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FREE AND CONJUGATED ZERANOL RESIDUES DETERMINED BY RADIO-IMMUNOASSAY IN URINE AND PLASMA OF CALVES TREATED WITH FORPLIX ®

J.P. DUCHATEL and G. MAGHUIN-ROGISTER

Laboratoire d'Analyse des Denrées Alimentaires d'Origine Animale, Faculté de Médecine Vétérinaire, Université de Liège, 45, rue des Vétérinaires, 1070 Bruxelles, Belgique

Résumé

RÉSIDUS LIBRES ET CONJUGUÉS DE ZÉRANOL MESURÉS PAR MÉTHODE RADIO-IMMUNOLOGIQUE DANS L'URINE ET LE PLASMA DE VEAUX TRAITÉS AU FORPLIX®. — Un antisérum, préparé chez le lapin traité au zéranol 7-hémisuccinate couplé à l'albumine bovine sérique, a été utilisé pour le dosage radio-immunologique des résidus libres et conjugués de zéranol dans l'urine et le plasma de veaux implantés au Forplix. Cet antisérum reconnaissait le zéranol ainsi que son métabolite zéaralanone et la mycotoxine zéaralénone. Les limites de détection, exprimées en équivalents zéranol, par cette méthode étaient de 100 pg/ml de plasma et de 1 ng/ml d'urine chez les animaux traités par des implants contenant du zéranol. Les concentrations plasmatiques en résidus de zéranol étaient trop faibles pour être détectées par cette méthode même après hydrolyse des protéines plasmatiques par la pronase. Les concentrations urinaires en résidus libres et conjugués étaient, quant à elles, facilement mesurables. Les résidus conjugués augmentaient sensiblement à partir du 25e jour après le traitement et étaient encore assez élevés (19 ppb) au 40e jour.

Zeranol, [3S, 7R] 3, 4, 5, 6, 7, 8, 9, 10, 11, 12-decadehydro-7, 14, 16-trihydroxy-3-methyl-1H-2-benzoxacyclotetradecin-1-one, a synthetic derivative of the mycotoxin zearalenone, is a constituent of several implants used for anabolisation of cattle or sheep (Ralston, 1978; Roche and Davis, 1978).

Measurement of residues resulting from zeranol administration has been impeded by the lack of assays sensitive in the ppb (part per billion) range. Recently, radio-immunoassays (RIA) for zeranol have been described (Dixon, 1980; Duchatel and Maghuin-Rogister, 1984) and were used for estimating residue levels in urine (Dixon and Russell, 1983; Gaspard *et al.*, 1983) and in meat (Baldwin *et al.*, 1983).

The specificities of RIA described to date are different. Dixon's antiserum (to zeranol 16-carboxypropylether -HSA raised in sheep) is specific for zeranol, but a cross reactivity of 30 % was observed for α -zearalenol, a possible metabolite of zearalenone. While Duchatel's antiserum not only recognises zeranol and its main metabolite, zearalanone, but also the mycotoxin zearalenone and its probable metabolites α and β zearalenol.

If Dixon's antiserum appears better adapted to official control purpose due to its relatively high specificity, our antiserum is better suited to studies of excretion patterns as it reacts not only with zeranol but also with its main metabolites.

Its application as a method for controlling residues in farm animals would require a purification step, for example by high performance liquid chromatography (HPLC) before the radio-immunoassay.

In this study, we have measured free and conju-

gated zeranol residues in urine of calves implanted with Forplix ®.

Materials and Methods

1. *Materials*

1.1. *Reagents and glassware*

Analytical grade methanol, ethyl acetate and dioxane were from Merck and were redistilled before use. Diethylether Aristar (BDH) from a freshly opened vial was used without further purification. Other reagents were charcoal (Sigma), dextran grade C (BDH), gelatine, sodium merthiolate, "RIA grade" bovine serum albumine (BSA) and succinic anhydride (Sigma).

A 0.2 M phosphate buffer, pH 7.0, 0.1 % gelatin, was used in the radio-immunoassay and for dilution of antiserum, labelled hormone and preparation of charcoal suspension.

Zeranol, zearalanone, zearalenone, taleranol, α and β - zearalenol were donated by the International Mineral Corporation (Terre-Haute, IN, USA). Their chemical purity was checked by thin-layer chromatography (silicagel 60, Chloroform-ethanol, 95:5, v/v).

Tritium-labelled zeranol was a gift from Dr Jouquey (Roussel-Uclaf), and was kept at 4 °C in toluol-ethanol (95:5, v/v).

β-glucuronidase-arylsulphatase *Helix pomatia* preparation was from Bœhringer, pronase B grade from Calbiochem. Scintillation cocktail was Ready solv. HP (Beckman). Radioactivity was measured in 6 ml polyethylene vials (LUMAC, Netherlands) using an "Inter technique SL 400" counter.

Disposable glass tubes (75 x 12 mm) were used in the assays. They were rinsed with methanol and dried in the oven before use. Glass-stoppered tubes (16 x 100 mm) used for the extraction of residues from plasma and urine were also methanol rinsed and dried.

1.2. *Animals*

Two series of veal calves (Norman male calves, average weight 51.6 kg) were used for the anabolisation. They were fed with milk replacer. First series: 8 animals were implanted with FORPLIX^R (140 mg trenbolone acetate, 36 mg zeranol) at the base of one ear. Blood samples were collected once daily for 9 days following treatment. Second series: 5 animals were injected with 5 ml Gynoestryl Retard fort^R (Roussel-UCLAF, France) (4 mg 17 ß oestradiol hexahydrobenzoate per ml). After 30 days, they were implanted with FORPLIX^R.

Samples (n = 20) of control plasma and urine (without possible zeranol residues) were taken from the second series of animals at regular intervals for four weeks preceding Forplix treatment. After FORPLIX implantation, blood and urine samples were collected on day 6, 13, 20, 27, 34 and 41.

2. *Methods*

2.1. *Preparation of the antiserum*

Antibodies were raised in rabbits treated with a zeranol 7-hemisuccinate-bovine serum albumin conjugate.

2.1.1. *Preparation of zeranol 7-hemisuccinate*

Zeranol (100 mg) and succinic anhydride (100 mg) dissolved in dry pyridine (3 ml) were left at 25 °C for 48 h. Zeranol 7-hemisuccinate was purified according to Dixon (1980) and ethyl acetate extract was dried on sodium sulphate and evaporated to dryness.

2.1.2. *Coupling to bovine serum albumin*

The dry residue of zeranol 7-hemisuccinate was taken up in 3 ml of dry dioxane and coupled to 470 mg bovine serum albumin by the mixed anhydride method described by Erlanger *et al.* (1957). The reaction was left to proceed at pH 9 for 4 h at 4 °C. After overnight dialysis, the antigen was isolated by precipitation at pH 4.3 using hydrochloric acid. The precipate was dissolved in 1 % sodium bicarbonate and dialysed against 9 ‰ sodium chloride.

By UV spectrophotometry (Erlanger, 1957), it was determined that 40 molecules of zeranol hemisuccinate were linked per molecule of serum albumin.

2.1.3. *Immunization of rabbits*

Three rabbits were immunized by intradermal injection (Vaitukaitis *et al.*, 1971) of the immunogen solution (2 mg per rabbit and per ml of 9 ‰ sodium chloride) emulsioned in complete Freund adjuvent. The rabbits received a total of seven injections, one injection every 15 days.

The titer of antibodies to zeranol was followed in blood samples taken during the immunization period. One hundred and twenty two days after the first injection, serum taken from one rabbit (D.7851) diluted at 1/40 000 was able to bind 65 % of tritium labelled zeranol. This antibody was diluted in phosphate buffer (1/100, v/v) and kept frozen at − 18°C in 1 ml aliquots.

2.2. *Assay procedure*

Free (unconjugated) residues were extracted by Vortex mixing (10 sec) with diethylether (5 ml) from unbuffered plasma (0.5 ml) or urine (0.02 ml) diluted in 2 ml distilled water.

Glucuronate and sulphate conjugated residues contained in the aqueous phase were hydrolysed as described below (2.2.1.) before extraction and assay.

In order to take into account a possible binding of residues to plasma proteins, plasma samples (0.5 ml) were also incubated for 24 h at 37 °C with 0.04 ml of pronase (1 mg) in 0.05 M Tris-chloride buffer pH 7.7, 0.015 M in calcium chloride. After incubation, samples were extracted by 5 ml of diethylether as described above.

2.2.1. *Hydrolysis of conjugated residues*

Conjugated residues contained in the aqueous phase (2.5 ml for plasma, 2.02 ml for urine) were hydrolysed for 2 h at 37 °C using *H. pomatia* β -glucuronidase-arylsulphatase (5 µl per tube). Hydrolysates were then extracted by 5 ml of diethylether and dried under a stream of nitrogen.

2.2.2. *Radio-immunoassay*

Dried ether extracts were taken up in 100 µl RIA buffer. Tritium labelled zeranol (20,000 dpm in 100 µl buffer) and antiserum diluted at 1/40 000 (500 µl) were added successively.

The standard curve was established using tubes containing 0, 25, 50, 100, 200, 400, and 800 pg of zeranol in 100 µl buffer.

All the assays and standards were run in triplicate. After an overnight incubation at 4 °C, 0.5 ml charcoal-dextran suspension (2.5 g charcoal and 0.25 g dextran per liter of buffer) was added at 4 °C. Tubes were left at 4°C for 10 minutes and centrifuged at 3 000 rpm for 15 min at 4 °C. Five hundred µl of supernatant were mixed with 5 ml of scintillation cocktail and radioactivity was measured for 5 min.

Non specific binding was determined in tubes that did not contain antibody.

Results were computed from a logit-log standard curve (Rodbard, 1974) using an Apple II Europlus microcomputer.

3. Statistics

Statistical analysis of the significance between groups of samples was made by means of Student's t test.

Results

The specificity of D7851 anti-zeranol 7-hemisuccinate-bovine serum albumin antiserum is shown in table 1. The main metabolite of zeranol in the bovine species, zearalanone, cross-reacts at 100 %. A possible minor metabolite, β-zeranol (taleranol) shows a cross-reaction of 29 %.

Besides these cross-reactions useful for pharmacodynamic studies, the antiserum also recognises zearalenone, α-zearalenol and to a lesser extent β -zearalenol.

Standard curve covers the range 0-800 pg of zeranol. The presence of serum or urine extracts from untreated animals does not affect standard curves.

Table 1. — The percentage cross-reactivity of the antibody (D7851) to zeranol 7-hemisuccinate-bovine serum albumin raised in rabbit

Zeranol	100
Zearalanone	100
Zearalenone	53
α Zearalenol	54
ß Zearalenol	5
Taleranol	29
17 α-Estradiol	< 0.005
17 ß-Estradiol	< 0.005
Estrone	< 0.005
Estriol	< 0.005
Equilenin	< 0.005
DES	< 0.005
Dienestrol	< 0.005
Hexestrol	< 0.005
Testosterone	< 0.005
17 α-Methyl testosterone	< 0.005
Trenbolone	< 0.005
Progesterone	< 0.005
Cortisone	< 0.005
Dexamethasone	< 0.005
Stigmasterol	< 0.005
Biochanin A	< 0.005

The detection limit, calculated from the standard curve as the minimum amount of antigen that causes a significant decrease ($P < 0.05$) of bound radioactivity to antibodies, was 15 pg per tube.

Blank values, determined using plasma and urine extracts from untreated animals, were low enough to allow detection of 100 pg/ml of plasma or 1 ng/ml of urine ($P < 0.05$).

Intra-assay and inter-assay coefficients of variation were 9 and 11 % respectively.

Extraction yield was estimated as 95-98 % for both urine and plasma.

Radio-immunoassays of zeranol in plasma from veal calves treated with zeranol containing implants did not give results which were significantly different from those of untreated animals. This observation was verified even if blood was taken once daily for nine days following treatment.

No change was observed in the assay results due to proteolytic digestion of serum proteins by pronase before the extraction of zeranol residues. They were not significantly different from those of untreated animals.

In contrast, free and conjugated zeranol residues were easily measured in the urine from

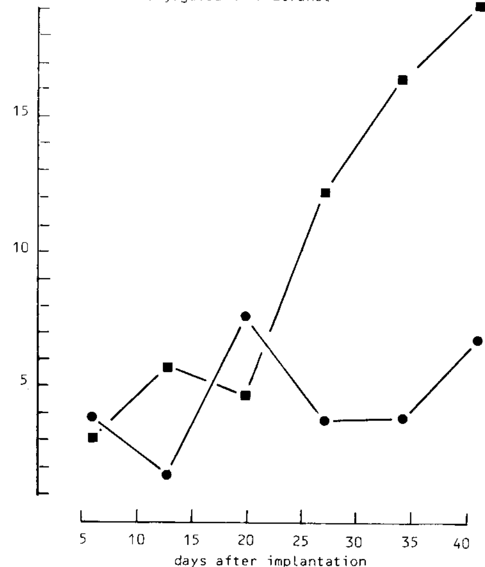

Fig. 1. — Evolution of free and conjugated zeranol residue concentrations in calf urine after treatment at time zero with Forplix®. Mean values of urinary concentrations determined by RIA for 5 animals are shown as a function of time (days after implantation).

treated animals, despite the higher blank values recorded for urine.

Figure 1 shows the evolution of free and conjugated residue concentrations in calf urine after implantation.

Before day 25, urine concentration of both free and conjugated residues were similar, showing a classical irregular pattern of residue concentration in urine after implant administration (Gaspard, 1983; Dixon *et al.*, 1983).

One interesting point which results from this experiment is that conjugated residues increased markedly only from the 20th day after treatment.

Discussion

The use of radio-immunoassay for the determination of zeranol residues in animals treated with zeranol containing implants has the advantage of lower detection limits. Other assay methods of biological samples, such as thin-layer chromatography, gas chromatography or high pressure liquid chromatography are less sensitive and more laborious than radio-immunoassays (Verbeke, 1979; Stephany and Van den Bosch, 1978; Frischkorn *et al.*, 1978).

Nevertheless, specificity is often the stumbling-block of radio-immunoassays. Either they are so specific that important metabolites are not detected, or they are not specific enough leading to false positive results. The latter problem must of course be absent in an assay designed for regulatory purposes, for example the detection of forbidden anabolic hormones in farm animals. In contrast, less specific antibodies, reacting not only with the parent drug but also with its main metabolites, would be very useful in studies of excretion patterns.

Our antibody belongs to this type as it reacts not only with α-zeranol but also equally well with its main metabolite in bovine species, zearalanone and to a lesser extent with possible minor metabolites such as β-zeranol (taleranol).

Of course the radio-immunoassay used in this work could not be used for regulatory purposes without the separation of zeranol and/ or its metabolites from cross-reacting substances, such as zearalenone. Indeed, if the lack of specificity of our antibody is not taken into account, false positive results would be recorded at least for animals nourished with zearalenone contaminated feed.

Detection limits of the present assay in the presence of urine or plasma extracts are lower than those described for other zeranol radio-immunoassays (Dixon, 1980; Dixon and Russel, 1983; Thouvenot and Morphin, 1983).

Despite the high assay sensitivity, plasma values from treated animals did not appear to be significantly different from control values, even when zeranol concentrations in plasma are measured a few hours after implantation and then for several days afterwards.

Plasma proteins could bind zeranol, or one of its metabolites, with high affinity. This phenomenon would impede extraction and RIA determination of residues in the blood of treated animals.

Plasma incubation in the presence of pronase before this extraction step did not change the results: residue concentrations determined by RIA were still not significantly different from those of untreated animals.

From these observations, we can assume the existence in plasma of an unknown zeranol metabolite that would not be recognized by our antiserum (D 7851).

Detection and identification of this hypothetical metabolite are still in progress.

Evolution of urine concentration with time is easily followed using our assay. Forty to fifty days (Gaspar *et al.*, 1983) after treatment, high residue levels (> 6ppb) in urine are still measurable.

An interesting point was the observation of non synchronized excretion patterns for free and conjugated residues in urine. There is a delayed increase in conjugated residues, the excretion rising only from the 25th day after treatment.

Assays more specific for zeranol are now under study. These assays will be required for the regulatory control of the use of anabolic agents in meat producing animals.

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Summary

Anti-zeranol 7-hemisuccinate-bovine serum albumin was raised in rabbits. This antiserum was used in a radio-immunoassay of zeranol residues in urine and plasma of calves implanted with Forplix^R. The antibody was specific for zeranol but cross-reacted with its metabolite zearalanone and the mycotoxin zearalenone. Detection limits in plasma and in urine were 100 pg/ml and 1 ng/ml respectively. In veal calves treated with zeranol containing implant, no residues were detected in plasma even if plasma proteins were hydrolysed with pronase before the radio-immunoassay. Free and conjugated residues in urine were easily measured. The urine concentration of conjugated residues increased markedly after the 20th day of treatment and was still high (19 ppb) at day 40.

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