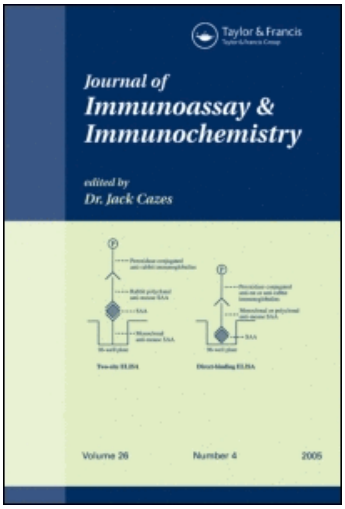

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A Specific Radioimmunoassay for the Detection of 19-Nortestosterone Residues in Urine and Plasma of Cattle

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A SPECIFIC RADIOIMMUNOASSAY FOR THE DETECTION OF 19-NORTESTOSTERONE RESIDUES IN URINE AND PLASMA OF CATTLE

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ABSTRACT.

19-Nortestosterone (NANDROLONE) is illegaly used as a growth promoter for meat producing animals . This work describes a radio-immunoassay (RIA) to detect, in cattle urine, NANDROLONE and its metabolites.

The antiserum was specific for 19-nortestosterone and its main metabolites, and to a lesser extent for TRENBOLONE (17B-hydroxyestra-4,9,11-trien-3-one).

The sensitivity of this assay was 6 ug/l at 90 % B0/T binding.

This assay can be used in forensic control of illegal treatment with 19-nortestosterone in meat production.
(KEY WORDS: 19-NORTESTOSTERONE, NANDROLONE, RADIO-IMMUNOASSAY OF, ANTISERUM, MEAT PRODUCTION).

INTRODUCTION.

The illegal administration of hormonal anabolic preparations in animal production is common in Belgium.Since the reinforcement of the official control program for "stilbenic compounds", the detection frequency of diethylstilbestrol (DES) has drastically decreased (1).

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A previous report has shown that 19-nortestosterone (NANDROLONE) frequently occured in illicit hormonal preparations (2). Several pharmaceutical preparations containing NANDROLONE are probably used as growth promoter by the farmers. In the Netherlands, the analysis of injection sites has shown that 84 % of 573 samples contained 19-nortestosterone (3). Until now, the methods to detect 19-nortestosterone residues in urine were thin layer chromatography (4) or gas chromatography/mass spectrometry (5). A radioimmunoassay (RIA) would therefore be useful to perform screening on large numbers of samples.

MATERIALS AND METHODS.

Reagents

Diethylether (Aristar,BDH) was used without further purification; tritiated 19-nortestosterone (19 Ci/mmol) was purchased from Amersham ,U K; the steroid standards 19-nortestosterone, testosterone, androsterone, 17 -testosterone, dihydrotestosterone, methyltestosterone, 17 -estradiol, progesterone were purchased from Steraloid ,USA. Trenbolone and 17 -trenbolone were gifts from Roussel UCLAF, France. Metabolites of 19-nortestosterone were kindly provided by Dr. Kuys from Organon Scientific Group (OSS, The Netherlands).

The RIA buffer was phosphate buffer, 20 mmol/L, pH 7.4, containing gelatin ,5 g/L.

The hydrolysis buffer was phosphate buffer, pH 7.0, 100 mmol/L, containing B-glucuronidase-arylsulfatase from H. pomatia

(Boerhinger) (100 µl/15 ml) and (^{3}H)-19 nortestosterone (2.5 * 10^{5} cpm/15 ml) as internal standard for evaluation of extraction recovery.

Preparation of 19-Nortestosterone-Bovine Serum Albumin Conjugate.

19-Nortestosterone (500 mg) and succinic anhydride (500 mg) were dissolved in 20 ml dry pyridine and heated under reflux for 4 hours.19-Nortestosterone hemisuccinate was purified according to Dixon (6) and the final ethyl acetate extract was evaporated to dryness. The dry residue (250 mg) was taken up in 20 ml of dry dioxan and coupled to 1.2 g of bovine serum albumin according to the mixed anhydrid method (7). The resultant precipitate was dissolved in sodium chloride , 9 g/L.

Immunization of Rabbits.

Four rabbits were immunized according to the procedure of Vaitukaitis (8). The immunogen solution (2 mg per rabbit) was emulsified in complete Freund's adjuvent.The rabbits received a total of seven injections, at intervals of 15 days. The serum of one rabbit showed acceptable binding properties. It was diluted with RIA buffer (1:100) and stored in 1 ml aliquots at -20°C.

Samples.

A pool of blank urine was prepared from cattle that had been maintained under controlled conditions.

Eighty four urine samples were collected (from June to July 1985) by the official veterinary inspectors and were examined for

their 19-nortestosterone content. Urines were stored at -20°C until assayed.

Hydrolysis and Extraction.

Three hundred µl of hydrolysis buffer was added to 100 µl of plasma or urine samples and the mixture incubated 1 h at 37°C. Steroids were then extracted by mixing with 2 ml diethylether and the organic phase evaporated to dryness under a stream of nitrogen. The dry residue was dissolved in 500 µl of RIA buffer. A 100µl aliquot was mixed with 5 ml of scintillation fluid (Beckman Ready-solv HP) and counted in a LS1800 Beckman Scintillation Counter for the estimation of extraction recovery.

Radio-Immunoassay.

One hundred µl of (^{3}H) 19-nortestosterone (10000 cpm) in RIA buffer and 100 µl of antiserum (initial dilution 1/20000) were added to the 300 µl sample extract.

All tubes were incubated at 37°C for 30 minutes and subsequently for 2 hours at 4°C to equilibrate the reaction. Dextran coated charcoal suspension (5 g Norit charcoal, dextran 0.5 g/L of RIA buffer) was added (500 ul) and incubated for 10 minutes in order to separate bound and free steroid. After centrifugation, 500 ul of supernatant was added to 5 ml scintillation fluid and the radioactivity was determined.

The results were calculated from a calibration curve after logit-log transformation and linear regression analysis .

TABLE 1

Relative Cross Reactivities of Antiserum against 19-Nortestosterone expressed as Percentages.(9)

Chemical (trivial) name	
17β-Hydroxy-4-estren -3-one (19-nortestosterone)	100
17α-hydroxy-4-estren-3-one (19-norepitestosterone)	3.25
4-estren-3,17-dione (19-norandrostendione)	46.3
5 -estrane-3,17-dione (19-norandrostandione)	20.1
17β-hydroxyandrost-4-ene-3-one (testosterone)	<0.03
17α-hydroxyandrost-4-ene-3-one (epitestosterone)	<0.01
5β-androstan-17β-ol-3-one (dihydrotestosterone)	<0.01
4-androsten-17α-methyl-17β-ol-3-one	<0.01
Estra-1,3,5(10)-tiene-3,17β-diol (17β-estradiol)	<0.01
Pregn-4-en-3,20-dione (progesterone)	<0.04
5α-androstan-3 -ol-17-one (androsterone)	<0.02
17β-hydroxyestra-4,9,11-trien-3-one(TRENBOLONE)	9.09
17α-hydroxyestra-4,9,11-trien-3-one(epiTRENBOLONE)	4.29
Diethylstilbestrol	<0.01

To correct for the additionnal counts in the biological samples resulting from the internal standard, the procedure of Hoffmann (9) was applied.

RESULTS.

Specificity of the Antiserum.

Assay cross reactivity determined according to Abraham (10) of the antiserum towards various steroids and anabolic compounds are given in table 1. Metabolites of 19-nortestosterone and TRENBOLONE showed an appreciable cross reactivity. All other related compounds showed a cross reaction lower than 0.05 %.

TABLE 2.

Quality Control Parameters of 19-Nortestosterone Radioimmunoassay

Amount of NANDROLONE (µg/l)		Coefficient of variation (%)	
Added	Measured (A)	Intra-assay n= 3	inter-assay n= 4
Urine:			
0.5	0.51 ± 0.08	12.9	12.4
1.0	1.11 ± 0.15	10.5	12.4
1.5	1.69 ± 0.10	4.7	5.0
2.0	2.21 ± 0.12	2.8	5.6
Plasma			
0.5	0.46 ± 0.07	14.4	13.8
1.0	1.07 ± 0.13	5.6	13.0
1.5	1.55 ± 0.12	2.1	8.3
2.0	2.04 ± 0.26	8.5	12.1

(A): mean ± s.d.(n= 12).

Assay Parameters.

Initial binding (B_0/T) was 40 + 4 %; non specific binding 1.2 + 0.3 %; slope -0.9345 + 0.049 ; correlation coefficient 0.9993 ; sensitivity at 90 % B/B_0 binding 6 + 3 pg; concentration at 50 % binding 53 + 6 pg (mean + s.d.). The mean of extraction recoveries of 19-nortestosterone from urine was 89 + 4. % (n= 96). To estimate intra and inter assay variability , urine and plasma containing 0.5, 1.0, 1.5 and 2.0 ug/l of 19-nortestosterone were analyzed in triplicate over four assays. The results are shown in table 2.

TABLE 3.

Determination of 19-Nortestosterone Content in Urine Samples collected at the slaughterhouse.

	ND	0-0.5 µg/l	0.5-1 µg/l	1-3 µg/l	) 3 µg/l
Number of samples	44	7	12	14	7
%	52.4	8.3	14.3	16.6	8.3

ND= not detectable.

The interference of the biological matrix for urine and plasma taken from control untreated animals were respectively 0.05 + 0.01 ug/L (n=14) and 0.03 + 0.01 ug/L (n=16).

Determination of 19-Nortestosterone Content in Urine Samples.

Eighty four urine samples collected at the slaugtherhouse were analyzed using the RIA procedure. Their results are given in table 3.

Thirty three samples (39 %) showed a 19-nortestosterone content higher than 0.5 ug/l.

DISCUSSION.

Analytical control of meat producing animals in farms or in slaugtherhouses must detect the parent drug and/or its main metabolites. Cross reactivity of the antiserum with metabolites of

the drug is thus a useful characteristic of this RIA as it measured not only parent drug but also main metabolites although with lesser sensitivity. 17 B-hemisuccinate derivative was selected for the coupling to bovine serum albumin, rather than 19-nortestosterone-3-carboxymethyloxime in order to obtain higher cross reactivity against metabolites.

Our antiserum had a high specificity: it did not cross-react with endogenous hormones. This was not the case in a previous study (11) where an antibody raised with the same steroid derivative as the present work shows significant cross reactivities with the natural sex hormones and their metabolites. Concentrations of these hormones could be high enough for even low cross reactivity to result in false positive results.

Recently, a specific RIA for 19-nortestosterone which does not cross react with 19-nortestosterone metabolites has been described (12). This antiserum wich was prepared against a 19-nortestosterone-7 -carboxyethylthioether shows a higher cross reaction with testosterone (1.7 %) and estradiol (1.6 %) than our antiserum.

Cross reactions with TRENBOLONE and its main metabolite in bovine species (17α-trenbolone) were found to be 9 % and 4.3 % respectively. Jansen et al (12) also described a cross reaction of their antiserum with TRENBOLONE. These observations can be explained by the lack of C-19 methyl group in TRENBOLONE and in 19-nortestosterone. If improved specificity were needed, urine samples could be purified by HPLC procedure (13) in order to separate 19-nortestosterone and TRENBOLONE residues.

On the basis of the characteristics of the antiserum and the assay described here, it can be concluded that this RIA is applicable to the screening detection of illegal administration of NANDROLONE.

Gas chromatography-mass spectrometry gives of course definitive results about the presence of anabolic residues in treated animals. Nevertheless, the cost of the GC-MS determination and the relative scarcity of laboratories trained in this method in Belgium impeed control of large numbers of samples.

In our country, Thin Layer Chromatography (TLC) is used for anabolic residues detection in animals since 1979 (4); it is fiable in well trained hands.

RIA is proposed as a screening method as it is already applied for stilbene residues determination (14). Positive RIA results must be confirmed by TLC before being considered as "officialy" positive.

A collaboration study (unpublished data) is still in progress wich involves RIA and TLC methods indicates a good correlation between both methods when the levels of nortestosterone (or metabolites) is over 1 µg/L. Under this value, the TLC method appears not sensitive enough to confirm RIA results.

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