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In vitro liver models are important tools to monitor the abuse of anabolic steroids in cattle†

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Current veterinary residue analysis mainly focuses on the monitoring of residues of the administered parent compound. However, it is possible that larger amounts of metabolites are excreted and that they can have a prolonged excretion period. In order to unravel specific metabolic steps and to identify possible biological markers, two *in vitro* liver models were used, *i.e.* monolayer cultures of isolated hepatocytes and liver microsomes, both prepared from liver tissue of cattle. Clostebol, boldenone, norethandrolone (NE) and ethylestrenol (EES) were used as model substrates. Results show that the metabolic profiles derived from *in vitro* experiments are predictive for the *in vivo* metabolic pathways of the steroids evaluated in this study. By means of this strategy, it is possible to identify 17α-ethyl-5β-estrane-3α,17β-diol (EED) as a common biological marker for NE and EES. By *in vivo* experiments it was shown that EED is particularly important for the detection of the abuse of NE or EES because of its high excretion levels and its prolonged presence as compared with the parent compounds or any other metabolite.

Aim of investigation

A knowledge of the metabolic pathways of a particular synthetic anabolic steroid can be important to improve the screening for its abuse in cattle. Therefore, the investigation of the metabolism of some of these steroids *in vitro* was initiated. The liver is quantitatively the main organ responsible for a wide variety of biotransformations of xenobiotics. There are several possibilities for using liver preparations to assess the hepatic metabolism, *e.g.*, perfusion of liver as such, incubation of subcellular fractions, liver slices, liver tissue preparations and cells.[1,2]

The phase I oxidative biotransformations are the most important for the metabolism of steroids. The enzymes involved are almost exclusively localised in the endoplasmic reticulum. The microsomal fraction represents the 'pinched off' and vesiculated fragments of the original endoplasmic reticulum that retain most of their enzymatic activity.[1,3] The microsomal fraction was therefore used to study the oxidations of the steroid nucleus in a low matrix environment.

Primary cultures of hepatocytes isolated from cattle livers allow a more accurate quantitative study of the different metabolic routes. In addition, the formation of cytosolic metabolites can be monitored directly within the system.[3] The phase I and II metabolic conversions, the metabolic routes of the endoplasmic reticulum and the cytosol, can be studied in combination.

Our results in both cattle liver microsomal preparations and primary cultures of isolated hepatocytes made it possible to clarify and predict the structure of *in vivo* metabolites of clostebol (CLT), norethandrolone (NE) and ethylestrenol (EES).

As the metabolites of CLT in cattle urine were identified in a previous study,[4] CLT was used as a reference compound to evaluate the *in vitro* assays to approximate the *in vivo* situation. The metabolism of NE and EES in cattle was not yet known. The predictive value of the *in vitro* methods was further confirmed by the successful unravelling of the metabolism of NE and EES. *In vivo* experiments and case studies in control procedures to detect forbidden growth promoters verified these conclusions.

Experimental

Experimental animals and samples

Microsomes were prepared from livers of four different animals: 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 1 year old.

For the *in vivo* experiments, 36 mg of EES were administered intramuscularly to a 12 month old heifer (±100 kg) and 250 mg of NE to a 15 month old heifer (±300 kg). A 50 mg amount of EES was dissolved in 10 ml and 250 mg of NE in 12 ml of a 1 + 1 (v/v) mixture of pharmaceutical grade Miglyol and *N*-methyl-2-pyrrolidone (Sigma, St. Louis, MO, USA). Urine samples from animals treated with 17β-boldenone undecanoate were obtained from RIKILT-DLO (Wageningen, The Netherlands). The urine metabolites of 4-chlorotestosterone were identified in an earlier study in our laboratory.[4] For this study, two 18 month old heifers and two 10 month old bulls were

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injected intramuscularly with different amounts of 4-chlorotestosterone acetate.

Chemicals and standards

All reagents and solvents were of analytical-reagent grade. All anabolic steroids were obtained from the Belgian Reference Laboratory (Wetenschappelijk Instituut voor Volksgezondheid-Louis Pasteur, Brussels, Belgium). Tri-Sil-TBT (Pierce, Rockford, IL, USA), which consists of trimethylsilylimidazole, *N,O*-bis(trimethylsilyl)acetamide and 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). 17α-Ethyl-5β-estrane-3α,17β-diol (EED) was provided by the Institut für Biochemie, Deutsche Sporthochschule Köln, Germany. All reagents used for the chemical synthesis of the metabolites were from Aldrich (Milwaukee, WI, USA).

In vitro experiments

Microsomes were prepared and incubated with steroids as described previously.[5] Protein concentrations were assessed by the method of Lowry *et al.*[6] using bovine serum albumin as a standard. Hepatocytes were isolated according to Van't Klooster *et al.*,[3] based on the method of Seglen.[7] Cells were cultured at a density of 4.106 cells per 60 mm culture dish (Greiner, Alphen a/d Rijn, The Netherlands) in 4 ml of Williams' E supplemented with 4% newborn calf serum, 1.67 mmol l^{-1} glutamine, 50 mg ml^{-1} gentamicin sulfate, 1 mmol l^{-1} hydrocortisone, 1 mmol l^{-1} insulin, 0.5 mmol l^{-1} $CaCl_2$ and 0.5 mmol l^{-1} $MgCl_2$. Cells were incubated for 4 h in a humidified atmosphere of air (95%) and CO_2 (5%) at 37 °C. The medium was then replaced by a medium without serum, $CaCl_2$ and $MgCl_2$. After incubation for 20 h, the hepatocytes were incubated with either 100 mmol l^{-1} of the steroid for 6 and 24 h or with 10 mmol l^{-1} for 24 h. The steroids were dissolved in methanol (final concentration of methanol 0.1%).

Sample preparation

To isolate steroids from microsomal incubation mixtures a liquid–liquid extraction of 1 ml of the incubation mixture with 6 ml of diethyl ether and subsequently with 6 ml of *tert*-butyl methyl ether was performed. 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 analyzed by HPLC and GC-MS as described below.

A 2 ml volume of the liver cell incubation mixtures of hepatocytes was adjusted to pH 5.2 with acetate buffer (0.2 mol l^{-1}, pH 4.8) after which 50 μl of *Succus Helix Pomatia* were added and the mixture was incubated overnight at 37 °C. The mixture was then loaded on to a Chem-Elut™ column and the steroids were eluted by additions of 5 ml of *tert*-butyl methyl ether and 5 ml of chloroform. All extracts were analyzed by GC-MS, as described below.

The clean-up of urine and faeces samples by solid-phase extraction (SPE) and HPLC fractionation was carried out as described previously.[4] For the detection of the 6-hydroxy metabolites of 17β-boldenone (17β-BOL) and 1,4-androstadiene-3,17-dione (ADD), the HPLC fractionation was expanded with three additional fractions. These were collected from the drain prior to the actual collection of the standard steroids. The collector was operated in the time mode. The three fractions were collected with time windows, relative to naphthalene as internal standard, ranging from 0.23 to 0.29, from 0.29 to 0.35 and from 0.35 to 0.46.

Gas chromatography-mass spectrometry

In order to obtain ethoxime-trimethylsilyl (EO-TMS) derivatives, dry residues were derivatised with ethoxyamine and a silylating mixture (Tri-Sil-TBT).[5] The GC-MS analyses were performed on a Finnigan GCQ system (San Jose, CA, USA). The GC column used was a DB-5 MS 30 m × 0.32 mm id fused-silica column with a 0.25 μm film thickness (J&W Scientific, Folsom, CA, USA). Temperature settings were as follows: injector, 260 °C; transfer line, 275 °C; ion source, 200 °C; oven program: from 50 °C (held for 20 s) to 190 °C at 50 °C min^{-1}, then to 320 °C (held for 4 min) at 4.6 °C min^{-1}. The carrier gas was helium at a velocity of 40 cm s^{-1} for the GCQ. Aliquots (1 μl) were injected on a split–splitless injector in the split mode.

Results and discussion

CLT

CLT was used as a reference compound. Its urine metabolites were identified in an earlier study on three animals.[4] For several years, these metabolites have been successfully used in routine analysis to detect the misuse of CLT in cattle breeding. The isotope ratios of the chlorine group present in the CLT molecule facilitated the location of formerly unknown chlorinated metabolites and are an excellent marker for their exogenous character. 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 present in microsomal liver preparations and incubation mixtures of hepatocytes. Although epimerisation is a common *in vivo* biotransformation route, no 17α-CLT was found. Only a trace amount of an important hydroxylated urine metabolite, 4-chloroandrost-4-ene-3ξ,17ξ,ξ-triol, was found *in vitro*.[4] In addition, new compounds, not yet described in the urine of CLT treated animals, were present in the microsomal preparations yielding mass spectra with similar electron impact (EI) fragmentation patterns to those of some of the hydroxylated metabolites found in cattle urine, but eluting at different retention times.

EES and NE

Incubation of microsomal liver preparations from four different animals with EES, revealed NE as the major biotransformation product [Fig. 1(A)]. Because of its strong hydrophobic nature, the oxidation of EES is of major importance to eliminate it from the body. NE itself was used as substrate in an incubation experiment with liver microsomes. Only minor hydroxylated products were tentatively identified.

Additional experiments on the metabolism of NE were performed with isolated liver cells. To identify possible metabolites, NE was incubated for 24 h at 100 and 10 μmol l^{-1}, and for 6 h at 100 μmol l^{-1}. The major conversion product was the reduced form 17α-ethyl-5β-estrane-3α,17β-diol (EED). Hydroxylation products were of minor importance. A typical chromatogram and the EI mass spectrum of EED are shown in Fig. 1(B) and 2, respectively. The two fragment ions of the D-ring at *m/z* 144 and 157 relate the product to NE and EES (Fig. 2) and prove its exogenous character. This makes it very useful as a biological marker for NE and EES. The loss of two [M − 90] is typical of the presence of two trimethylsilylhydroxy groups. The molecular ion of the trimethylsilyl derivative (M_r 450) is not present owing to the loss of the ethyl group [M − 29] at position 17. This generates the ion at *m/z* 421 in the spectrum. The ions at *m/z* 331 and 241 originate from the subsequent loss of the two trimethylsilylhydroxy groups.

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

Urine and faeces samples of the heifers treated with EES and NE were collected at regular intervals and analyzed by GC-MS.

EES itself was not detectable in urine or faeces. The metabolite NE was only detectable in urine samples for 3 d after a 3 d lag period at concentrations lower than 2 ng ml^{-1}, whereas faeces samples remained negative for NE. However, EED could be detected in both urine and faeces. EED was the only conversion product in faeces that points to the administration of EES.

After administration of NE, large amounts of the parent compound were excreted in urine during the first week. NE was present in faeces up to 36 h after NE administration. EED prolonged the detection of NE treatment in faeces for almost 1 week.

The longer detectability of EED in faeces as compared with NE can be explained by the fast conversion of NE to EED in liver. The latter metabolite was seen in incubations of primary cultures of hepatocytes. Therefore, it was concluded that EED is an excellent biological marker to reveal the abuse of NE and EES in cattle, particularly when faeces samples are screened.

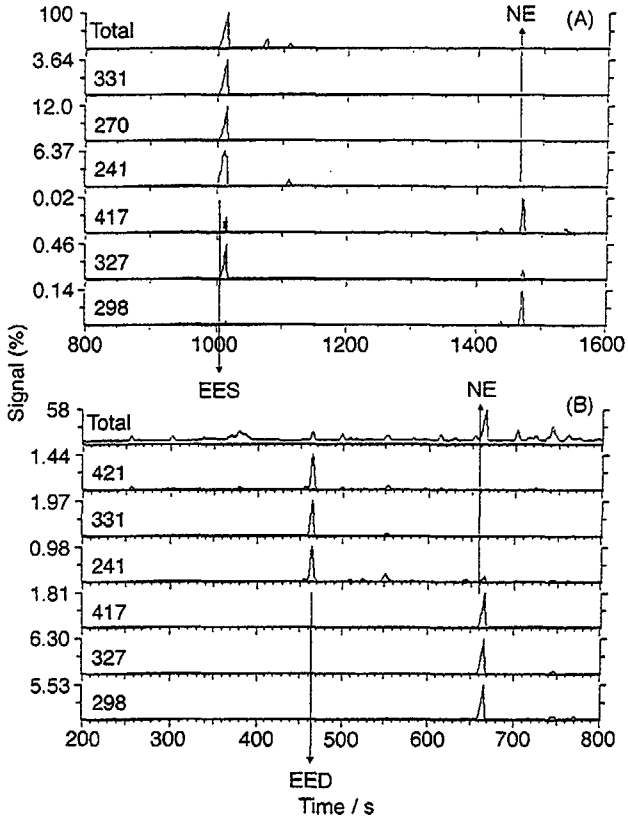

Fig. 1 (A) Total ion current and single ion chromatograms are shown for the three most important diagnostic ions of EES and NE, pointing towards NE as the most important metabolite of EES after incubation with liver microsomal preparations. (B) EED as the major *in vitro* metabolite after a 6 h incubation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

17β-BOL

The oxidation of 17β-boldenone (17β-BOL) to ADD is the major metabolic pathway in microsomes. Additionally, small amounts of 6-hydroxy-17β-boldenone and 6-hydroxy-1,4-androstadiene-3,17-dione are formed. The 6-hydroxy metabolites were synthesised according to the method of Schänzer.[8] After 24 h of incubation with isolated liver cells, a significant portion of 17β-BOL and ADD is hydroxylated at position 6. Only a small portion of either 17β-BOL or ADD is subjected to further reductions of the unsaturated bonds and/or keto functions. α-Epimerisation to 17α-boldenone (17α-BOL) is also detected. Incubations of 17β-BOL with liver microsomes and isolated hepatocytes indicate that hydroxylations of the androstadiene skeleton, especially at the allylic position 6, are important metabolic pathways.

To study the *in vivo* situation, urine samples obtained after application of 17β-boldenone undecanoate were screened for the presence of 17β-BOL, the earlier detected *in vitro* metabolites of 17β-BOL and 5β-androst-1-ene-3,17-dione (5β-AED). 5β-AED was reported as a metabolite of 17β-BOL in human urine,[8] but was not found after *in vitro* incubations. 5β-AED was synthesised according to the method of Schänzer and Donike.[8,9] 17β-BOL, 17α-BOL, ADD and 5β-AED were detected in cattle urine. In addition to 5β-AED, several reduction products were tentatively identified. These included all the spectra found in the incubations with hepatocytes, but also some additional isomers. Also, a small amount of 6-hydroxy-17β-boldenone was found. All urine metabolites found were predicted by the *in vitro* screening except for 5β-AED and some of the isomers of the reduction products.

Conclusions

The search for metabolites that could act as biological markers for the illegal use of anabolic steroids as growth promoters in

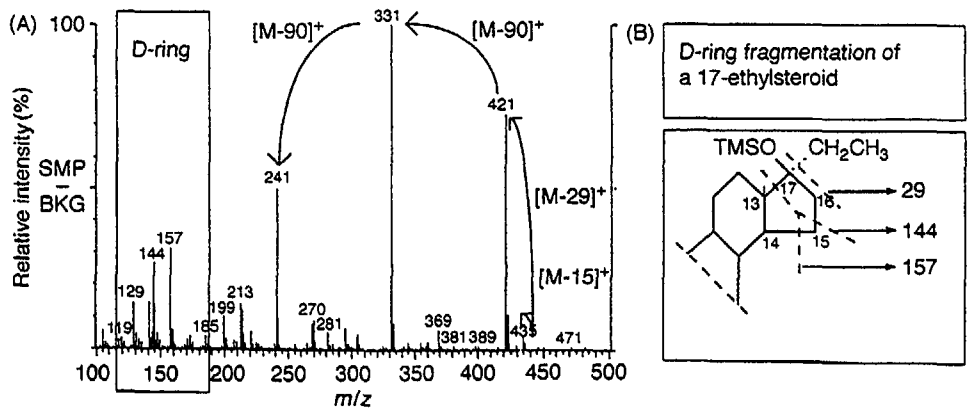

Fig. 2 (A) Full scan EI mass spectrum of EED. (B) D-ring fragmentation in the trimethylsilyl derivative of EED gives rise to the fragment ions at *m/z* 157 and 144 present in the spectrum of EED. Immediate loss of the ethyl group at position 17 causes a loss of the molecular ion at *m/z* 450.

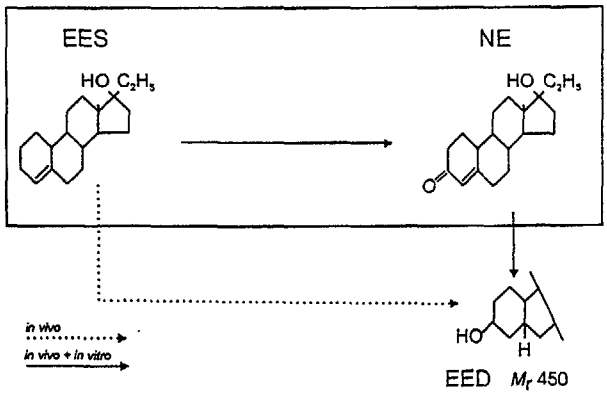

Fig. 3 *In vitro* metabolic conversion of EES and NE to EED. NE is an intermediate in the conversion of EES to EED.

cattle led to *in vitro* techniques, such as microsomal preparations and monolayer cultures of intact liver cells, prepared from liver tissue of slaughtered cattle. The combined use of the two techniques allowed a study of the prominent changes of the steroid skeleton by the liver. For all synthetic steroids included in this study, the liver microsomes were able to predict the major hydroxylations and oxidations of the steroid skeleton by membrane-bound mixed function oxidative enzymes, the cyt P450 systems. Hepatocytes more closely reflect the *in vivo* situation, since in addition to the oxidations and hydroxylations, reductions of the unsaturated bonds are also performed.

In conclusion, *in vitro* studies reduce the need for *in vivo* experiments and provide a low matrix environment for fast localisation of diagnostic metabolites, as illustrated by the example of EED.

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