces after intramuscular injection of 36 mg of EES in a 12-month-old heifer. (B) Excretion profile of EED and NE in urine and faeces after intramuscular injection of 120 mg of NE in a 15-month-old heifer.

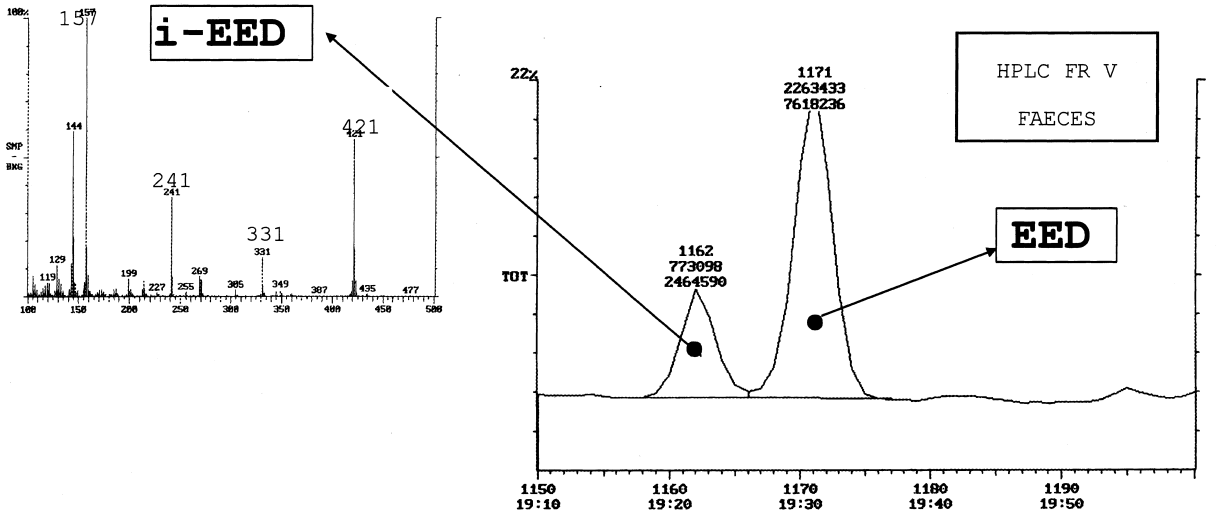

Fig. 5. The isomer i-EED, in the HPLC fraction V of a faeces extract, as a minor in vivo metabolite in cattle after administration of NE.

that EED is an excellent biological marker to reveal the abuse of NE and EES in cattle, particularly for the screening of faeces samples. It is shown hereafter that our findings were immediately applicable in daily practice.

3.3. Identification of the common biological marker of NE and EES

The identity of EED was confirmed by chemical synthesis, ^{1}H-NMR spectroscopy and comparison with the reference standard by GC–MS and HPTLC.

3.3.1. Selectivity of the reaction

For the synthesis of EED, NE was used as substrate for catalytic hydrogenation of the double bond and reduction of the 3-keto function.

Catalytic hydrogenation, a widely used approach in steroid chemistry, has been shown to be of great value for the selective and stereospecific reduction of various functional groups, because the stereochemical course of a catalytic hydrogenation depends on several factors: hydrogen availability to the catalyst, the catalyst itself and the reaction medium [31].

In the hydrogenation of polarised double bonds, the stereochemistry of the product is markedly influenced by the nature of the reaction solvent [31,32]. Δ^4-3-Keto steroids almost exclusively give rise to the 5β-isomer in basic media, whereas varying mixtures of the 5α- and 5β-isomers are produced in neutral solvents [18,33,34].

It has been suggested that in basic mixtures the reaction occurs by hydrogenation of the initially formed enolate anion, which is irreversibly adsorbed on the catalyst surface (Fig. 6). Addition of a hydride ion from the Pd catalyst gives the adsorbed dianion. Protonation of this dianion completes the hydrogenation of the polarised double bond. In the presence of strong base, Δ^4-3-Keto steroids form the heteroannular enolate anion (compound 1 in Fig. 6). Steric hindrance of the C- and D-ring of the steroid will favour the trans adsorption of this form. The enolate is adsorbed on the catalyst from the least hindered side. Protonation of the latter species from the solution leads to the *cis* or 5β-isomer [35].

In very dilute base or in the presence of weak bases, the homoannular enolate (compound 2 in Fig. 6) is formed. This can be adsorbed in either trans or cis manner. In this case, the presence of the C- and D-ring of the steroid can only result in slightly favouring trans adsorption and thus lead to the formation of a slight excess of the *cis* or 5β-isomer. For this reason, sodium hydroxide (NaOH) was added to the solvent ethanol for the catalytic reduction of norethandrolone.

The main methods to reduce ketones to alcohols comprise the use of metal hydrides. Lithium aluminium hydride ($LiAlH_4$) is the most powerful

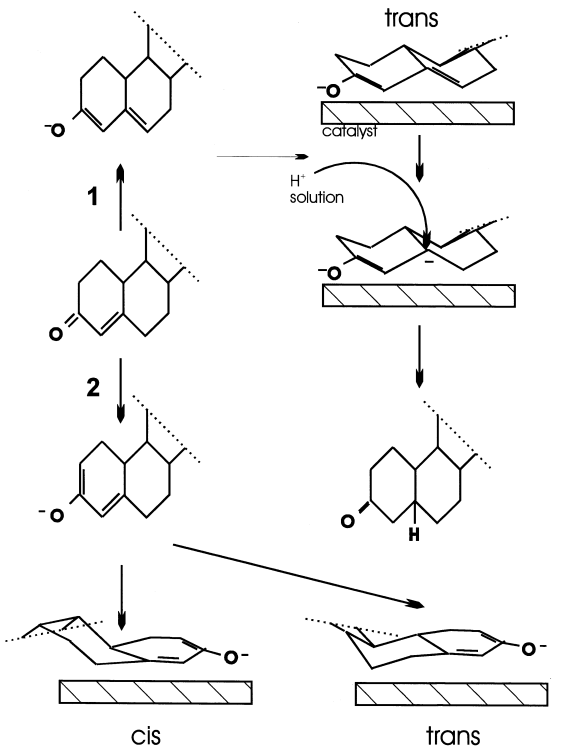

Fig. 6. The selective catalytic hydrogenation of steroids on a Palladium/C catalyst in the presence of a strong base.

hydride reagent, whereby the reductions can be done at room temperature. The proportion of epimers depends mainly on the steric environment. The percentage or ratio of the epimers formed in a number of reductions covering a wide variety of steroids were published earlier: a 3-ketone in the 5β-series gives mainly the 3α-ol [18,31].

3.3.2. ^{1}H-NMR spectroscopic analysis

Theoretically, four possible isomers can emerge from the total reduction of norethandrolone: 17α-ethyl-5β-estrane-3α,17β-diol, 17α-ethyl-5α-estrane-3β,17β-diol, 17α-ethyl-5β-estrane-3β,17β-diol and 17α-ethyl-5α-estrane-3α,17β-diol. The latter two isomers were excluded by ^{1}H-NMR (Fig. 7).

Only the proton at position 3 (H_3) and the C-18 and C-21 methyl signals can be assigned. Resonance of the skeleton protons give a broad envelop devoid of information ranging from δ 0.9 to 2.0. The coupling pattern of H_3, reveals an axial orientation of the proton. The septet results from the axial–axial ^{1}H,^{1}H-coupling (Jaa=10 Hz) and the axial–equatorial ^{1}H,^{1}H-coupling (Jae=5 Hz) with the neighbouring protons, pictured in Fig. 7.

The axial orientation of H_3 excludes the 3α,5α and the 3β,5β forms, which possess an equatorial H_3. The isomers left over as possible candidates for the synthesised product are the 3α,5β and the 3β,5α isomers. Considering earlier published results, the selectivity of the chemical synthesis as described above includes the 3β,5α configuration only as a minor product [1,18,31] The 3α,5β isomer should correspond with the major reduction product.

3.3.3. Comparison with the pure standard 17α-ethyl-5β-estrane-3α,17β-diol

^{1}H-NMR analysis reduced the number of possibilities to two for the identity of the synthesised product: 17α-ethyl-5β-estrane-3α,17β-diol, 17α-ethyl-5α-estrane-3β,17β-diol. Therefore, GC–MS and HPTLC characteristics of the synthesised product were compared with the pure standard EED, purchased from the Institut für Biochemie (Köln).

All GC–MS and HPTLC data of this pure standard matched the data of the synthesised product and the common in vivo metabolite for NE and EES (Table 2), both eluting in the HPLC fraction V. The appearance of the mass spectrum was not influenced by using only the silylating reagent TBT or the combination of the ethoxime reagent and TBT excluding the presence of oxo functions. The trimethylsilylated tertiary alcohol function on position 17 (TMSO in Fig. 2B) induces a typical fragmentation for 17α-ethyl steroids like NE, EES and EED. The two fragment ions of the D-ring at m/z 144 and m/z 157 in the spectrum of the EOTMS derivative correspond to similar fragments in NE and EES (Fig. 2B). This makes it very useful as a biological marker for NE and EES. The loss of two [M−90] is typical for the presence of two hydroxy-trimethylsilyl (HOTMS) groups. The M_r 450 is not present due to the loss of the ethyl group at position 17.

The 4×4 mode of the HPTLC detection allowed one to distinguish EED as a yellow–white spot from the matrix present in HPLC fraction V of urine and faeces samples (Table 2). UV detection of EED takes place after colouring with 5% sulphuric acid solution in acetic anhydride, which is normally used to visualise the 3-keto, Δ4 steroids such as NE.

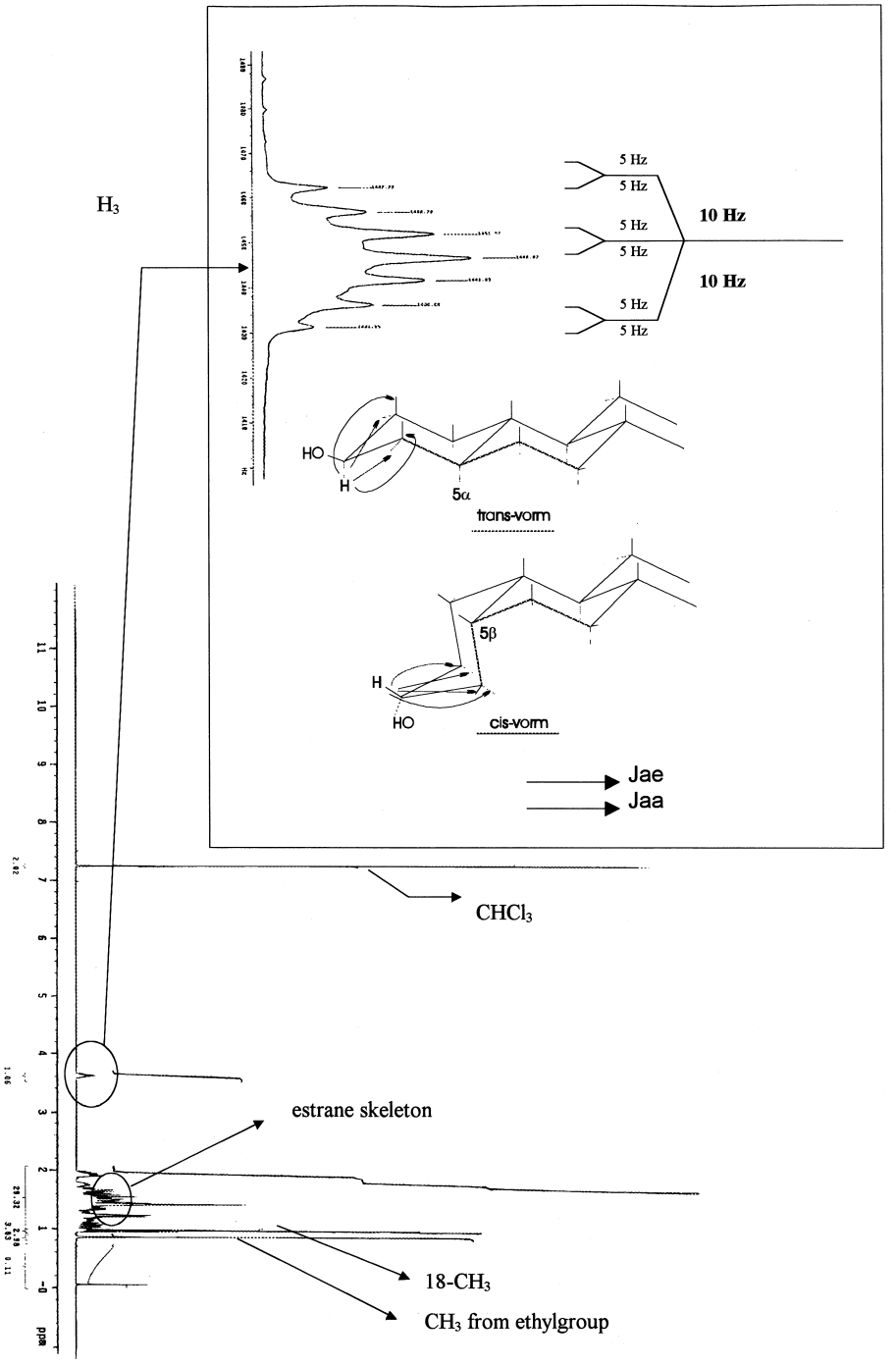

Fig. 7. ^{1}H-NMR spectrum of EED, recorded at 400 MHz in $C^{2}HCl_{3}$ on a Varian system, showing the coupling of H_{3} with his adjacent protons for the 17α-ethyl-5β-estraan-3α,17β-diol and 17α-ethyl-5α-estraan-3β,17β-diol isomers. Jae=Axial–equatorial coupling and Jaa=axial–axial coupling.

Table 2
Analytical data for GC–MS and HPTLC detection of NE, EES, EED

Substance	GC–MS data[b]			HPLC data Fraction	HPTLC data		
	M_r EOTMS	M_r	MU EOTMS		RRf (I)[a]	RRf (II)[a]	Appearance (366 nm)
NE [$C_{20}H_{30}O_2$]	417	302	29.50	IV	1.46	1.22	Grey–blue
EES [$C_{20}H_{32}O$]	360	288	24.52	>VIII	–	–	–
EED [$C_{20}H_{34}O_2$]	450	306	26.96	V	1	1.31	Yellow–white

[a] RRf (...)=Relative to β-testosterone for solvent I or II.
[b] Abbreviations: M_r=molecular mass, MU=methylene unit values, EOTMS=ethoxime-trimethylsilyl derivative.

3.4. The merits of EED as a biological marker in routine residue analysis

The identification of a specific metabolite of EES and NE contributes to the detection of EES and NE abuse in residue analysis. Based on this approach, our laboratory has found three major cases of NE abuse and several routine samples, both urine and faeces, were found to be positive for EED.

In a first batch of NE treated cattle, 175 faeces samples were screened for the presence of NE by HPTLC and GC–MS: 7.5% of the samples were positive for NE. The NE positive samples and 80 randomly chosen ''negative'' samples were then reanalysed by GC–MS for the presence of EED. All NE positive faeces samples were positive for EED and the EED levels always exceeded the NE levels. Moreover, if the presence of EED was accepted as a biological marker for NE abuse, 77% of the ''negative'' faeces samples were found to be positive, too.

Shortly after this first case, a second batch of cattle was found positive for NE. Ten urine samples were analysed by HPTLC and GC–MS: two were positive for NE and EED and three others for EED alone. Two weeks later, 70 additional urine and faeces samples from the same farm were submitted for analysis. NE was no longer detectable, but EED was still present in two samples as evaluated by GC–MS analysis.

In a third case, where cutaneous administration of NE was suspected, samples from four different animals were found to be positive for EED, and negative for NE.

For one month, 92 randomly chosen routine samples (59 faeces and 33 urine samples) were screened for the presence of NE and EED. Two urine and four faeces sample were found to be positive for EED, whereas none of these samples revealed any NE.

Fig. 8 shows that routine samples positive for NE, always reveal a higher concentration of EED. Along with the longer detection period of EED, as compared to NE, the higher levels of EED confirm the importance of EED in routine control analysis. This corresponds to our findings in in vitro and in vivo experiments.

As mentioned earlier, the in vivo urine and faeces samples of the NE treated animal revealed an isomer of EED in fraction V. This isomer was only found in samples with high concentrations of EED. At these high concentrations of EED, NE was always detected. This means that this isomer is of minor importance for the detection of NE and EES abuse in cattle.

Levels of 2 ng/ml for urine and 10 ng/g for faeces samples – generally accepted as decision limits for other anabolic steroids – were readily

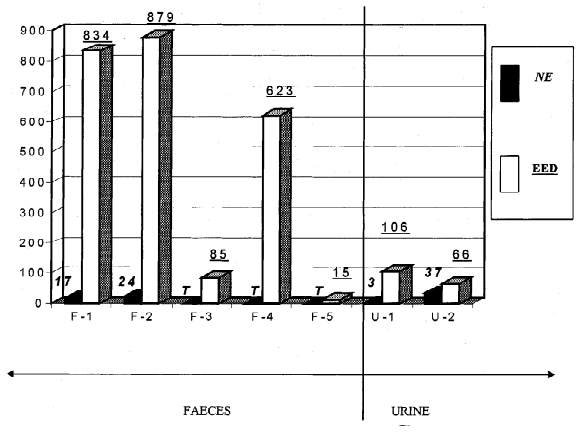

Fig. 8. The higher concentration levels of EED in routine urine and faeces samples positive for NE.

detectable, as illustrated by Fig. 9 [5,23,26,36–39]. For every batch of 9–14 samples of both the in vivo experiments and the routine samples one blank sample spiked at the above mentioned concentration levels was analysed to control the overall method. In total, 15 blank urine and 15 blank faeces samples from different origin were spiked and analysed. For both matrices the average recoveries of the spiked amounts of NE and EED were determined. The mean recoveries of EED were approximately 64% in urine and 19% in faeces. The same semi-quantitative method allowed one to estimate approximate mean

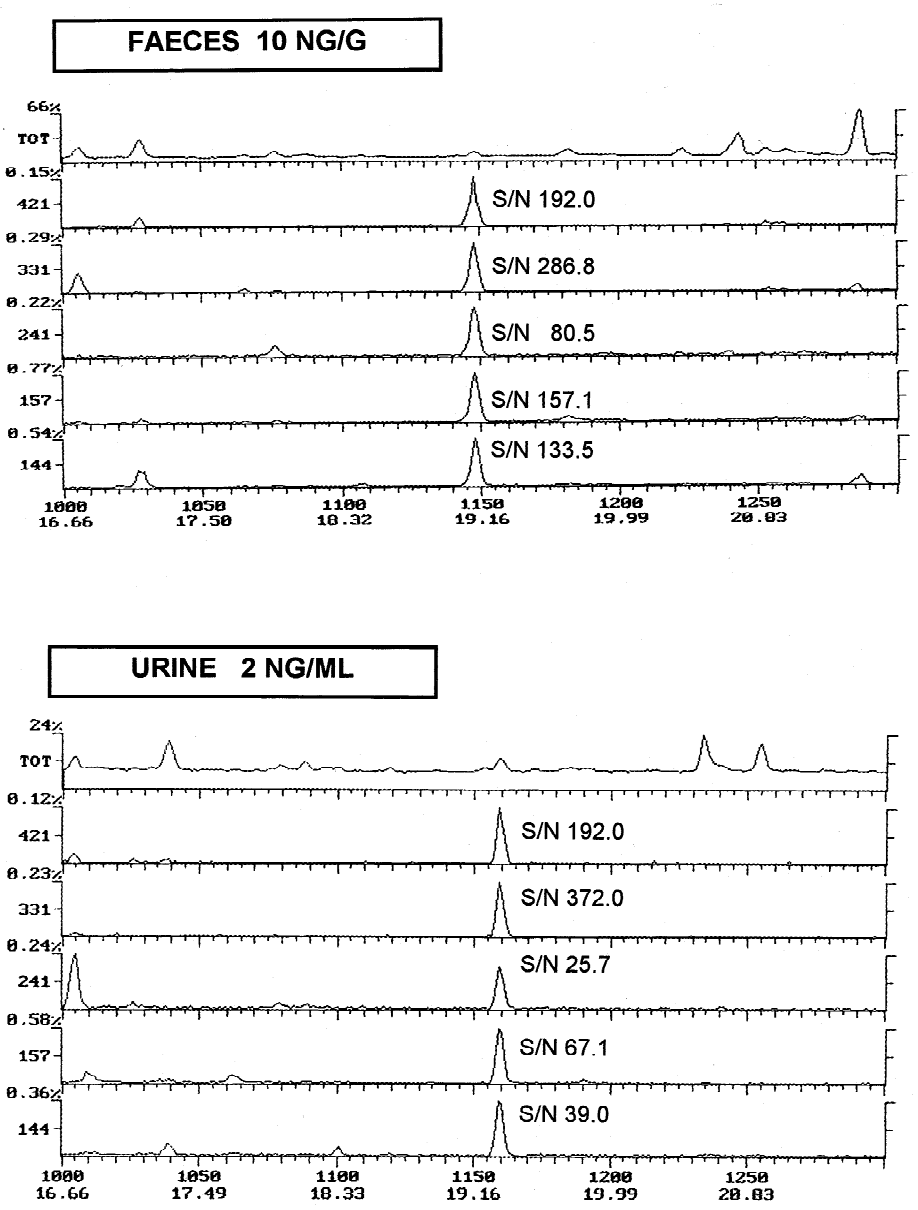

Fig. 9. Total ion current chromatogram and single ion chromatograms of a faeces extract fortified with 10 ng/g EED and an urine extract fortified with 2 ng/ml EED.

recoveries of 55% in urine and 25% in faeces for NE.

Additionally to the analysis of the blank samples of the two in vivo experiments, 13 blank urine and 13 blank faeces samples coming from different bovine animals were analysed for the presence of EED. In HPLC fraction V no EED nor any other mass spectrum including the two typical D-ring fragment ions were found.

These characteristics and the results of the routine screening clearly show the value of EED as a biological marker for the detection both in urine and faeces.

4. Conclusions

A better understanding of the metabolism of EES and NE in ruminants opens perspectives for a powerful detection method for EES and/or for a serious improvement of the detection of NE, in both urine and faeces samples.

The search for metabolites, that could act as biological markers for the illegal use of growth promoters in cattle, led to in vitro techniques, such as microsomal preparations and monolayer cultures of intact liver cells, prepared from liver tissue of slaughtered cattle. These in vitro studies reduced the need for in vivo experiments.

The higher amounts and prolonged presence of EED in excreta, as compared to the excretion of any other metabolite or the parent compounds NE and EES, were shown in both urine and faeces, but were most pronounced in faeces. Since in Belgium, faeces samples constitute the largest part of the routine samples submitted for regulatory control, this biological marker is of great importance for the detection of the abuse of NE and/or EES. The cases of NE abuse, detected in the margin of the routine analyses in our laboratory, illustrate the power of EED screening.

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