

**BIOTIN-STREPTAVIDIN ENZYME IMMUNOASSAY FOR THE DETERMINATION
OF OESTROGENS AND ANDROGENS IN BOAR FAECES**

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INTRODUCTION

Measuring sex steroids in faeces for monitoring the reproductive status is a tool in veterinary medicine (1-3). A non-invasive method of sampling is especially useful in zoo- and wildlife animals. Sex-steroids are metabolized in the liver and excreted via faeces in unconjugated form. As the metabolites differ between species, group specific assays have to be established. To avoid time consuming clean up procedures sensitive enzymeimmunoassays (EIA) were developed using the biotin-streptavidin system.

EXPERIMENTAL

Synthesis of the steroid-DADOO-biotin conjugates:

The following steroid derivatives (Steraloids Inc.) were used to synthesize biotinylated labels:

5 α -androstane-3 β ,17 β -diol-3-hemisuccinate (HS), 5 α -androstane-2-bromo-3,17-dione and 17 β -oestradiol-17-glucuronide. A 5 α -androstane-3,17-dione-thioether was prepared from the 2 α -bromo-steroid by the method described by Kohen et al. (4). All were coupled to N-biotinyl-1,8-diamino-3,6-dioxaoctane (DADOO-biotin; Boehringer Mannheim, 1119818) using a mixed anhydrid reaction (5) as follows:

Solvent A: 10 mg of the steroid derivative were dissolved in 1 ml dimethylformamid (DMF, Merck, 2937) and cooled under constant stirring to -18°C. Subsequently 10 μ l methylmorpholin (Merck Nr. 805894) and 5 μ l isobutylchloroformiate (Merck, 802358) were added.

Solvent B: 5 mg biotinyl-DADOO in 1 ml solvent, consisting of 2 parts DMF and 1 part sodiumphosphate buffer (0.1 mol/l; pH 8.0) were cooled to -18°C.

After 30 min solvent A and B were mixed under constant stirring. The mixture was incubated for 3 hours (light protected). Afterwards 10 ml of Aqua dest. were added and the sample was passed through a primed Sep-pak C18 cartridge (Millipore Waters Ass., WAT 51910). The cartridge was washed with 5 ml water and the conjugate eluted with methanol (4 ml). It was separated from the precursors by HPLC using a C18 column (Novapak C18; 3,9 x 150 mm; Millipore Waters Ass.) with a linear methanolic gradient starting with 20% and ending with 100%. Fractions of 1 ml were collected and analyzed for streptavidin-binding products. With the fractions containing the immunoreactive biotin conjugate checker board titrations with displacement were performed as described by Meyer (1989) to find optimal dilutions of antibody and label.

EIA procedure

The EIAs were performed on microtitre plates with a double antibody technique as described by Möstl et al. (1987). The antisera were raised in rabbits against testosterone-3-CMO-bovine-serum-albumin (BSA) or 5 α -androstane-3 α -ol-17-one-HS:BSA and in sheep against 17 β -oestradiol-17-HS:BSA. Testosterone, epiandrosterone and oestrone were used as standards. Horse-radish-peroxidase (POD) linked streptavidin (Boehringer Mannheim, 1089153) bound in a second incubation step to the biotinylated label (diluted 1:40000, incubated for 45 min at 4°C on a shaker). Substrate for the subsequent color reaction was tetramethylbenzidine (Fluka, 87748).

Extraction and determination of oestrogens and androgens in boar faeces

Faecal samples from 9 yearling boars (Dt. Edelschwein) were collected on a daily basis from the day of castration up to 1 week later and two times a week for another 5 weeks. The oestrogens were extracted with KOH/chloroform/n-hexane (2) and androgens with 55% methanol. After shaking and centrifugation an aliquot of the supernatant was directly assayed after dilution with assay buffer.

Separations using HPLC (Silicagel 60, 25 x 0.4 cm; androgens: n-hexane/chloroform 70/30, flow: 4 ml/min; oestrogens: n-hexane/chloroform 50/50, flow: 2 ml/min) were performed to find immunoreactive substances present in faeces of boars.

RESULTS

The dilutions of antibody and label, the sensitivity (defined as 2 standard deviations from the signal given by the zero blank) and the reproducibility (calculated from multiple measurements of a pooled faecal sample of one boar) of the EIAs are shown in Tab.1. The crossreactivity of different steroids (Tab. 2-4) was calculated at 50% tracer displacement.

Tab. 1. Characteristics of the established biotin-streptavidin EIAs

Assay	17 β -OH-androgens	17-oxo-androgens	oestrogens
Label (DADOO-biotin coupled with:)	5 α -androstane-3 β ,17 β -diol-3-HS	5 α -androstane-3,17-dione-thioether	17 β -oestradiol-17-gluc.
Antibody against: (coupled with BSA)	testosterone-3-CMO	5 α -androstane-3 α -ol-17-one-HS	17 β -oestradiol-17-HS
Standard	testosterone	epiandrosterone	oestrone
Antibody ($\times 10^3$)	1:75	1:120	1:300
Label ($\times 10^3$)	1:5000	1:36	1:5000
Sensitivity (fmol/well)	1	34	0,7
Intraassay-CV (n=20)	7.9	10.1	12.4
Interassay-CV (n=50)	12.9	15.8	14.3

Tab. 2. Specificity of the rabbit anti-testosterone-3-CMO:BSA antibody as determined by the biotin-streptavidin-EIA

Crossreactivity with	(%)
4-Androsten-17 β -ol-3-one (Testosterone)	100
5 α -Androstan-17 β -ol-3-one (5 α -dihydrotest.)	23.7
5 β -Androstan-17 β -ol-3-one (5 β -dihydrotest.)	12.3
4-Androsten-3 β ,17 β -diol	7.6
5 α -Androstan-3 α ,17 β -diol	5.5
5 α -Androstan-3 β ,17 β -diol	1.3
5 β -Androstan-3 α ,17 β -diol	1.1
4-Androsten-17 α -ol-3-one (Epitestosterone)	<0.1
5 α -Androstan-3 α -ol-17-one (Androsterone)	<0.1
5 α -Androstan-17 α -ol-3-one	<0.1

Tab. 3. Specificity of the rabbit anti-5 α -androstane-3 α -ol-17-one-HS:BSA antibody as determined by the biotin-streptavidin-EIA

Crossreactivity with	(%)
5 α -Androstan-3 β -ol-17-one (Epiandrosterone)	100
5 α -Androstan-3,17-dione (Androstandione)	800
5 α -Androstan-3 α -ol-17-one (Androsterone)	40
4-Androsten-3,17-dione (Androstendione)	15.6
4-Estren-3,17-dione (Norandrostendione)	10.4
5-Androsten-3 β -ol-17-one (Dehydroepiandrosterone)	7.4
5-Androsten-3,17-dione	7.0
5 β -Androstan-3,17-dione	2.3
5 α -Androstan-3 α ,17 β -diol	1.4
5 α -Androstan-3 β ,17 β -diol	1.3
5 α -Androstan-17 β -ol-3-one (5 α -dihydrotest.)	1.3
5 α -Androstan-17 α -ol-3-one	1.1
5 α -Androstan-3 α ,17 α -diol	0.2
5 α -Androstan-3 β ,17 α -diol	0.1
5 β -Androstan-3 α -ol-17-one (Etiocholanolone)	<0.1
1,3,5(10)-Estratrien-3-ol-17-one (Oestrone)	<0.1

Tab. 4: Specificity of the sheep anti-17 β -oestradiol-17-HS antibody as determined by the biotin-streptavidin-EIA

Crossreactivity with	(%)
1,3,5(10)-Estratrien-3-ol-17-one (Oestrone)	100
1,3,5(10)-Estratrien-3,17 α -diol (Oestradiol-17 α)	19
1,3,5(10)-Estratrien-3,17 β -diol (Oestradiol-17 β)	70
1,3,5(10)-Estratrien-3,16 α ,17 β -triol (Oestriol)	129
1,3,5(10),7-Estratetraen-3,17 β -diol	20
1,3,5(10),7-Estratetraen-3-ol-17-one	87
1,3,5(10),6,8-Estrapentaen-3,17 β -diol	0.8
1,3,5(10),6,8-Estrapentaen-3-ol-17-one	1.1
1,3,5(10)-Estratrien-3-ol-17-on-3-sulfat	<0.01
1,3,5(10)-Estratrien-3-ol-17-on-3-glucuronide	<0.01

The main steroids measured in boar faeces with the EIAs showed the same elution pattern as testosterone, dihydrotestosterone (DHT), epiandrosterone (Fig.1), oestrone and oestradiol-17 β (Fig. 2). Therefore the concentrations of the measured androgens and oestrogens were expressed as equivalents of testosterone, epiandrosterone or oestrone respectively.

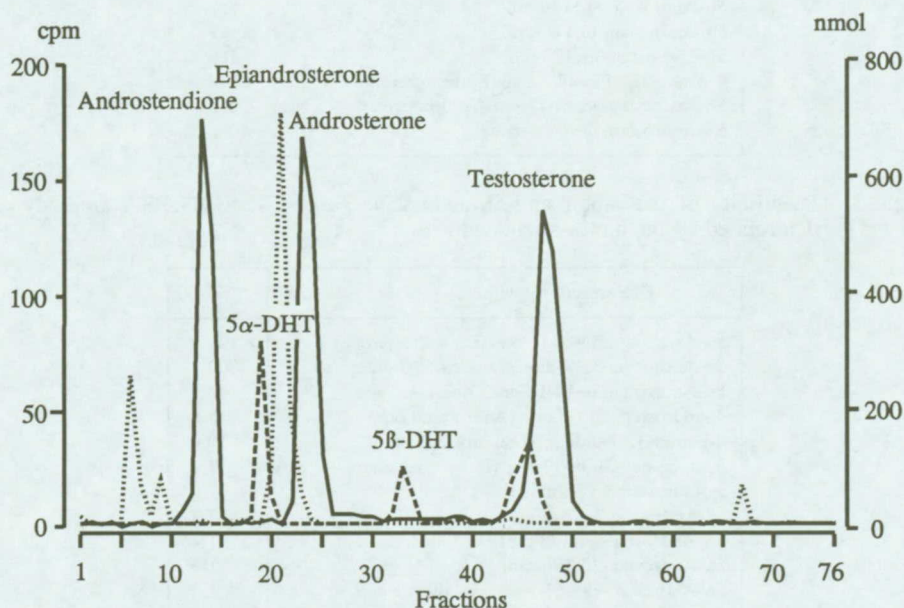


Fig.1. Immunoreactive 17 β -OH-androgens (- - - -) and 17-oxoandrogens (.....) in boar faeces (nmol/fraction) and radioactivity (cpm/fraction) of added 3 H-steroids (—)

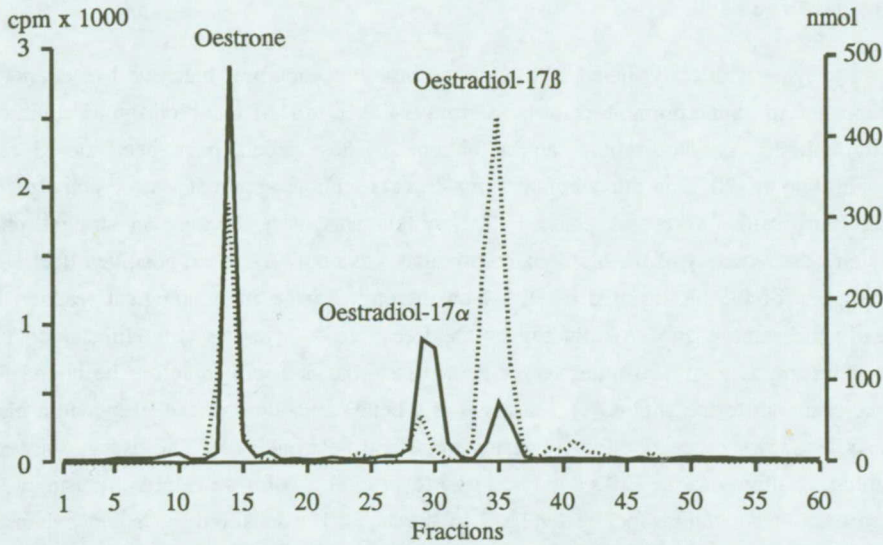


Fig.2. Immunoreactive oestrogens (.....) in boar faeces (nmol/fraction) and radioactivity (cpm/fraction) of added ^3H -steroids (—)

The concentrations of all measured steroids in boar faeces declined to basal levels after 8 to 10 days. In nearly all boars a period of increased faecal steroid concentration was seen between 0-4 days after castration reaching maximum values at Day 2 or 3 (Fig.3).

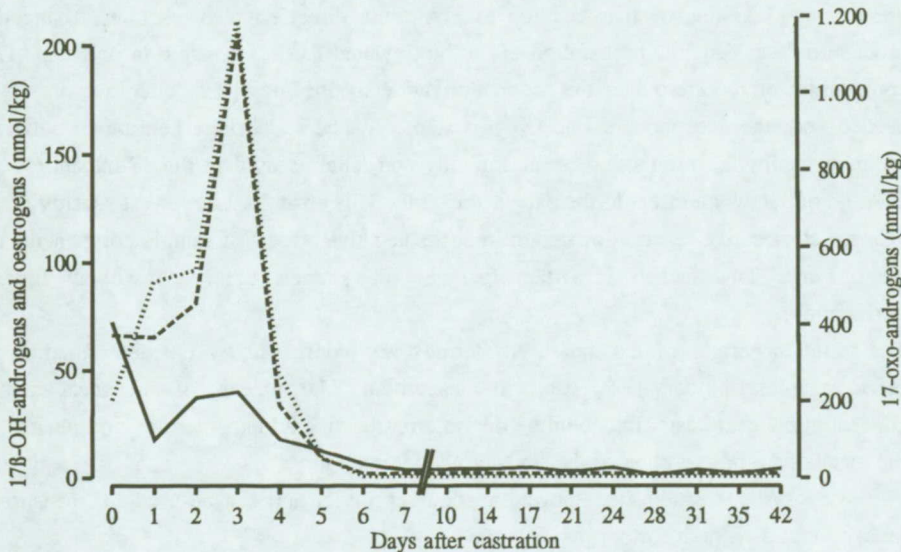


Fig.3. Concentration (nmol/kg) of 17 β -OH-androgens (—), 17-oxoandrogens (.....) and oestrogens (—) in faecal samples of a boar after castration

DISCUSSION

If an enzyme is directly linked with a steroid hormone each new batch will have another proportion of bound hormones. As the steroid is linked to DADOO-biotin in an equimolar ratio a better standardization can be obtained. These labels have been stored as a lyophilisate at -20°C in our laboratory for 3 years without apparent loss of activity. For their purification a reverse phase HPLC system was used, because in straight phase systems the recovery of the biotinylated products was poor. A second positive effect is the small size of the biotinylated label, which therefore in the immunological reaction has nearly the same chances of binding as the free hormone. (Labels with directly coupled enzymes are at least 100 times larger than the steroid and will therefore be bound in a smaller amount to the antibody.) The result is a better sensitivity of the streptavidin-biotin EIAs as shown especially for the testosterone and oestrone-EIA. The assays showed a similar sensitivity as the EIAs described by Meyer et al. (7) for oestrogens by using a two step reaction procedure for the synthesis of the label. The sensitivity was better than that of the EIA described by Gómez-Sánchez et al. (8) or Dressendörfer et al. (9).

Due to the incubation step with streptavidin:POD the procedure of the color reaction takes about one hour more than in the direct EIA. This disadvantage is compensated by the fact that only one reagent (e.g. streptavidin:POD) is required for the measurement of different substances. In addition each streptavidin is marked with two or three molecules of the enzyme, which results in an amplification of the color reaction. The accuracy of these assays was improved in relation to EIAs with direct coupled enzymes. Using the procedure described, the preparation of the biotinylated label is possible in one step. The conjugation of the steroid to the biotin derivative by the previously cited authors (7-9) needed with the exception of one (8) two steps. A side and bridge heterology between immunogen for the production of an antibody and label as used in the 17-oxoandrogen-EIA did not show an effect to the expected extent. This assay has the lowest sensitivity. Using a consecutive reaction procedure reduces negative effects of sample components on the enzyme. This fact is important for assaying extracts of faeces without further purification.

The testicular origin of the measured steroids was confirmed by the determination in faecal samples of boars before and after castration. The increase of the faecal steroid concentration after castration could be due to crossreacting adrenal steroids or a change in the metabolism or excretion of the sex-steroids after surgery.

The assay systems established showed a good sensitivity and can be used for measuring faecal samples without further purification.

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