Journal of Chromatography, 489 (1989) 157–171
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4613

DETERMINATION OF β-19-NORTESTOSTERONE AND ITS METABOLITE α-19-NORTESTOSTERONE IN BIOLOGICAL SAMPLES AT THE SUB PARTS PER BILLION LEVEL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE IMMUNOAFFINITY SAMPLE PRETREATMENT

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SUMMARY

An immunoaffinity precolumn (immuno precolumn) packed with Sepharose-immobilized polyclonal antibodies against the anabolic hormone 17β-19-nortestosterone (β-19-NT) was used for the selective on-line pretreatment of raw extracts of urine, bile and tissue samples by high-performance liquid chromatography. Using UV detection (247 nm), β-19-NT and its metabolite 17α-19-nortestosterone (α-19-NT) can be determined in biological samples with a detection limit of 0.05 μg/kg. Owing to the high clean-up efficiency of the immuno precolumn and the large sample volumes used, confirmation by gas chromatography–mass spectrometry is possible at this level. In urine samples from a calf treated with 19-nortestosterone 17β-laurate, the maximum concentrations of β-19-NT (1.3 μg/l) and α-19-NT (3.1 μg/l) were found seven days after intramuscular administration. In a bile sample from this calf only α-19-NT (55 μg/l) was detected In meat samples from three treated calves, the concentration of β-19-NT varied from 0.1 to 1.6 μg/kg and no α-19-NT could be detected. In liver samples from these calves, the concentrations of β-19-NT and α-19-NT were less than 0.05–0.1 and 0.5–0.9 μg/kg, respectively. In the corresponding kidney samples, the concentrations of β-19-NT and α-19-NT were 0.4–0.5 and 0.5–1.6 μg/kg, respectively. The application of the same immuno precolumn to the determination of 17β- and 17α-trenbolone, two structurally related steroids, is also demonstrated.

INTRODUCTION

Anabolic preparations are attractive means of improving the growth rate and feed conversion of animals in livestock breeding [1]. In all European Community

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countries, however, the use of growth promoters has been banned since 1988 [2]. Within The Netherlands, urine is chosen as the matrix to screen for the presence of these compounds using gas chromatography combined with mass spectrometry (GC–MS). With GC–MS several anabolics can be determined simultaneously in urine above the 1–5 μg/l level [3]. However, owing to metabolism [4,5], the residual amount of the parent compounds, in this instance 17β-19-nortestosterone (β-19-NT), in urine is often below 1 μg/l, especially one week or more after administration. Therefore, for more effective monitoring of the presence of β-19-NT in urine, a method with a detection limit far below 1 μg/l is necessary or, alternatively, a more abundant metabolite, e.g., 17α-19-nortestosterone (α-19-NT), as proof of the use of β-19-NT, may be determined [6].

Using radioimmunoassay (RIA), β-19-NT can be determined in urine at the 1 μg/l level [6]; however, with such a method cross-reacting compounds can interfere, which may lead to false-positive results. Combining RIA with high-performance liquid chromatography (HPLC) yields a more specific assay with a detection limit of 0.2 μg/l [7,8]. In The Netherlands, however, positive results obtained with such screening methods have to be confirmed with a highly specific spectrometric method such as GC–MS. Owing to the lower sensitivity of GC–MS, relatively large sample volumes have to be analysed. In addition, we found that the large number of interfering substances in urine samples have to be removed by extensive sample treatment [3].

In order to simplify the clean-up procedure, the preconcentration has to be highly selective. For this reason, an immunoaffinity precolumn (immuno precolumn) packed with Sepharose-immobilized polyclonal antibodies against β-19-NT is used for selective on-line sample pretreatment in HPLC [9]: β-19-NT and α-19-NT are selectively preconcentrated on this precolumn from a relatively large volume of urine (25 ml). Subsequently, the analytes are separated by HPLC and detected by UV absorption at 247 nm. The detection limit of this method is low (0.05 μg/l) and the total analysis takes 45 min. The method was applied only to the determination of free β-19-NT and α-19-NT in spiked non-hydrolysed urine samples [9]. In the urine of most species, however, anabolics are mainly excreted as conjugates of glucuronic and sulphuric acids [10–12]. Hence for the determination of the analytes in urine from treated animals, hydrolysis of the conjugates to free steroids is necessary because the conjugates cannot be detected by conventional methods.

This paper demonstrates the application of HPLC with on-line immunoaffinity chromatography to the determination of β-19-NT and α-19-NT in hydrolysed urine samples and also in biological samples such as bile, meat, liver and kidney of calves treated with 19-nortestosterone 17β-laurate. The applicability of the method to the determination of the related steroids 17β- and 17α-trenbolone is also shown.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Merck–Hitachi (Darmstadt, F.R.G.) Model 655-A-11 pump for the analytical column, a Kratos (Ramsey, NJ, U.S.A.) Model

400 pump for sample handling, two Kratos Model Must valve-switching units (containing one solvent-selection valve and three high-pressure six-port rotary valves), a Kratos Spectroflow 450 solvent programmer, a Merck–Hitachi L-4200 UV–VIS detector set at 247 nm and a Merck–Hitachi Model D2000 integrator. The analytical column was a Chrompack (Middelburg, The Netherlands) 100 mm × 3 mm I.D. glass column packed with Chromspher (5 μm) C_{18} and protected with a Chromsep reversed-phase guard column (Chrompack). The C_{18} precolumn was a 10 mm × 2 mm I.D. Chrompack reversed-phase preconcentration column. Packing and testing of the immuno precolumn (10 mm × 10 mm I.D.) have been fully described elsewhere [9].

The GC–MS system (Hewlett-Packard, Rockville, MD, U.S.A.) consisted of a Model 5890 gas chromatograph, a Model 5970 mass-selective detector and a Model 7673-A auto-injector. A CP-Sil-5 CB column (25 m × 0.25 mm I.D.) (Chrompack) with a film thickness of 0.12 μm was used with helium as the carrier gas.

Chemicals

Cyanogen bromide (CNBr)-activated Sepharose 4B was obtained from Pharmacia (Woerden, The Netherlands). HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, F.R.G.) and β-19-NT and norgestrel (NG) from Sigma (St. Louis, MO, U.S.A.). Gifts were received of α-19-NT from Organon (Oss, The Netherlands), 17α-trenbolone from the National Institute of Public Health and Environmental Hygiene (RIVM, Bilthoven, The Netherlands) and 17β-trenbolone from Roussel Uclaf (Paris, France).

Stock solutions of the steroids were prepared in methanol and stored at 4°C until used. *Helix pomatia* digestive juice was obtained from Merck and contained a minimum of 40 U/ml β-glucuronidase and 20 U/ml arylsulphatase. Protease was obtained from Sigma and contained 11.6 U of subtilopeptidase A per milligram of solid. Amberlite XAD-2 was purchased from Serva (Heidelberg, F.R.G.) and was purified by washing with 2 *M* sodium hydroxide, 2 *M* hydrochloric acid, acetone, ethanol and water.

NG was purified by preparative HPLC: 1 mg of NG was dissolved in 200 μl of methanol and injected onto a Hibar (Merck) LiChrosorb RP-18 (7 μm) column (250 mm × 10 mm I.D.) with acetonitrile–water (55:45) as the mobile phase. Demineralized water, prepared with a Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used. All eluents were filtered through 0.45-μm Millipore filters. All other chemicals were of analytical-reagent grade.

Antibody preparation and immobilization

19-Nortestosterone 17β-hemisuccinate–bovine serum albumin (BSA) was prepared according to Kyrein [13] and used to raise antibodies in rabbits. The collected antiserum was purified according to the method of Steinbuch and Audrian [14]. The isolated immunoglobulin G (IgG) fraction was stored freeze-dried (RIKILT Batch 726-3). Cross-reactivities determined by radioimmunoassay were about 70, 20, 40 and less than 1% for α-19-NT, NG, 17β-trenbolone and β-testosterone, respectively.

The IgG fraction was immobilized on CNBr-activated Sepharose 4B as rec-

ommended by the manufacturer [15]; 10 mg of freeze-dried IgG were used per millilitre of gel. The immunosorbent was stored at 4°C in 0.1 *M* sodium phosphate buffer (pH 7.2) containing 0.02% sodium azide until packing of the precolumn.

Sample materials

Most of the samples were obtained from two animal experiments.

In experiment I, a male veal calf (calf No. 13), aged 26 weeks and weighing 183 kg, was injected intramuscularly in the neck with 200 mg of 19-nortestosterone 17β-laurate and 20 mg of estradiol 17β-benzoate (dissolved in 4 ml of arachis oil containing 10% benzyl alcohol). Another male veal calf of the same age and weight (calf No. 12) served as a control. From both calves samples of urine were collected from two days before to nineteen days after administration of the steroids. Samples of bile, liver, kidney and meat were taken nineteen days after administration. This experiment was performed in March 1987 at the Institute for Animal Nutrition Research (ILOB) at Wageningen, The Netherlands.

In experiment II, two male veal calves (Nos. 2 and 6), both aged nine weeks and weighing 90 kg, were treated as described in experiment I. A third male veal calf of the same age and weight (No. 7) served as a control. Meat samples were taken fourteen days after administration. This experiment was performed in January 1985 at the Institute for Livestock Feeding and Nutrition Research (IVVO, Lelystad, The Netherlands).

Blank samples of urine, bile and meat were also obtained from IVVO. All samples were stored at −20°C until analysis.

Sample preparation

*Urine.*Urine (50 ml) was mixed with 6 g of Amberlite XAD-2 for 15 min and the mixture was transferred into a glass column (100 mm×9 mm I.D.). The settled Amberlite XAD-2 was washed with three portions of 10 ml of water and dried under a stream of nitrogen. The steroids and the conjugates were eluted with 25 ml of methanol–ethyl acetate (50:50) and the eluate was evaporated to dryness at 50°C. To the residue 2 ml of 0.25 *M* acetate buffer (pH 4.8) and 50 μl of *Helix pomatia* juice were added and the mixture was incubated at 37°C for 2 h. Next, four drops of 6 *M* hydrochloric acid and 20 ml of ethyl acetate were added. After mixing for 15 min, the water layer was removed and the ethyl acetate layer was incubated for 1 h at 37°C and subsequently washed with two 3-ml portions of 10 wt.% sodium hydrogencarbonate and 3 ml of water. The ethyl acetate layer was evaporated to dryness, the residue was dissolved in 70 ml of water–acetonitrile (95:5) and 53 ml of the solution were analysed by HPLC as described below.

*Bile.*To 2 ml of the bile sample 2 ml of a 0.25 *M* acetate buffer (pH 4.8) and 50 μl of *Helix pomatia* juice were added and the mixture was incubated for 2 h at 37°C. Subsequently, the same procedure was used as described for urine samples, starting with the extraction with ethyl acetate.

*Meat, liver and kidney.*To 20 g of a minced sample 80 ml of 0.1 *M* Tris buffer (pH 9.5) containing 20 mg of protease were added and the mixture was incubated for 3.5 h at 60°C. Next, the mixture was filtered over glass-wool and 6 g of Am-

berlite XAD-2 were added to the filtrate. After mixing for 15 min, the mixture was transferred to a glass column (100 mm × 9 mm I.D.). The settled Amberlite XAD-2 was washed with 10 ml of water and dried under a stream of nitrogen. The analytes were eluted with five portions of 10 ml of methanol and the methanol was evaporated. For meat samples the residue was dissolved in 70 ml of water–acetonitrile (95:5) and 53 ml of the solution were transferred on to the immuno precolumn. The residues of liver and kidney samples were hydrolysed in the same way as urine samples after elution from the Amberlite XAD-2.

High-performance liquid chromatography

The first step involved preconditioning of the immuno precolumn with water (20 ml), then the sample (53 ml) was introduced by the sample-handling pump via the solvent selection valve. Next, the immuno precolumn was flushed with water (10 ml) to displace the remaining sample and to remove non-specifically bound impurities. In the next step, the C_{18} precolumn was switched off-line from the analytical column and preconditioned with water (10 ml). Subsequently, the immuno precolumn, which now contained the trapped analytes, and the C_{18} precolumn were switched in series. Desorption of the analytes from the immuno precolumn and refocusing on the C_{18} precolumn were accomplished with 20 ml of an aqueous solution containing 250 μg/l NG and 5% acetonitrile. After the complete transfer of the analytes to the C_{18} precolumn, the actual separation was started by switching the C_{18} precolumn on-line with the analytical column; β-19-NT, α-19-NT and NG were then separated on the analytical column, using acetonitrile–water (35:65) as the mobile phase at a flow-rate of 0.4 ml/min. Simultaneously, the immuno precolumn was reconditioned by flushing with methanol–water (70:30).

A more extensive description of the automated HPLC procedure and a scheme of the HPLC system have been reported elsewhere [9].

Gas chromatography–mass spectrometry

After the analytical separation, fractions containing β-19-NT and α-19-NT were collected in a 4-ml vial and the eluent was evaporated to dryness at 40°C under a stream of nitrogen. The analytes were converted into monotrimethylsilyl derivatives by adding 100 μl of a freshly prepared mixture of 100 μl of trimethylchlorosilane (TMCS), 1 ml of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 9 ml of pyridine and heating for 1 h at 60°C. After evaporation under a stream of nitrogen (40°C), the residue was dissolved in 25 μl of an external standard solution containing 2 μg of PCB 138 (2,2′,3,4,4′,5′-hexachlorobiphenyl) per millilitre of isooctane–*n*-decane (4:1). For analysis, 5 μl were injected in the splitless mode into the GC–MS system utilizing selected-ion monitoring (SIM). The analytes were considered to have been positively identified if the GC retention times of the derivatives and the ratios of the intensities of the fragments with *m/z* values of 215, 256, 331 and 346 (base peak) agreed with those of the standards to within ±5 s and ±10%, respectively.

RESULTS AND DISCUSSION

Immuno precolumn characteristics

In our experience, for quantitative determinations the total amount of the analytes should not exceed 60–70% of the binding capacity (cf. dilution of bile discussed below). The capacity of the immuno precolumn was determined by loading 53 ml of a standard solution of β-19-NT (20 μg/l) and comparing the peak area with that obtained by direct loading of the same standard solution on the C_{18} precolumn. When this procedure was performed two weeks after packing the immuno precolumn, that is, when about 50 samples and standards had been analysed, the binding capacity was found to be 250 ng of β-19-NT. After about 600 samples and standards had been run over a five-month period, the capacity had decreased to 60 ng. In our laboratory this capacity is now checked once every week.

The cross-contamination in a blank, run directly after loading the immuno precolumn to its maximum capacity (β-19-NT), is less than 0.2%.

Urine

In an earlier study [9], the immuno precolumn technique was only applied to spiked urine samples. In this study, urine samples from experiment I were first analysed directly, i.e., non-hydrolysed. Here, 25 ml of urine were diluted with 25 ml of acetonitrile–water (10:90) before loading on to the immuno precolumn. In these samples no β-19-NT and α-19-NT, i.e., less than 0.05 μg/l, could be detected. In other words, according to expectations, the concentrations of free β-19-NT and α-19-NT in urine from a treated calf are very low. As reported in the literature [10–12] also, most of the analytes are present as conjugates of glucuronic and sulphuric acids.

Hydrolysed urine

At first, experiments were performed in which 100 μl of *Helix pomatia* juice were added directly to 100 ml of urine (adjusted to pH 4.8). After incubation at 37°C for 2 h, 20 ml of the hydrolysed urine were diluted with 80 ml of water and 53 ml (corresponding to 10.6 ml of urine) were loaded on to the immuno precolumn. In these samples, however, many interfering compounds were observed, which allowed the detection of β-19-NT and α-19-NT only at levels above 1 μg/l. Flushing the loaded immuno precolumn with 20 ml of acetonitrile–water (5:95) prior to desorption gave only a minor improvement. The additional use of a liquid–solid sorption procedure involving Amberlite XAD-2 [10,16] prior to hydrolysis also did not give a significant improvement. Obviously, the hydrolysed urine contains much more interfering compounds than does the non-hydrolysed urine. One explanation is that hydrolysed urine will contain many deglucuronidated and desulphated compounds, which can interact either specifically with the immobilized antibodies or non-specifically with the stationary phase (Sepharose), the coupling group or even the large non-selective surface of the immobilized antibodies. In addition, impurities introduced by the *Helix pomatia* juice can cause interferences.

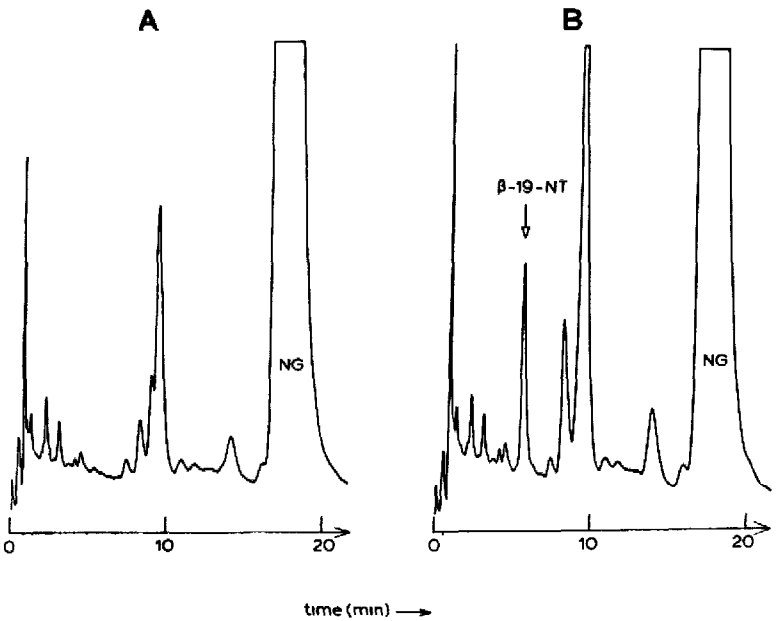

Fig. 1. Chromatograms of a hydrolysed urine sample (0.004 a.u.f.s.); 13.25 ml of urine sample were loaded on to the immuno precolumn. (A) Blank urine; (B) blank urine spiked with 1 μg/l β-19-NT. For further conditions, see the section on the HPLC procedure.

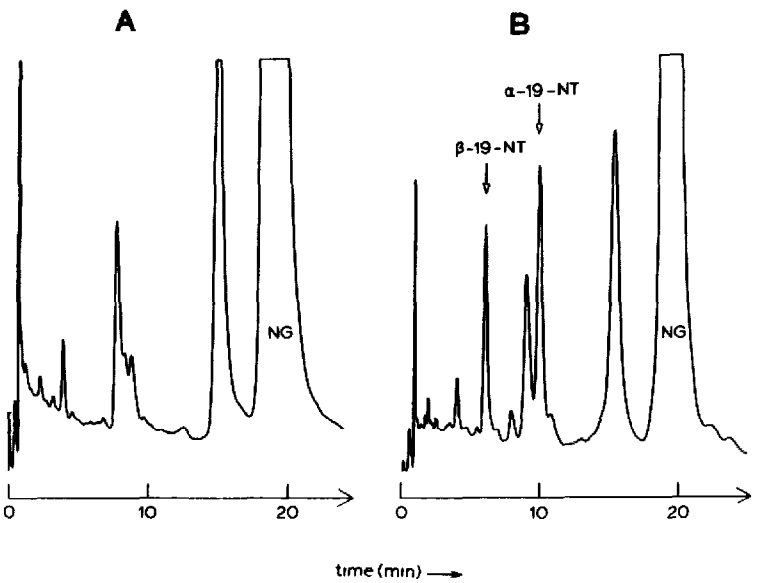

Fig. 2. Chromatograms of hydrolysed urine samples obtained from a calf treated with 19-nortestosterone 17β-laurate (experiment I); 36.5 ml of urine sample were loaded on to the immuno precolumn. (A) Urine taken before treatment (day 0; 0.004 a.u.f.s.); (B) urine taken seven days after treatment (0.008 a.u.f.s.). The concentration of β-19-NT was 1.3 μg/l and that of α-19-NT 3.1 μg/l. For further conditions, see the section on the HPLC procedure.

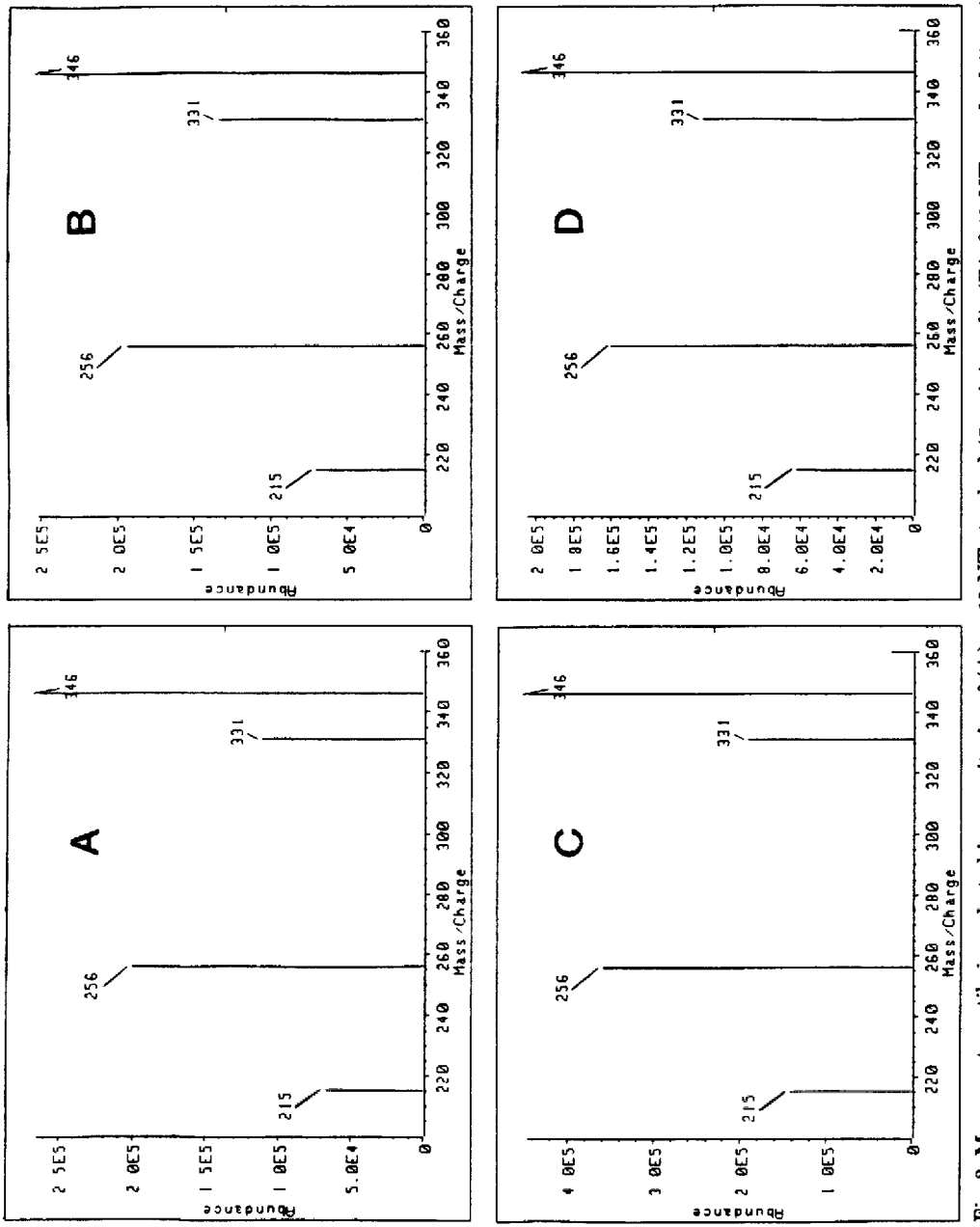

Fig. 3. Mass spectra, utilizing selected-ion monitorig, of (A) α-19-NT standard (5 ng injected), (B) β-19-NT standard (5 ng injected), (C) α-19-NT present in the HPLC fraction of urine taken from a calf (No. 13, experiment I) on the fifth day after treatment and (D) β-19-NT present in the HPLC fraction of the same urine as in (C).

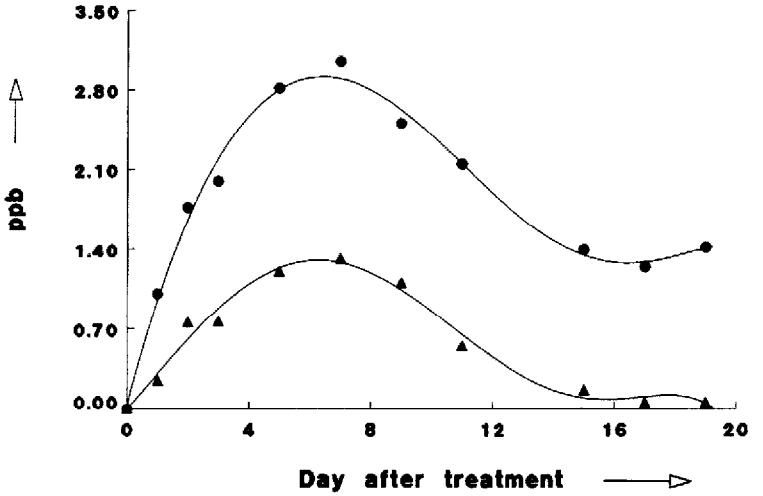

Fig. 4. Concentrations of β-19-NT (▲) and α-19-NT (●) in hydrolysed urine samples (experiment I) plotted against the number of days after treatment of the calf.

Final procedure. The sample pretreatment was further extended to include an extraction with ethyl acetate of the acidified hydrolysed urine sample, a procedure often used to hydrolyse steroid sulphates [3,10]. The ethyl acetate extract was incubated at 40°C for 1 h and traces of acid were removed by subsequent washings with sodium hydrogencarbonate and water. The ethyl acetate was evaporated to dryness, the residue was dissolved in 70 ml of acetonitrile–water (5:95) and 53 ml of the solution were transferred to the immuno precolumn. Using this procedure, most of the interfering compounds were removed and, as shown in Fig. 1, β-19-NT can now be determined far below the 1 μg/l level, whereas the recovery of β-19-NT added to blank urine at the 1 μg/l level was still $82 \pm 9\%$ ($n=10$). As shown, however, several compounds eluting between 8 and 11 min will disturb the detection of α-19-NT (retention time 9.2 min). These interfering compounds occur in urine samples in varying concentrations. Some of them were isolated, derivatized and analysed according to the procedure described under *Gas chromatography–mass spectrometry*. However, identification could not be achieved, probably because of the absence of hydroxy and carbonyl groups.

The presence of β-19-NT and α-19-NT in the HPLC fractions could be confirmed by GC–MS above the 0.05 μg/l level. As demonstrated in Fig. 2, β-19-NT and α-19-NT could be detected and confirmed (Fig. 3) in urine samples from experiment I. In Fig. 4 the results of the analyses of urine samples from this experiment are presented. The maximum concentrations of β-19-NT (1.3 μg/l) and α-19-NT (3.1 μg/l) were observed seven days after application. At the end of the experiment (day 19), the concentration of β-19-NT was just above the detection limit (0.05 μg/l), whereas the amount of α-19-NT was still 1.4 μg/l.

The results indicate that, under the conditions used in this experiment, β-19-NT can be detected unambiguously, i.e., at three times the detection limit, and the results confirmed by GC–MS for two weeks after intramuscular application of a preparation containing 19-nortestosterone 17β-laurate and estradiol 17β-

benzoate. Using the presence of the metabolite α-19-NT as proof of the use of such a preparation, control is possible for at least three weeks after application. For the latter procedure, however, the separation of α-19-NT from the interfering compounds should preferably be improved. Also, the absence of this metabolite in urine samples from non-treated calves still has to be established.

From the excretion study shown in Fig. 4, half-lives of two days for β-19-NT and fifteen days for α-19-NT were calculated. The results of this excretion study are in agreement with those described by Rattenberger et al. [7]. Tuinstra [17] and Van Ginkel et al. [18] found comparable concentrations of β-19-NT and α-19-NT in urine samples from treated calves.

Bile

Bile samples from the control calf (No. 12) and the treated calf (No. 13), both obtained from experiment I, were also analysed and the results are presented in Fig. 5. In the bile sample from the treated calf only α-19-NT was found, the concentration being 55 ± 2 μg/l ($n=4$). The recovery of α-19-NT, added to the blank bile sample at the 10–50 μg/l level, varied between 85 and 105% (mean 91%; $n=10$). Because of the large amount of α-19-NT in the positive bile sample, small sample volumes (2 ml) were used to prevent overloading of the immuno precolumn. The concentration of the metabolite in bile is high compared with that in urine samples. In ten bile samples from calves with proved administration of β-19-NT, analysed at the RIVM [18], similar concentrations of α-19-NT (18–62 μg/l; mean 43 μg/l) were found and also varying concentrations of β-19-NT (0.1–15 μg/l; mean 2.5 μg/l). In that study the mean ratio of the concentrations of α-19-NT and β-19-NT was calculated to be 79. Using the immuno precolumn

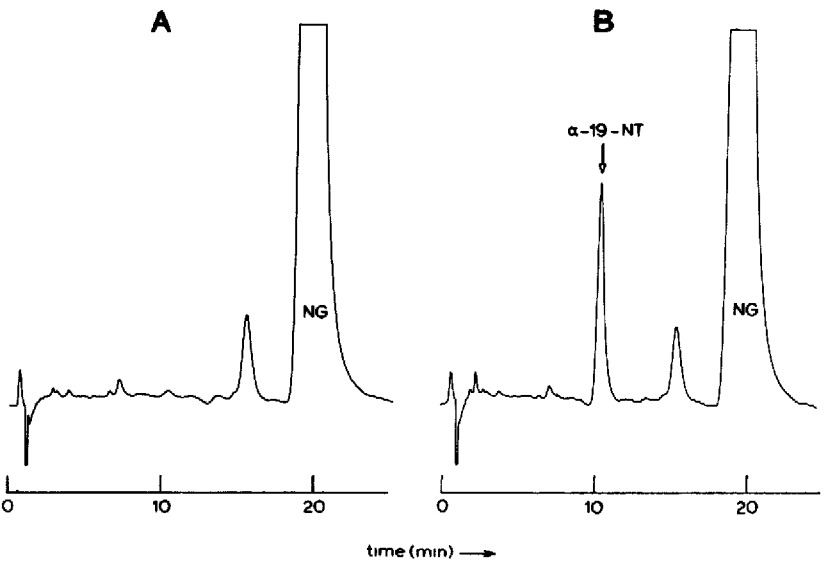

Fig. 5. Chromatograms of hydrolysed bile samples from experiment I (0.008 a.u.f.s.); 1.5 ml of bile sample was loaded on to the immuno precolumn. (A) Blank bile sample (calf No. 12); (B) bile from a treated calf (No. 13). For further conditions, see the section on the HPLC procedure.

with its high cross-reactivity for α-19-NT, it is difficult to detect small concentrations of β-19-NT in bile samples containing high concentrations of the metabolite. With small sample volumes, the amount of β-19-NT will often be below the detection limit and when using large sample volumes, the high concentration of α-19-NT will overload the immuno precolumn. Hence for the determination of β-19-NT in bile samples either an immuno precolumn with low cross-reactivity for α-19-NT or an immuno precolumn with a much higher binding capacity for the analytes has to be used.

Meat

Enzymatic digestion with a proteolytic enzyme has been used to obtain higher extraction recoveries of anabolics from tissue samples [19, 20]. Enzymatic deconjugation has not been applied because in muscle, hormones are mainly present as free compounds [21]. Using, for these reasons, a procedure involving the addition of protease only and, subsequently, an Amberlite XAD-2 sorption step, the recovery of β-19-NT, added to blank meat at the 2 μg/kg level, was $82 \pm 5\%$ ($n=4$) and the detection limit was 0.05 μg/kg. Meat samples from experiments I and II were analysed and only β-19-NT was found. The results are given in Table I and a typical example is shown in Fig. 6. The β-19-NT content of four of the six samples was found to be between 0.1 and 0.2 μg/kg. Although the calves had been treated similarly, the meat samples from calf No. 6 showed much higher concentrations of β-19-NT (up to 1.6 μg/kg). No values for the concentration of β-19-NT in meat samples could be found in the literature. However, in studies on trenbolone and zeranol in meat samples [22,23], similar concentrations were reported.

Liver and kidney

After enzymatic digestion with a proteolytic enzyme, the liver and kidney samples from experiments I and II were deglucuronidated and desulphated with *Helix pomatia* juice and treated as described for urine samples. The recoveries for β- and α-19-NT added to blank samples at the 1 μg/kg level were 51 ± 5 and $44 \pm 4\%$

TABLE I

CONCENTRATION OF β-19-NT IN MEAT SAMPLES FROM THREE CALVES TREATED WITH 19-NORTESTOSTERONE 17β-LAURATE

Muscle	Experiment	Calf No.	β-19-NT (μg/kg)
Diaphragm	I	13	0.1
M. longissimus dorsi	I	13	0.2
M. gluteus medius + M. semidendinosis	II	2	0.1
M. longissimus dorsi	II	2	0.2
M. gluteus medius + M. semidendinosis	II	6	0.9
M. longissimus dorsi	II	6	1.6

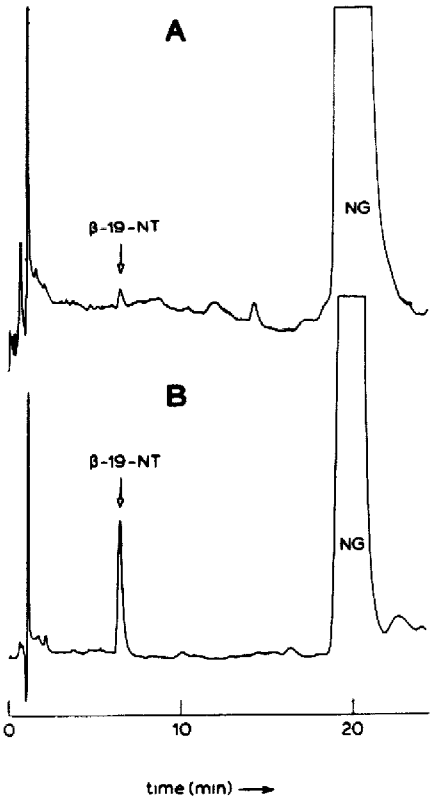

Fig. 6. Chromatograms of extracts from meat samples. The loaded extracts correspond to 15.1 g of meat. (A) Diaphragm from experiment I, containing 0.1 μg/kg β-19-NT (0.004 a.u.f.s.); (B) M. longissimus dorsi from experiment II, containing 1.6 μg/kg β-19-NT (0.008 a.u.f.s.). For further conditions, see the section on the HPLC procedure.

TABLE II

CONCENTRATIONS OF β-19-NT AND α-19-NT IN LIVER AND KIDNEY SAMPLES FROM THREE CALVES TREATED WITH 19-NORTESTOSTERONE 17β-LAURATE

Tissue	Experiment	Calf No.	β-19-NT (μg/kg)	α-19-NT (μg/kg)
Liver	I	13	0.1	0.8
Kidney	I	13	0.5	0.5
Liver	II	2	0.1	0.5
Kidney	II	2	0.5	1.6
Liver	II	6	<0.05	0.9
Kidney	II	6	0.4	1.2

($n=4$), respectively. The concentration of β-19-NT in the liver samples was low ($\leqslant 0.1$ μg/kg), whereas in kidney samples the concentrations were between 0.4 and 0.5 μg/kg (Table II). The concentration of α-19-NT in the liver and kidney samples varied between 0.5 and 1.6 μg/kg (Table II).

In the literature no data are reported for concentrations of β-19-NT and α-19-NT in liver and kidney tissue. The concentration of nortestosterone found in this study are of the same order of magnitude as the levels of natural steroids such as estradiol and testosterone [21], which are present both as conjugates and in the free form (accumulated in fat).

Trenbolone

Because of the relatively high cross-reactivity of the antibodies for 17β-trenbolone (40%), the simultaneous determination of this anabolic should be possible. Standard solutions of 17β-trenbolone and also 17α-trenbolone can indeed be preconcentrated on the immuno precolumn in the same way as nortestosterone (Fig. 7). Despite the poor separation of 17α-trenbolone from β-19-NT, both compounds can be determined simultaneously when using a dual-wavelength detector set at 247 and 340 nm.

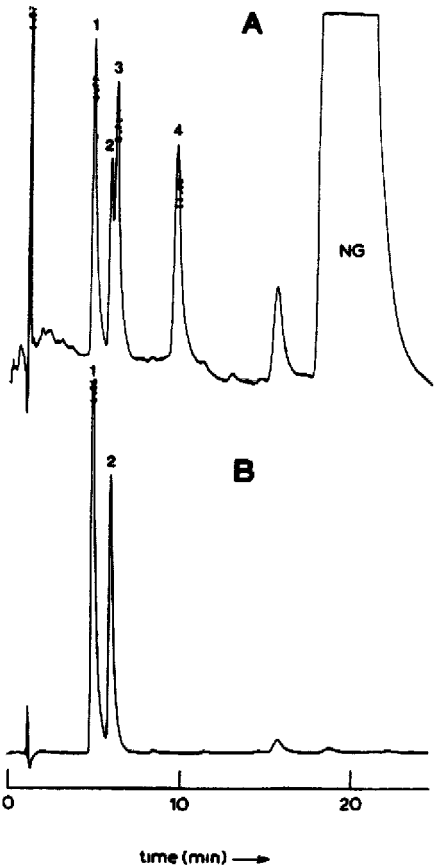

Fig. 7. Chromatograms of a mixture of (1) 17β-trenbolone (1 μg/l), (2) 17α-trenbolone (2 μg/l), (3) β-19-NT (0.2 μg/l) and (4) α-19-NT (0.3 μg/l); 53 ml of this mixture were loaded on to the immuno precolumn. (A) UV detection at 247 nm (0.002 a.u.f.s.); (B) UV detection at 340 nm (0.008 a.u.f.s.). For further conditions, see the section on the HPLC procedure.

CONCLUSIONS

The selectivity of an immuno precolumn, i.e., a precolumn packed with immobilized antibodies against β-19-NT, results in a high clean-up efficiency, which allows the determination of β-19-NT in biological samples at the sub μg/kg level using simple UV detection. At the same time, because of the high sample volumes, fractions can be used for confirmation by GC–MS.

By using an automated HPLC system including an immunoaffinity precolumn, the number of steps required for sample pretreatment is lower than that in the off-line procedure, which leads to a better reproducibility. With careful selection of the antiserum, more than one analyte can be determined and cross-reacting compounds such as, in this work, the metabolite α-19-NT and also trenbolone, a structurally related steroid, can be distinguished and determined. The immuno precolumn has been used for more than 600 runs. The cross-contamination in a blank, run directly after loading the immuno precolumn to its maximum capacity (β-19-NT), is less than 0.2%.

The analyte of major interest, the parent compound β-19-NT, was found in urine from a calf treated with 19-nortestosterone 17β-laurate, with a maximum concentration of 1.3 μg/l on the seventh day after intramuscular application. The analyte was also found in meat (0.1–1.6 μg/kg), liver (up to 0.1 μg/kg) and kidney (0.4–0.5 μg/kg) samples from treated animals. The metabolite α-19-NT was found in urine from a treated calf, with a maximum concentration of 3.1 μg/l and in bile (55 μg/l). In liver and kidney samples from treated calves the concentrations of α-19-NT varied from 0.5 to 0.9 and 0.5 to 1.6 μg/kg, respectively. Obviously, in biological samples from treated calves the concentration of the metabolite α-19-NT is much higher than that of the parent compound β-19-NT, especially in the bile sample. The absence of the metabolite from biological samples from non-treated calves has to be established before this more abundant metabolite can be used as an indicator or possible proof of the application of β-19-NT esters.

Future research will be focused on (i) designing a fully automated system by including an autosampler and a precolumn containing immobilized enzymes to achieve on-line hydrolysis and (ii) extending the range of analytes that can be determined in one run by using a mixture of antibodies or other group-selective (bio)molecules.

ACKNOWLEDGEMENTS

The authors thank P. Leenders for experimental assistance, W.A. Traag and H. Hooijerink for GC–MS measurements, P.L.M. Berende for performing the animal experiments and supplying sample materials, G.D. van Bruchem for determining the cross-reactivities and R.J.A. Paulussen for stimulating discussions. We are indebted to the National Inspection Service for Meat, Livestock and Animal Products for technical support and to Organon for a gift of 17α-19-nortestosterone, Roussel Uclaf for a gift of 17β-trenbolone and the RIVM for 17α-trenbolone.

REFERENCES

1 P.L.M. Berende and E.J. Ruitenberg, in L.J Peel and D.E. Tribs (Editors), Domestication, Conservation and Use of Animal Resources, Elsevier, Amsterdam, 1983, pp. 191–233.
2 Council of the European Communities, Council Directive 86/469/EEC of 16 September 1986, Off. J. Eur. Commun., L275 (1986) 36.
3 J.M. Weseman, H. Hooijerink, W.A. Traag, M.J. van Steenbergen, H.J. Korbee and R. Schilt, paper presented at the International Symposium on the Analysis of Anabolizing and Doping Agents in Biosamples, Ghent, May 16–19, 1988.
4 E. Houghton, Xenobiotica, 7 (1977) 683.
5 L.A. van Ginkel, H. van Blitterswijk, P.G. Langenbroek and R.W. Stephany, Berichten RIVM, Bilthoven, 1986, p. 234.
6 K. Vogt, Arch. Lebensmittelhyg., 38 (1987) 71.
7 E. Rattenberger, P. Matzke, P. Funke and H.H.D. Meyer, Arch. Lebensmittelhyg., 38 (1987) 73.
8 E.H.J.M. Jansen, R.H. van den Berg, G. Zomer and R.W. Stephany, J. Clin. Chem. Clin. Biochem., 23 (1985) 145.
9 A. Farjam, G.J. de Jong, R.W. Frei, U.A.Th. Brinkman, W. Haasnoot, A.R.M. Hamers, R. Schilt and F.A. Huf, J. Chromatogr., 452 (1988) 419.
10 C.H.L. Shackleton, J. Chromatogr., 379 (1986) 91.
11 M.C. Dumasia, E. Houghton and S. Sinkins, J. Chromatogr., 377 (1986) 23.
12 E. Houghton, M.C. Dumasia, P. Teale, M.S. Moss and S. Sinkins, J. Chromatogr., 383 (1986) 1.
13 H.J. Kyrein, Z. Lebensm.-Unters.-Forsch., 177 (1983) 426.
14 M. Steinbuch and R. Audrian, Arch. Biochem. Biophys., 134 (1969) 279.
15 Affinity Chromatography, Principles and Methods; Product Information, Pharmacia, Uppsala, 1983.
16 R. Verbeke, J. Chromatogr., 177 (1979) 69.
17 L.G.M.Th. Tuinstra, Report No. 86.07, RIKILT, Wageningen, 1986.
18 L.A. van Ginkel, H. van Blitterswijk, J. Zuydendorp, P.W. Zoontjes, H.J. van Rossum and R.W. Stephany, Report No. 368301 009, RIVM, Bilthoven, 1986.
19 G. de Groot and K.L. Wubs, J. Anal. Toxicol., 11 (1987) 175.
20 C. Van Peteghem, J. Chromatogr., 369 (1986) 253.
21 B. Hoffmann, Report No. VI/1533/88-EN, Commission of the European Communities, Directorate-General for Agriculture, Brussels, 1988.
22 S.N. Dixon, K.L. Russel, R.J. Heitzman and C.B. Mallinson, J. Vet. Pharmacol. Ther., 9 (1986) 353.
23 M. Rapp, Thesis, Technische Universität, Munich, 1986.