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ENZYME IMMUNOASSAY SCREENING PROCEDURE FOR THE SYNTHETIC ANABOLIC ESTROGENS AND ANDROGENS DIETHYLSTILBESTROL, NORTESTOSTERONE, METHYLTESTOSTERONE AND TRENBOLONE IN BOVINE URINE

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SUMMARY

Immunoassays are often used for the screening of anabolic residues in edible tissues and excreta (urine, faeces) from inspected animals. Radioimmunoassays have been used for ten years for the determination in biological samples of the main natural and synthetic anabolic estrogens and androgens. In order to simplify the sample preparation and analysis and to reduce the cost, competitive enzyme immunoassays (EIA) were developed for the main synthetic anabolics used illegally in livestock fattening. EIA are based on a competition between the analyte (hormone or metabolite) and the enzyme-labelled hormone for binding to specific antibodies immobilized in wells of a microtitration plate. Two enzymes were evaluated: horseradish peroxidase (HRP) and *Bacillus licheniformis* β-lactamase (BLL) using hydrogen peroxide-*o*-phenylenediamine or benzylpenicillin-starch-iodine as substrates, respectively. The same derivative was used for chemical coupling of the hormone to enzyme (tracer preparation) and to bovine serum albumin to produce specific antibodies in rabbits. Hormone doses that inhibited 50% of the tracer (HRP-hormone) binding to antibody (ID_{50}) were 18, 8, 6 and 11 pg per well for diethylstilbestrol, nortestosterone, methyltestosterone and trenbolone, respectively. These values were lower than those observed in RIA. The reproducibility and accuracy of EIA in urine analysis were similar to those of RIA. Very small amounts of urine were needed (2.5 μl). This simple method may require less than 2 h. With the BLL-hormone tracer, the enzymatic activity remaining in the wells and hence the hormone content of the sample could be estimated with the naked eye using benzylpenicillin-starch-iodine as substrate.

INTRODUCTION

Analytical methods able to detect subparts per billion levels of steroid hormones or metabolites in a complex biological matrix are not numerous. Table I summarizes methods that have been or are still applied in the control of anabolic agents that are used, legally or illegally, in meat-producing animals. The most suitable methods in terms of limits of detection are (1) physico-chemical meth-

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TABLE I

METHODS FOR THE CONTROL OF ANABOLIC AGENTS IN BIOLOGICAL SAMPLES

Method	Limit of detection	Ref.
Histology[a] (prostate, Bartholin gland)	-	
Bioassays	ppm	
Chromatography[a]		
GC	1–10 ppb	13
HPLC	1–5 ppb	14
TLC[a]	1–20 ppb	15
Mass spectrometry		
GC–MS	0.1–10 ppb	16
HPLC–MS	?	
MS–MS	?	
Immunoassays[a]		
RIA[a]	10–500 ppt	
EIA	10–500 ppt	5, 18
CLIA	?	3
Radioreceptor assay	1 ppb	17

[a]Methods used in official control in Belgium.

ods such as different types of chromatography [thin-layer (TLC), high-performance liquid (HPLC) and gas (GC)] and mass spectrometry and (2) biochemical methods such as immunoassays and radioreceptor assays.

The strategy of control in Belgium involves screening analysis by radioimmunoassay (RIA) [1]. Samples found positive for the presence of artificial hormones by RIA are examined by TLC. A TLC-positive result leads to the carcass being condemned. This two-stage system of control avoids excessive expense in the analysis of negative samples, the cost of analysis per sample and per hormone being lower for RIA.

RIA has been used for ten years for the detection of diethylstilbestrol in urine. Artificial anabolic agents, most often found in 'hormone cocktails' on the black market [2] or detected in injection sites drawn from carcasses by meat inspectors, can be assayed in urine by RIA. RIA has well known drawbacks due to the use of a radioactive reagent: worker security (especially pregnant women, who must stop working in the laboratory, radioactive waste, poor stability of the labelled ligand, an expensive and a slow method of measurement (especially for tritiated markers measured by liquid scintillation counting). For these reasons, we looked for suitable non-radioactive systems. Another problem encountered in the analysis of anabolic residues in biological samples is blank values. Samples for untreated animals give false-positive responses in the analytical measurement unless the samples have been extensively purified before the assay itself.

Chemiluminescence immunoassay (CLIA) has been applied to the detection of 19-nortestosterone [3] and 17α-methyltestosterone [4] in bovine urine and

tissues. Meyer and Hoffmann [5] described an enzyme immunoassay (EIA) of trenbolone in urine and biological samples using alkaline phosphatase as the label. EIA appeared to be a good alternative to RIA. We developed very simple systems with detection limits lower than those for RIA, allowing a considerable simplification of sample preparation with a simple dilution instead of a costly and time-consuming procedure of purification involving solid-phase extraction [3], elution and drying.

EXPERIMENTAL

Mono-O-carboxypropyldiethylstilbestrol (CP-DES), methyltestosterone 3-carboxypropyl oxime (MT-3-CMO) [7], trenbolone 17β-hemisuccinate (TBOH-HMS) [8] and 19-nortestosterone 17β-hemisuccinate (NT-HMS) [8] were conjugated to horseradish peroxidase (HRP) (Boehringer, Mannheim, F.R.G.) using the mixed anhydride procedure of Erlanger et al. [8]. TBOH-HMS was also conjugated to β-lactamase prepared from *Bacillus licheniformis* (BLL) by Dr. J.M. Frere (Laboratoire d'Enzymologie, Université de Liège, Liège, Belgium). The CP-DES derivative was prepared according to Jansen and Zomer [19]. Briefly, 2 mmol of DES, dissolved in 6 ml of dry dimethyl sulphoxide (DMSO), were added to a dispersion of 4 mmol of sodium-hydride in 10 ml of DMSO. After stirring the mixture for 15 min, a solution of ethyl 4-bromobutyrate (400 mg) in 10 ml of DMSO was added. The resulting mixture was stirred in the dark until TLC indicated that the reaction was complete (ca. 30 min), poured into ice–water (100 ml) acidified with 10% sulphuric acid and extracted with diethyl ether. The ether layers were washed with water to remove DMSO, dried and evaporated to yield the crude product.

Antisera are commercially available (Laboratoire d'Hormonologie Animale, Centre d'Economie Rurale, Marloie, Belgium). They were prepared in rabbits injected subcutaneously with an emulsion of complete Freund adjuvent and CP-DES, MT-3-CMO, TBOH-HMS and NT-HMS coupled to bovine serum albumin (BSA).

Microtitre plates (96 wells; NUNC, Roskilde, Denmark) were coated for 2 h at 37°C with antibodies using dilute solutions (100 μl) of antiserum in 0.05 *M* carbonate buffer (pH 9.6).

Urine (100 μl) was diluted with 300 μl of hydrolysis buffer [200 μl of β-glucuronidase–arylsulphatase from *Helix pomatia* (Boehringer) in 15 ml of 0.1 *M* phosphate buffer (pH 7.4) containing 0.9% NaCl] and incubated at 37°C for 1 h. The hydrolysed urine sample (400 μl) was diluted with 1.6 ml of EIA buffer [0.1 *M* phosphate buffer (pH 7.4)–0.1% BSA–0.9% NaCl–0.01% thimerosal] and 50 μl of the diluted hydrolysed urine were added to wells with 150 μl of diluted enzyme conjugate. A calibration graph was established using 50 μl of standard solutions of known hormone concentrations in the range 0.5–100 pg. Plates were incubated for 1 h for NT, MT and TBOH assays and 2 h for DES assay. After washing the plates with washing buffer (0.9% NaCl solution containing 500 μl/l Tween 20), enzymatic activity was determined.

Determination of HRP activity

The plates were incubated for 30 min in darkness at room temperature with 150 μl of substrate solution per well [60 mg of *o*-phenylenediamine and 10 μl of perhydrol (30% hydrogen peroxide) in 15 ml of citrate–phosphate buffer (pH 5) containing 0.01% thimerosal]. The enzymatic reaction was stopped by addition of 6 *M* sulphuric acid and the absorbance was read at 490 nm.

Determination of β-lactamase activity

The plates were incubated under the same conditions with 150 μl per well of the following substrate: 525 μg of benzylpenicillin (Glaxo, Greenford, U.K.) dissolved in 15 ml of starch–iodine solution [0.02% starch in 0.01 *M* phosphate buffer (pH 7.4)–0.9% NaCl–4.8 m*M* KI–0.12 m*M* iodine].

After incubation, the enzymatic activity was measured by the decrease in absorbance at 620 nm.

Radioimmunoassay

RIA methods have been described elsewhere [9–11].

RESULTS AND DISCUSSION

For the sensitive assay of low relative molecular mass substances, the number of suitable types of EIA is very limited. After an extensive literature survey, we concluded that the classical competitive EIA should reach the required limit of detection (less than 10 pg per well): (1) hormone-specific polyclonal antibodies were passively bound to polystyrene of microtitration plates; (2) the analyte competed with enzyme-labelled anabolic hormone for binding to specific antibodies; equilibrium was reached within 1–2 h; (3) the plates were washed; (4) the substrate solution was added for colour development.

A very simple homologous system was selected in which the same anabolic hormone derivative carrying a free carboxyl group was used to synthetize (1) hormone–serum albumin conjugate for immunization and (2) the hormone–enzyme conjugate. Formulae of the hormone derivatives used as starting substances for coupling to protein (enzyme or bovine serum albumin) by the mixed anhydride method are given in Fig. 1.

Horseradish peroxidase was selected as a marker enzyme for diethylstilbestrol, nortestosterone, methyltestosterone and β-trenbolone. Using hydrogen peroxide–*o*-phenylenediamine as substrate, the intensity of the yellow colour increased with increasing enzyme activity present in wells of the microtitration plate. This system required spectrophotometric measurement of the absorbance even for a semi-quantitative determination of the anabolic hormone present in urine samples. Calibration graphs are given in Fig. 2.

For the four HRP systems, the EIA curves showed hormone doses causing 50% binding inhibition that were five to ten times lower than those recorded using RIA (Table II). BLL–EIA was less sensitive than HRP–EIA but still showed a better detection ability than RIA.

Homologous EIA systems, using the same hapten derivatives for the immu-

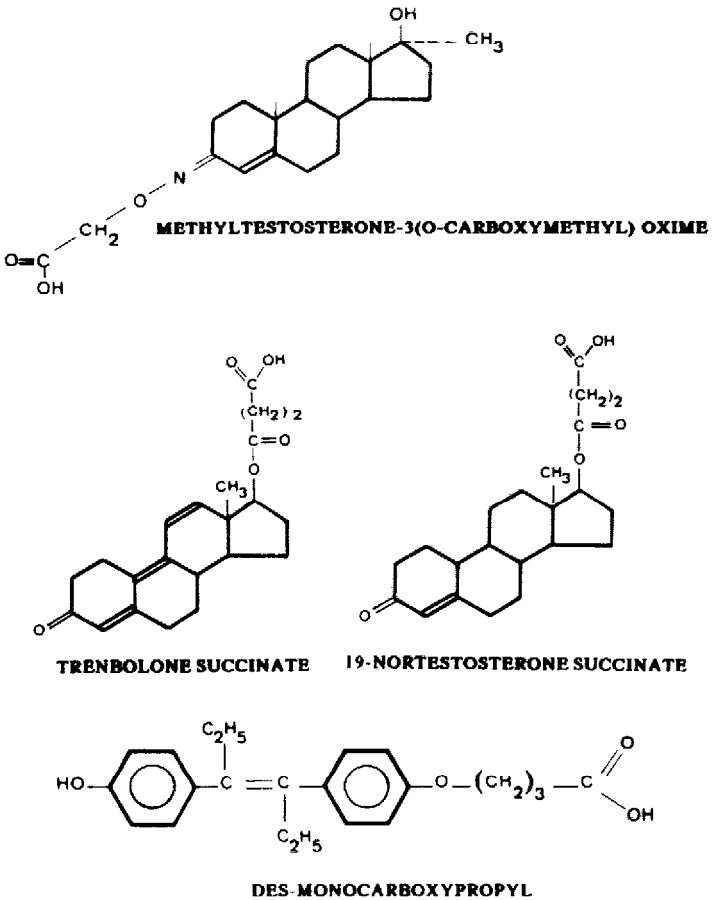
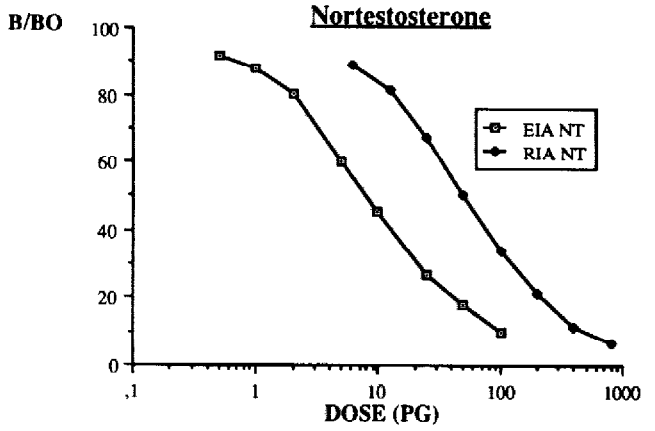


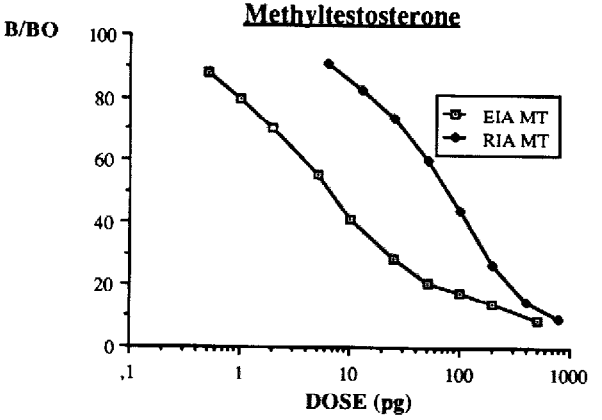
Fig. 1. Structures of anabolic derivatives used for coupling to enzyme and to bovine serum albumin.

nogen and enzyme–conjugate syntheses, generally have a poor reputation in terms of sensitivity and limit of detection as the common chemical bridge between the hapten and the proteins (BSA and enzyme) could be better recognized by the antibodies, the affinity being higher for the enzyme–hormone conjugate than for the free hormone analyte itself [12]. Nevertheless, our experience demonstrated that homologous systems are much easier to develop, needing the synthesis of only one hormone derivative, and they still allowed a sensitive detection of the analyte.

The intra- and inter-assay reproducibilities are given in Table III. The coefficients of variation correspond to those generally obtained with RIA. The accuracy was determined using urine samples enriched with known amounts of anabolic hormones (Table IV). The recoveries were similar to those generally observed using RIA.

The main advantage of EIA over RIA is the very small volume of urine sample needed for the analysis, i.e., the equivalent of 2.5 μl of crude urine in a well. This very limited amount of sample submitted to EIA decreased the problem of inter-


Nortestosterone
B/BO
100
80
60
40
20
0
,1
1
10
100
1000
DOSE (PG)
EIA NT
RIA NT


Methyltestosterone
B/BO
100
80
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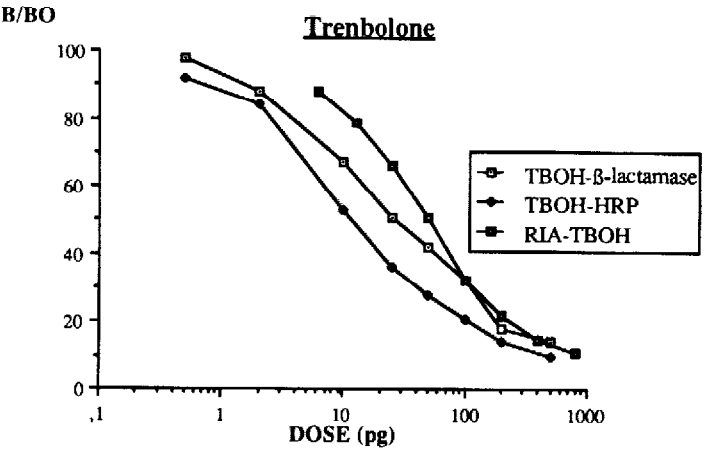
DOSE (pg)
EIA MT
RIA MT


Trenbolone
B/BO
100
80
60
40
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0
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DOSE (pg)
TBOH-ß-lactamase
TBOH-HRP
RIA-TBOH

Fig. 2.

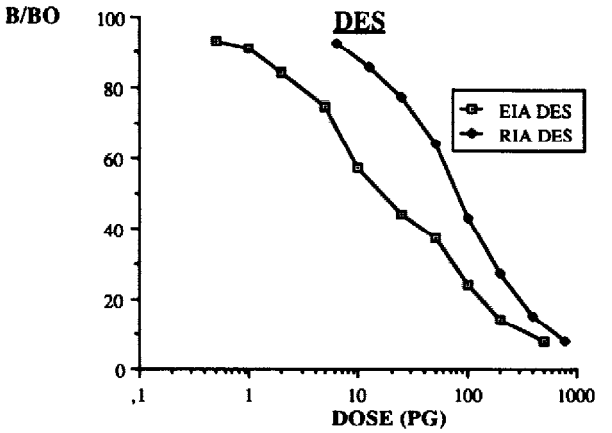

Fig. 2. Calibration graphs for EIA and RIA of nortestosterone (NT), methyltestosterone (MT), trenbolone (TBOH) using TBOH labelled with horseradish peroxidase (HRP) or β-lactamase and diethylstilbestrol (DES).

TABLE II

AMOUNTS OF ANABOLIC WHICH CAUSED A 50% REDUCTION OF THE INITIAL BINDING IN ASSAYS (RIA OR EIA) OF NORTESTOSTERONE (NT), METHYLTESTOSTERONE (MT), TRENBOLONE (TBOH) AND DIETHYLSTILBESTROL (DES)

Compound	Amount	
	EIA (pg per well)	RIA (pg per tube)
NT	8	50
MT	6	75
TBOH	11[a]; 28[b]	51
DES	18	85

[a]TBOH-HRP.
[b]TBOH-β-lactamase.

TABLE III

REPRODUCIBILITY OF EIA OF NORTESTOSTERONE (NT), METHYLTESTOSTERONE (MT), TRENBOLONE (TBOH) AND DIETHYLSTILBESTROL (DES)

Urine concentration (ppb)	Coefficient of variation (%)			
	NT	MT	TBOH	DES
Intra-assay (n=12)				
1.0	6.4	5.1	7.2	8.4
5.0	6.8	5.7	7.9	9.3
Mean:	6.6	5.4	7.5	8.8
Inter-assay (n=6)				
1.0	10.2	9.8	9.6	12.2
5.0	8.4	11.4	10.9	14.6
Mean:	9 3	10.6	10.2	13.4

TABLE IV

ACCURACY, ANABOLIC CONCENTRATION AND RECOVERY AFTER ADDITION OF KNOWN AMOUNTS OF NORTESTOSTERONE (NT), METHYLTESTOSTERONE (MT), TRENBOLONE (TBOH) AND DIETHYLSTILBESTROL (DES)

Values are means of twelve determinations (blank values subtracted), with recoveries (%) in parentheses.

Added (ppb)	Measured (ppb)			
	NT	MT	TBOH	DES
0.5	0.43 (86)	0.57 (114)	0.61 (122)	0.40 (80)
1.0	1.18 (118)	1.13 (113)	0.98 (98)	1.20 (120)
1.5	1.59 (106)	1.61 (107)	1.40 (93)	1.43 (95)
2.0	2.31 (115)	1.96 (98)	2.19 (109)	2.42 (121)

ference due to the biological matrix. It followed that the solid-phase extraction purification step that is essential when RIA is used could be avoided with EIA. The result is a very simple and rapid assay. A first semi-quantitative screening may even be performed on an unhydrolysed urine sample with a practical limit of detection of ca. 3 ppb.

As soon as the reagents needed for the EIA screening procedure described in this paper become commercially available, EIA could advantageously be used instead of RIA in the Belgian official control strategy.

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