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Development of a competitive enzyme immunoassay for 17α-19-nortestosterone

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ABSTRACT

Because 17β-19-nortestosterone and its esters are frequently used anabolic steroids in cattle in Europe, there is a need for an assay that can also detect certain metabolites. The enzyme immunoassay described here involves the detection and quantitation of the major metabolite 17α-19-nortestosterone in urine. The assay is based on the coating of an antibody raised in a rabbit against 17α-19-nortestosterone-3-carboxymethyloxime–bovine serum albumin (17α-19-NT-3-CMO-BSA), the competitive incubation of 17α-19-NT and the 17α-19-nortestosterone-3-CMO–horseradish peroxidase label, followed by the detection of the blue colour developed by the action of the enzyme on tetramethylbenzidine. The 3-CMO conjugate of 17α-19-nortestosterone was used to produce an antibody with selective affinity for the 17α-epimer. For the optimization of the assay, different coatings and incubation conditions were tested. The standard curve ranged between 0.98 and 4000 pg per well, with a B/B_0 50% of ± 65 pg per well. Colour was measured with a microtitre plate reader. The method was validated by means of certified blank and spiked cattle urine samples.

INTRODUCTION

17β-19-Nortestosterone (17β-19-NT) is an anabolic steroid with an efficacy comparable with that of the naturally occuring hormone testosterone. From the regulatory controls, it is well known that 19-nortestosterone (19-NT) and its esters are still the most frequently used anabolizing agents in cattle production.

However, after injection of 19-NT or its esters, the 17α-19-NT epimer is the predominant metabolite found in urine [1]. Therefore a reproducible and sensitive detection method is required for this compound. Although radioimmunoassays are widely used for similar purposes, the development of non-isotopic methods is

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desirable because they are less costly, have no problems associated with the use and disposal of radioisotopes, and use labels that are easy to synthesize and with long shelf-lives. Since enzyme immunoassays (EIA) have been introduced in steroid analysis, assays in microtitre plates are probably the most useful alternatives presently available.

Microtitre plate EIAs have been described for progesterone [2], trenbolone [3], 17β-19-NT [4,5], methyltestosterone and diethylstilbestrol [6] and zeranol [7].

The competitive immunoassay described here makes use of a highly specific antibody against 17α-19-NT and a horseradish peroxidase label conjugated to 17α-19-NT. The method is fast and sensitive enough to detect sub-ppb levels of the metabolite in cattle urine.

EXPERIMENTAL

Reagents and instrumentation

17α-19-Nortestosterone was kindly provided by Prof. Martens (LUC, Diepenbeek, Belgium). All other steroids were purchased from Steraloids (Wilton, NH, U.S.A.) or Sigma (St. Louis, MO, U.S.A.). All organic solvents were of analytical grade.

The HPLC equipment consisted of the following components: an automatic injector (Model 231, Gilson, Meyvis, Bergen op Zoom, The Netherlands), two solvent-delivery systems (Model 2150, LKB, Woerden, The Netherlands) controlled by a gradient controller (Model 2152, LKB), a UV–VIS detector (Model 200, Linear, Analytica, Maasdijk, The Netherlands) and a computing integration system (Model CI-10B, LDC/Milton Roy, Interscience, Breda, The Netherlands). Bond Elut C_{18} columns (1 ml) were purchased from Analytichem International (Harbor City, CA, U.S.A.), and PD-10 columns from Pharmacia (Woerden, The Netherlands).

Horseradish peroxidase (HRP) and *Helix pomatia* juice were obtained from Boehringer (Mannheim, Germany). Gelatine (type AAA) was purchased from N.V. Delft (Delft, The Netherlands). Flat-bottomed eight-well strips (type Maxisorp) were obtained from Life Technologies (Ghent, Belgium). All strips were read with a microtitre plate reader of Eurogenetics (Tessenderlo, Belgium). Quantitative results were calculated with a Tulip personal computer (Compudata, Malines, Belgium) with the available ELISA-aid software (Eurogenetics).

Preparation of 17α-19-nortestosterone antiserum

The 17α-19-nortestosterone-3-carboxymethyloxime conjugate was prepared as follows. To a solution of 17α-19-nortestosterone (20 mg) in dry pyridine (1 ml), carboxymethoxyamine hemihydrochloride (14 mg) was added. The mixture was stirred at room temperature for 24 h. The pyridine was removed by distillation under reduced pressure. The residue was dissolved in chloroform (25 ml). The organic layer was washed four times with distilled water (10 ml), dried over

anhydrous sodium sulphate, and removed by distillation under reduced pressure. The resulting white foam was triturated with diethyl ether to afford 17α-19-nortestosterone-3-carboxymethyloxime as a white powder (17 mg, 85%). The purity was checked by ^{1}H NMR. The NMR spectrum (Jeol 270 MHz, C^2HCl_3) shows an almost 1:1 mixture of the *syn* and *anti* isomers of 17α-19-nortestosterone-3-carboxymethyloxime conjugate. In the *anti* isomer the vinylic C-4 proton absorbs at 5.86 ppm (relative to tetramethylsilane), and the *syn* isomer absorbs at 6.50 ppm. The two isomers also show different absorptions for the N–O–CH_2–COO protons at 4.60 and 4.59 ppm. The C-17 proton gives a doublet (J = 6 Hz) centred at 3.77 ppm. The C-18 methyl group absorbs at 0.71 ppm.

The 17α-19-nortestosterone-3-carboxymethyloxime conjugate of bovine serum albumin (BSA) was prepared as follows. To a solution of 17α-19-nortestosterone-3-carboxymethyloxime (6.6 mg) in dry dioxane (500 μl), 220 μl of a solution of N-hydroxysuccinimide in dry dioxane (10 mg/ml) were added. The reaction mixture was cooled to 0°C, and 12 μl of a solution of dicyclocarbodiimide (330 mg/ml) in dry dioxane was added. The reaction mixture was kept at 4°C for 18 h. After centrifugation of the white crystals, 300 μl of the supernatant were added to a solution of 50 mg of BSA in 3 ml of phosphate-buffered saline (0.1 *M*, pH 8.5). The reaction mixture was kept at 4°C for 60 h, then dialysed against distilled water.

The immunization procedure was performed as follows. The primary injection (rabbit No. 57) consisted of 0.5 ml of a solution of 0.6 ml of the BSA conjugate in saline (4 mg/ml) and 0.8 ml of Freund's adjuvant complete. The booster injections consisted of the same solution with Freund's adjuvant incomplete at six and twelve weeks after the first injection. At week 14 the animals were treated with 1.5 ml of nembutal followed by collection of the blood by heart puncture. After standing for 3 h at room temperature, the serum was obtained by centrifugation (1000 *g*, 15 min).

The immunoglobulin G (IgG) fraction of the antiserum was prepared by precipitation with sodium sulphate (315 mg of sodium sulphate with 1.75 ml of serum). After centrifugation (1000 *g*, 5 min), the precipitate was washed twice with 1.75 ml of a solution of 18% sodium sulphate. The final precipitate was dissolved in 2.5 ml of distilled water and applied to a PD-10 column in order to exchange the buffer [0.1 *M* phosphate-buffered saline (PBS), pH 7.0] and to remove the sodium sulphate. The final volume was 3.5 ml, which was stored at −20°C.

Synthesis of the 17α-19-nortestosterone-3-carboxymethyloxime conjugate with horseradish peroxidase

The 17α-19-nortestosterone-3-carboxymethyloxime conjugate of horseradish peroxidase (HRP) was prepared as follows. To a solution of 17α-19-nortestosterone-3-carboxymethyloxime (1.0 mg) in dry dioxane (250 μl), 25 μl of a solution of N-hydroxysuccinimide in dry dioxane (10 mg/ml) were added. The reaction

mixture was cooled to 0°C, and 5 μl of a solution of dicyclocarbodiimide (250 mg/ml) in dry dioxane were added. The reaction mixture was kept at room temperature for 24 h. The course of the reaction was followed by high-performance liquid chromatographic (HPLC) analysis of the reaction mixture. The HPLC column (150 mm × 4.6 mm I.D.) with Valco fittings and 2-μm frits (Chrompack, Middelburg, The Netherlands) was packed with Hypersil ODS (5 μm) (Shandon, Zeist, The Netherlands) using a column packing instrument according to the manufacturers' instructions. Gradient elution was applied with two mobile phases. Mobile phase A was methanol–water (50:50, v/v) and mobile phase B was methanol. The elution conditions were: from 0 to 8 min, a linear gradient from 0 to 100% B; from 8 to 10 min, elution with 100% B to clean the column from tightly bound components; from 10 to 10.5 min, a linear gradient from 100 to 0% B; from 10.5 to 15 min, 100% A to equilibrate the column for the next injection. Detection occurred at 240 nm. From the reaction mixture, 1 μl was diluted with 200 μl of mobile phase A by the autoinjector. From this mixture 25 μl were injected into the HPLC column. With these conditions, the starting 17α-19-nortestosterone-3-carboxymethyloxime eluted as a rather broad peak at a retention time of 2.89 min, which probably consisted of a mixture of the *syn* and *anti* isomers. After 24 h the starting reagent had disappeared completely and eluted as 17α-19-nortestosterone-3-carboxymethyloxime-N-hydroxysuccinimide at a retention time of 10.75 min.

After centrifugation of the white crystals, 80 μl of the supernatant (five equivalents) were added to a solution of 5 mg of HRP (one equivalent) in 920 μl PBS (0.1 *M*, pH 8.5). The same procedure was performed with a 10:1 equivalent ratio of active ester to HRP. The reaction mixtures were kept at 4°C for 72 h, followed by desalting on a PD-10 column to remove unreacted low-molecular-mass compounds. The eluates of the PD-10 columns were monitored for peroxidase activity by colorimetric assay with tetramethylbenzidine.

Buffers and EIA solutions

PBS solution [(0.01 *M*, pH 7.25) in 0.9% NaCl] was used for coating the antibody to the microtitre plate wells. Another PBS solution (0.02 *M*, pH 7.2), to which 0.05% Tween 20 was added, was used for the washing steps. A more concentrated PBS solution (0.1 *M*, pH 7.0) with 0.1% gelatine was used as blocking reagent and as incubation buffer. Hydrolysis buffer consisted of 100 μl of *Helix pomatia* juice in 5 ml of PBS (0.1 *M*, pH 7). The substrate solution was prepared by addition of 2 ml of sodium acetate buffer (1.1 *M*, pH 5.5), 300 μl of tetramethylbenzidine (6 mg per 100 ml of dimethyl sulphoxide) and 4 μl of hydrogen peroxide (30%) to 18 ml of bidistilled water. This solution was prepared freshly before use. The stop reagent was 1 *M* H_2SO_4. Standards were made up freshly for each assay a stock solution of 17α-19-NT in methanol (1 mg/ml).

Urine samples

Certified blank control samples were spiked with 0.5, 1 or 2 ppb. Positive samples, obtained from different slaughterhouses, were previously analysed by radioimmunoassay [8] for 17α-19-NT.

Extraction of the urine samples

To 0.5 ml of urine, 300 μl of hydrolysis buffer were added. This solution was incubated for 1 h at 37°C. After dilution with 4 ml of bidistilled water the hydrolysed urine was brought onto a C_{18} column, previously conditioned with two 1-ml volumes of methanol and two 1-ml volumes of water. After addition of the urine sample, the column was washed with 1 ml of water and 17α-19-NT was eluted with two 1-ml volumes of ethyl acetate. This solvent was evaporated under a gentle stream of nitrogen at 37°C in a water-bath. Incubation buffer (1 ml) was added, and the tube was vortexed for 10 s. A 0.05-ml aliquot of the extract was used in the assay, corresponding to 0.025 ml of the urine sample.

EIA protocol

Microtitre plates were coated with 17α-19-NT rabbit antiserum in 100 μl (dilution 1:4000) of coating buffer per well for 2 h at 37°C. The plates were washed three times with 300 μl of washing buffer. The remaining active sites were saturated with blocking reagent for 90 min at 37°C.

After drying, the plate was incubated with standards or sample extract (0.05 ml) and HRP label (0.05 ml, dilution 1:50 000) in incubation buffer for 90 min at 37°C. The plate was washed again three times with washing buffer and dried on absorbent paper, and the enzyme activity was determined by addition of 200 μl of substrate solution. After 20 min reaction time, the colour development was stopped by addition of 50 μl of 1 *M* H_2SO_4. The absorbances were measured at 450 nm in a microtitre plate reader.

RESULTS AND DISCUSSION

Synthesis of label and immunogen

In order to obtain an immunoassay specific for the metabolite 17α-19-NT and with a low affinity to its 17β stereoisomer, the 3-position of the steroid moiety was chosen as site of attachment. To obtain a homologous immunoassay both the immunogen and the enzyme conjugate were coupled at the 3-position. From ^1H NMR analysis it appeared that the 17α-19-nortestosterone-3-carboxymethyloxime consisted of a mixture of the *syn* and *anti* isomers at the 3-position in a ratio of *ca.* 1:1. The occurrence of different isomers apparently did not influence the specificity of the immunoassay. As far as possible the synthesis of the starting reagent, 17α-19-nortestosterone-3-carboxymethyloxime-N-hydroxysuccinimide, was followed by HPLC analysis. In order to investigate the influence of the extent of substitution of the amount of active ester to HRP, an excess of five or ten times

of active esters was used to prepare the enzyme conjugate. Since no difference was observed in binding between the antiserum and both conjugates, further experiments were performed with the HRP conjugate, which was prepared with a ratio of 1:5.

Specificity of the antibody

The cross-reactivity of the antibody was determined by comparison of the responses of 17α-19-NT with structurally related compounds. The percentage cross-reactivity was determined as the ratio of the amount of cross-reacting material that yields 50% inhibition of binding with the amount of standard giving the same inhibition. The affinity was calculated from logit–log plots. The results are shown in Table I.

As could be expected on a theoretical basis, the specificity of the antiserum for 17α-19-NT was excellent. All the tested steroids but one showed cross-reactivities of 0.2% or less. It appeared that a combination of two structural characteristics, the 17α-hydroxyl group and the lack of the 19-methyl group, predominantly determined the specificity. Only 5α-estrane-3β,17α-diol, which has both structural characteristics, showed a substantial cross-reactivity (11.3%). Furthermore, the presence of an aromatic *A*-ring also destroyed nearly all the affinity for estradiol.

Dilution curves of enzyme label and antibody

To determine the optimal concentration of the enzyme label and antibody, a two-dimensional titration curve was established. Enzyme dilutions from 1:25 000 to 1:100 000, and antibody dilutions from 1:500 to 1:16 000, were used. The two-dimensional titration of 17α-19-NT antibody and 17α-19-NT-3CMO–HRP is given in Fig. 1.

For all further experiments an enzyme dilution of 1:50 000 and an antibody dilution of 1:4000 were chosen.

TABLE I

CROSS-REACTIVITIES OF STRUCTURALLY RELATED COMPOUNDS

Compound	Cross-reactivity (%)
17α-19-Nortestosterone	100
17β-19-Nortestosterone	0.078
17α-Testosterone	0.2
17β-Testosterone	0.001
17α-Estradiol	0.2
17β-Estradiol	0.001
5α-Estrane-3β,17α-diol	11.34
19-Noretiocholanolone	0.03
17α-Methyltestosterone	0.002
Trenbolone	0.007

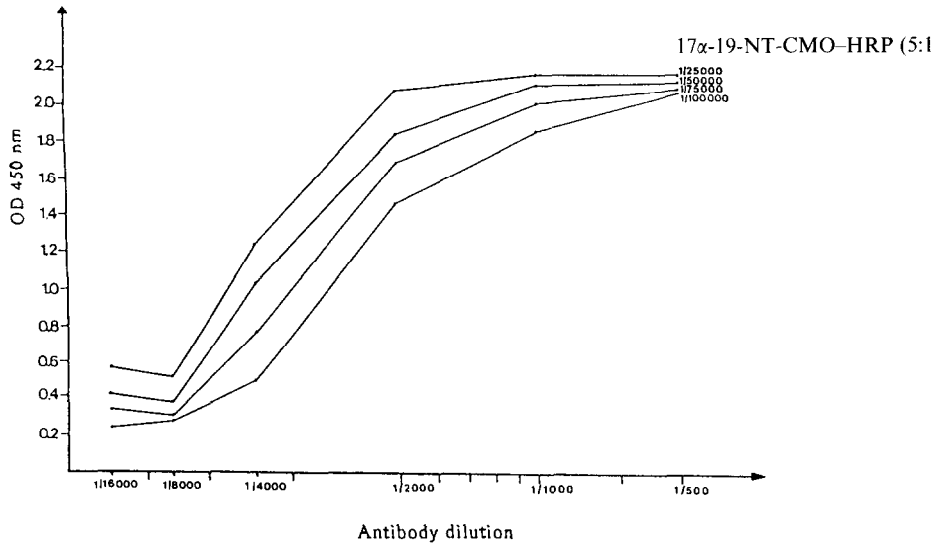

Fig. 1. Two-dimensional titration of enzyme label and antibody.

Standard curve

The dose–response curve covering the range 0–4 ng is shown in Fig. 2. The working range for 17α-19-NT is between 7 pg per well (90% B/B_0) and 1000 pg per well (10% B/B_0). The apparent concentrations of 17α-19-NT in various volumes of urine were determined by diluting a high positive cattle urine in the series 1:2, 1:4, 1:8 and 1:16. A plot of the apparent 17α-19-NT concentration (y) against

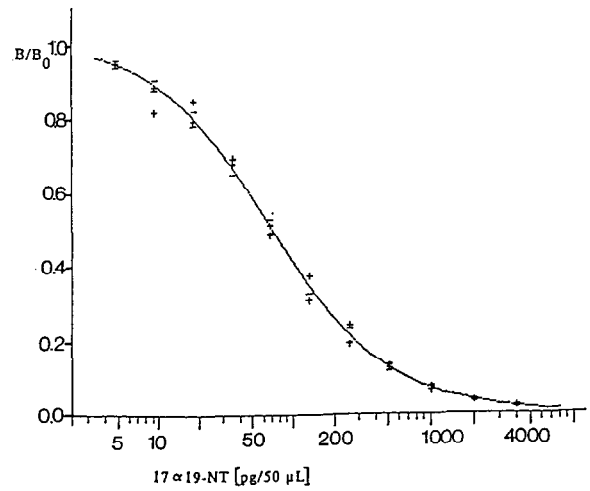

Fig. 2. Standard calibration curve for the EIA of 17α-19-NT.

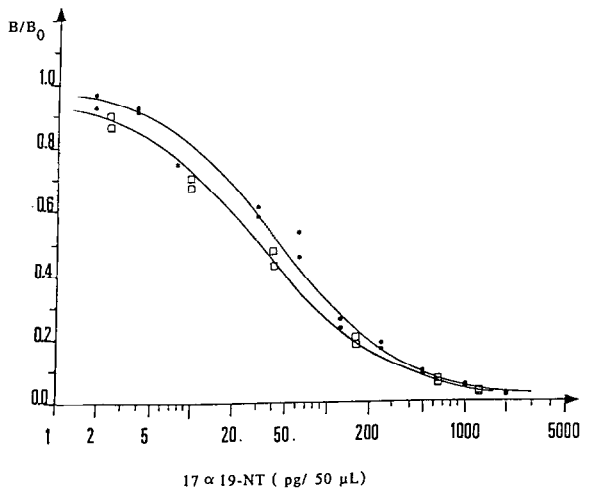

Fig. 3. Comparison of standard calibration curves for EIA in the presence (□) and absence (●) of urine.

volume of urine extract (x) shows is linear, given by the equation $y = 8.463 + 0.128\,x$, and the regression of these data gives a correlation coefficient of 0.9966.

The parallelism between a standard curve obtained with standards and spiked urine samples was checked. The results are shown in Fig. 3.

The lowest amount of 17α-19-NT that could be determined from a blank urine was 2.5 pg per well or 0.1 µg/l.

Repeated assays established the precision of the assay. The day-to-day variation ($n = 6$) of spiked urine samples at the 1.2- and 2.5-ppb levels were 10.6 and 13.3%, respectively. The within-day variation ($n = 4$) of the spiked urine samples at the 0.4- and 2-ppb levels were 12.1 and 14.5%, respectively.

In conclusion, it has become clear that the competitive EIA for 17α-19-NT described here is very specific and sensitive to low levels of 17α-19-NT. Although only a limited number of urine samples have been tested, the applicability for the determination of urinary 17α-19-NT looks very promising.

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