A fast immunoassay for the screening of alfa-trenbolone in bovine urine

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Abstract

Brazil is one of the many countries that forbids the use of anabolic compounds, which generates difficulties on monitoring its use, once it has one of the biggest cattle herd. Therefore, several anabolic compounds are used, including trenbolone acetate. With the agreement of "Ministério da Agricultura, Pecuária e Abastecimento", an ELISA based test was done with the production of polyclonal antibodies in rabbits and testing in steers urine which received trenbolone acetate implants. The test showed to be cheap, easy and reliable to use in bovine urine to determine alfa- trenbolone, trenbolone acetate major metabolite, released in the animals urine until 60 days after implant was been injected. The results in comparison to the ones obtained by the commercial kit used by the Brazilian "Ministério da Agricultura, Pecuária e Abastecimento" were similar, with no significant differences.

Key-words:
Trenbolone.
ELISA.
Cattle.

Introduction

The trenbolone acetate (TBA) is one of the anabolic substances used to increase muscle and weight gain in steers and there are several commercial products with trenbolone as major substance. According to the Brazilian "Ministério da Agricultura, Pecuária e Abastecimento", the use of any substance, natural or not, with anabolic activity to aim weight gain or growing is forbidden ("Instrução Normativa n.10, de 27 de abril de 2001"). The same law is applied in the European Union Community[1]. The authorities concern is the legal use of anabolic compounds in USA and Argentine, from where it comes in an illegal way.

The monitoring of these substances is done by Reference Laboratories, using imported Kits to certify the meat for exportation and it is applied to only a few hundreds of animals because the high cost of these tests.

The mecanism of action of the anabolic substances happens by the protein deposition on tissues[2]. According to Hayden, Bergen and Merkel[3] the treatment with trenbolone acetate combined or not with other substances does not affect the insulin, cortisol and growth hormone but increases its action, increasing the number of liver hormone cell receptors. The residues of such substances in our body, as final consumers, is not well known but it can leads to hormone problems, as seen in studies with DES[4]. The trenbolone acetate, in bovine metabolism, becomes alfa-trenbolone in urine and beta-trenbolone in muscles. This work aimed to develop an immunoassay, based on polyclonal antibodies produced in rabbits, to identify alfa-trenbolone from bovine urine, with the simplest handling of samples, which is traduced into lower costs and less time consuming. Identify alfa-trenbolone in urine represents a non-invasive technique and it means monitoring the cattle before the death, which is desirable to control the use and to discharge contaminated animals for population consumption[5].

Material and Methods

Immunogen preparation

The trenbolone molecule can not induce antibody response itself, needing a conjugation to a protein, so the hapten (TBA) linked to BSA (protein) can together be immunogenic. TBA (Sigma) was used in the Carbodiimide-BSA method[6]. The immunogen was injected in female rabbits, to produce polyclonal antibodies, in a 15 days schedule for three months, where the first injection was with Complete Freund Adjuvant, subcutaneously, the second with Incomplete Freund Adjuvant, also subcutaneously and the other injections were intramuscle, with PBS. A blood sample was token before each immunization. After this period, the rabbits were bled and the sera collected.

ELISA Optimization and Validation

The titer optimization was done using indirect PTA-ELISA[7] (Plate Trapped Antigen). Microtiter plates were coated with TBA for one hour, at 37 ^{0}C. The plates were quenched with 200 mL of PBS-BSA 1%, for another hour at 37 ^{0}C, 100 mL of diluted sera samples were added to the microplates, incubated for one hour, at 37 ^{0}C.Plates were washed three times with PBS-T-G solution (PBS buffer, pH7,4; 0,5 % of tween 20 and 2,5 % of gelatin). 100 mL of secundary antibody (goat anti-rabbit HRP) was incubated for one hour, at 37 ^{0}C. Enzyme substrate was OPD. The reaction was stopped by adding 100 mL aliquots of HCl 4N. The coloured product of peroxidase reaction was measured at 480 nm, by Microplate Reader, Packard Spectra Count^{TM}.

After rabbit serum optimizations, an ELISA competition assay was performed. Trenbolone molecules were bind to HRP, using protocols[8,9] from the Residue Laboratory, Veterinary Science Division, Belfast, Northern Ireland. The same ELISA was used for the standard curves for alfa and beta trenbolone molecules. The enzyme substrate used was TMB, stopped by adding 100 mL of HCl 1N. Coloured peroxidase reaction was measured at 450 nm.

The ELISA validation (sensibility, specificity[8]; accuracy or exactness and precision[6]) was done as the standards specified by FAO. For the determination of specificity, cross-reacting profiles of antiserum against testosterone, beta-19 nortesterone, alfa-19-nortestosterone and 17-beta-oestradiol, trenbolone acetate and beta-trenbolone were performed. The curves were plotted as log concentration against relative percentage absorbance measured at 450 nm. Cross reaction was calculated by interpolating the concentration of the relevant hapten and each of the competitors that was required to give 50 % displacement, in the following formula.

$$(50\%) = \frac{\text{concentration of trenbolone hapten needed for 50 \% displacement}}{\text{Concentration of competing hapten needed for 50 \% displacement}}$$

Bovine urine test

Six steers received the pellets implants (commercial drug: Finaplix - trenbolone acetate) and six steers were used as controls. The animal age ranged from 60 to 80 days, from different breeding origins. The pellets were introduced in the back ear, as following the fabricant recommendations. Urine samples were collected in plastic bags each 4 days, until the recommended period of 60 days[5], all in the morning period. The protocol used was the same established in the "Laboratory of Hormonology" commercial Kit (CER – Marloie – Belgic). A filtration was done in Whatman paper no. 1, to discharge rude residues (hair, dust) and the urine samples were centrifuged (5000 rpm for 5 minutes) and 100 mL was used in ELISA microplates.

Results and Discussion

To optimize the ELISA tests with the sera produced in rabbits, calibration curves were done using SIGMA standards for alfa and beta trenbolone (Figure 1). The anti-trenbolone serum was diluted 1:1000 to be

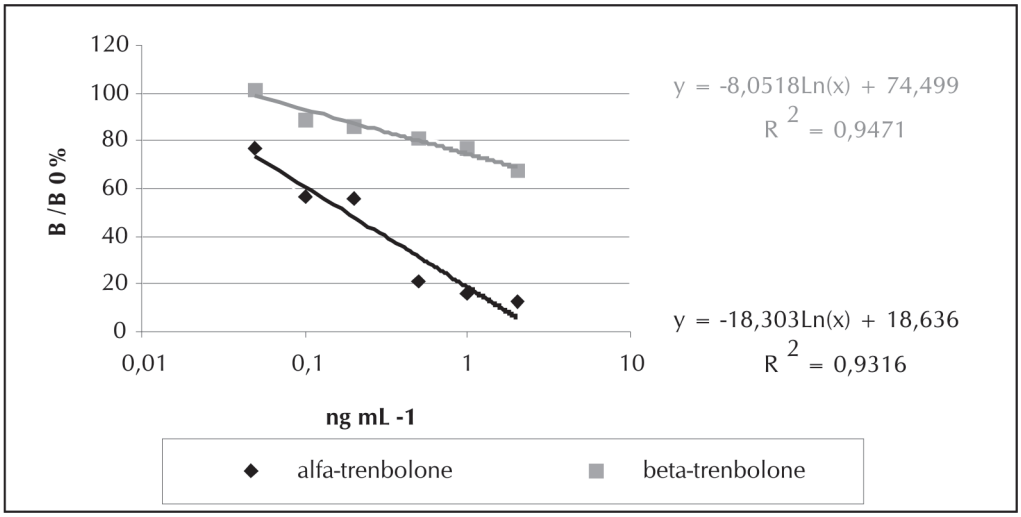

Figure 1 – Calibration curves using alfa-trenbolone and beta trenbolone standards. Rabbit Serum diluted 1:1000 in a Competition ELISA

used in the assay. The competition assay was done using our own sera and trenbolone–HRP liked molecule.

By using the Kit to obtain the curves, our equations and R^2 did not differ, in the alfa trenbolone curve, as in table 1. Despite the R^2 from the kit showed to be lower than our curve (Figure 1), neither value can be used for urine samples, since the major metabolite in urine is alfa-trenbolone.

The quality of our curves showed to be in the established limits (around 0.98 $\pm$ 0.02), according to Van De Wiel and Koops[10].

The exactness of our ELISA could be measured by the difference between values of "R" for our sera and the commercial Kit for both trenbolone metabolites, with 0.05% for alfa- trenbolone and 9.1 % for beta-trenbolone. According to Plazier[11], the exactness can be up to 10%.

The urine samples were immediately frozen as they were collected and by the end of 60 days, all the samples were centrifuged and used in the competition ELISA. Comparing to the standard curves, D.O. measurements were converted to ng of trenbolone. mL^{-1} and, especially on figure 2, we can observe the curve of alfa-trenbolone residues on the animal urines, along the period.

As shown in figure 3, the beta-trenbolone residues could not be quantified in urine samples, as been a muscle residue, according to Dubois et al.[12]. Only the alfa-trenbolone can be quantified in urine samples using the simplified method for extraction (one-step centrifugation of the urine collected). The animals were maintained for more 90 days before incineration.

The specificity was determined by Cross-reaction[8], as shown on table 2.

Sensibility

By the standard curves and the urine samples, the ELISA sensibility was 1 ng per

Table 1 – Equations and R^2 values obtained with the antibodies and HRP-linked trenbolone in comparison to the commercial Kit

		curve equation	R^2	R
alfa-trenbolone	our curve	Y=-18,303Ln(x) + 18,636	0.9316	0.96519
	commercial kit	Y=-37,683Ln(x) + 88,987	0.9409	0.97000
beta-trenbolone	our curve	Y= -8,0518 Ln(x) + 74,499	0.9471	0.97319
	commercial kit	Y= -39,285 Ln(x) + 107,74	0.7948	0.89152

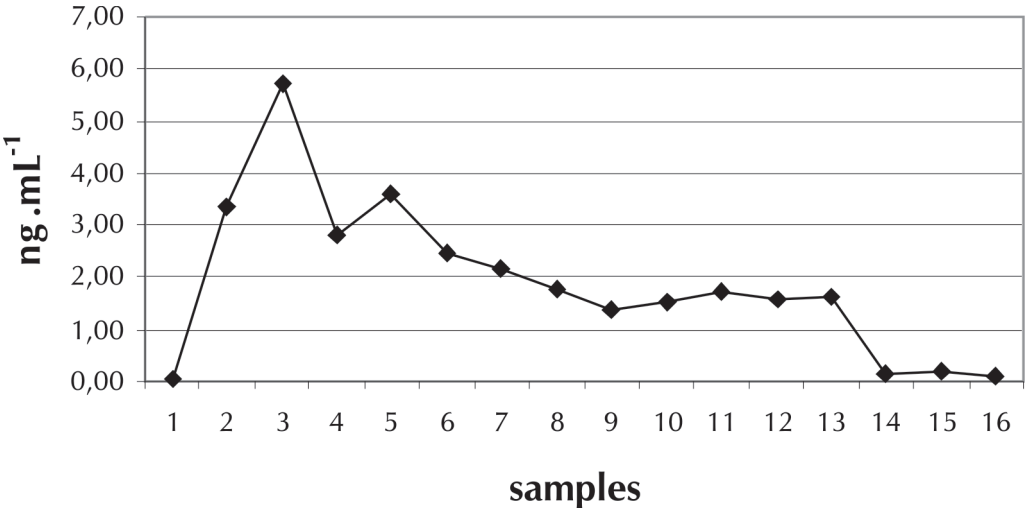

Figure 2 – Alfa-trenbolone residues from urine samples collected during 60 days (average of 6 replicates). Data converted from D.O. 450 nm in Competition ELISA. The samples were collected each 4 days

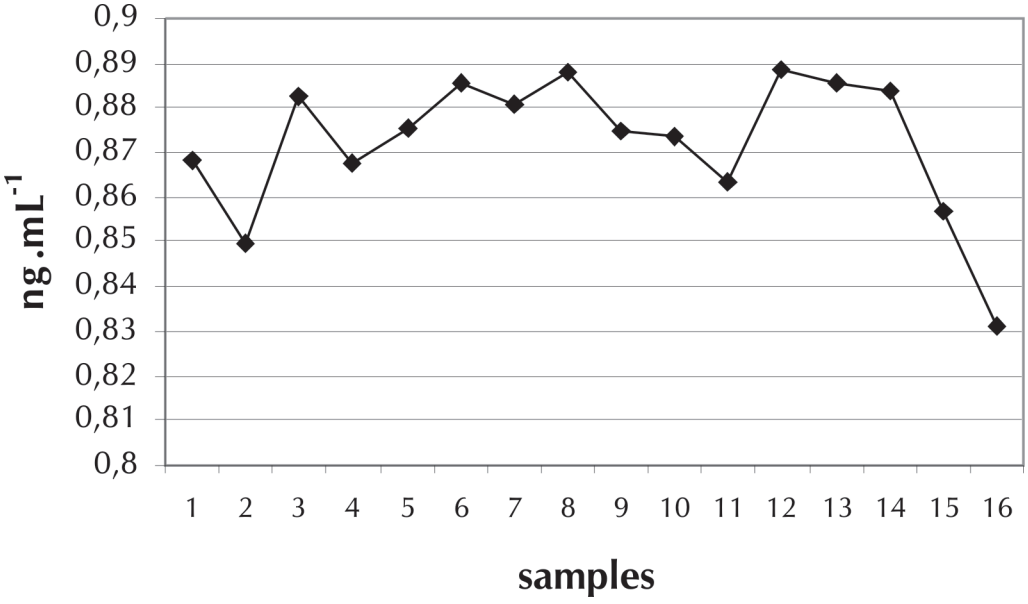

Figure 3 – Beta-trenbolone residues from urine samples collected during 60 days (average of 6 replicates). Data converted from D.O. 450 nm in Competition ELISA. The samples were collected each 4 days

Table 2 – Cross-reacting haptens to trenbolene, expressed by %

cross-reacting haptens	% cross reaction
Trenbolone	100
beta-trenbolone	98
beta-19-nortestosterone	49.21
alfa-19- nortestosterone	18.86
17-beta-oestradiol	19.40
testosterone	13.16

mL (Figure 1) for alfa-trenbolone, which showed to be a safe value to work with urine samples (Figure 2). Detection Limits can only be lower with the use of chromatography[13], which increases the costs of the test, only recommended for a confirmatory test.

The precision of this Competition ELISA is done by the repetibility and

reproducibility of the results. The reproducibility of the assay was not determined since the license to use the trenbolone was given for one experiment. The repetibility was reached by the samples repetitions done in all assays, with no significative differences on the data for urine samples and the use of all sis steers data.

Conclusions

Such results can disable this test for monitoring animals with suspect of anabolic use, by collecting urine samples[14], lowering the cost for the test and the time consuming to prepare the samples in comparison to the methodologies described by several authors including Dubois et al[12]. To analyse drug residues in livestock, any effort has to be done in charge to make available low-cost tests for the countries whose law forbids the use and for the countries whose meat quality has been a priority for the population. There is a need to develop practical, economic, and very accurate methods for residues detection, particularly those originating in products chemically different from the natural steroid hormones and their derivatives[5]. The ELISA test presented was developed to help Brazilian Ministry Authorities to use our own tests to certify our products, as a screening test, to aim better Veterinary Sanity and Food Quality.

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Um imunoensaio rápido para a detecção de alfa-trembolona em urina bovina

Resumo

O Brasil está entre os países que mantêm o uso de anabolizantes proibido, o que gera dificuldades muito grandes no monitoramento destas substâncias, uma vez que possui o maior rebanho bovino. Contudo, diversas substâncias são largamente utilizadas, entre as quais o acetato de trembolona. Com o consentimento do Ministério da Agricultura, Pecuária e Abastecimento, foram produzidos anticorpos policlonais para um teste baseado em ELISA ("enzyme linked immunosorbent assay") capazes de detectar acetato de trembolona na urina de bovinos tratados com trembolona. O teste apresentou baixo custo e de fácil execução para a detecção de alfa-trembolone, o principal metabólito na absorção do acetato de trembolona, liberado na urina dos animais. Os resultados foram similares aos obtidos com o "kit" comercial usado pelo Ministério da Agricultura, Pecuária e Abastecimento do Brasil.

Palavras-chave:
Trembolona.
ELISA.
Bovinos.

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