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DETERMINATION OF TRENBOLONE IN CATTLE MEAT WITH ELISA METHOD

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ABSTRACT

In recent years, hormones and hormone like substances have been recently used in livestock production to obtain a high yield performance in a shorter period of time.

These anabolic agents are used to increase the weight gain, to improve the food efficiency, storing protein and to decrease fatness. But, depending on the use of anabolic in animal feed, anabolic residues that may occur in meat and meat products present risks to human health. The present study was undertaken to detect and quantify the levels of trenbolone residues (a potent synthetic analog of testosterone) in meat in Republic of Macedonia. Cattle meat samples were collected within period of 12 months as they were delivered by the authorised veterinary inspectors. A total of 82 samples of cattle meat were analyzed for level of trenbolone by Enzyme-Linked Immunosorbant Assay method. The detection limit was 91.5 ppt for the assay. The overall recoveries and the coefficients of variation (CVs) were in the range of 83.7%-99.6% and 4.1%-8.2%, respectively, a working range between 25 to 400 ppt, and the regression equation of the final inhibition curve was: $y = -0.2451x + 1.6221$, $R^2 = 0.9928$. The average experimental value of trenbolone in meat was 152.2 ppt. This value gave no evidence for the illegal use of hormones in Republic of Macedonia, but these results do not exclude the possibility of misuse of these potentially harmful chemicals in future. Therefore it is necessary to conduct permanent control of these hormones as a food quality and health safety measure.

Key words: Trenbolone, ELISA, validation, residues, cattle meat.

INTRODUCTION

In recent years, hormones and hormone like substances have been recently used in livestock production to obtain a high yield performance in a shorter period of time.

These anabolic agents are used to increase the weight gain, to improve the food efficiency, storing protein and to decrease fatness. But, depending on the use of anabolic in animal feed, anabolic residues that may occur in meat and meat products present risks to human health (1-4). Trenbolone acetate is a powerful synthetic steroidal androgen, which is used as a growth promoter in cattle. It is rapidly hydrolyzed to its metabolite 17 β- trenbolone after administration to cattle. It is thought to act on skeletal muscle, either through androgen receptors to increase protein synthesis or through glucocorticoid receptors to reduce the catabolic effects of glucocorticoids. Trenbolone acetate decreases the rate of both protein synthesis and degradation, and when the rate of degradation is less than the rate of synthesis, muscle protein rate increases (5). In many countries outside the EU trenbolone acetate is licensed as growth promoter for steers and heifers. In addition, its tremendous effectiveness in dry and lactating cull cows (38 % increased weight gain accompanied by lower fat deposition) was reported (6). The European Economic Community (EEC) banned the use of anabolic compounds as growth accelerators in food animals while the United States Food and Drug Administration (USFDA) permitted the limited use of some hormones with natural origins (such as estradiol and testosterone) and some synthetic hormones such as trenbolone in animal husbandry (7-8). The permitted limit values for trenbolone is 1 ppb in muscle (CRL GUIDANCE PAPER (7 December 2007)). In Republic of Macedonia, the use of hormones as growth promoters has been made illegal too. The aim of this study was to detect the levels of trenbolone residues in the meat in Republic of Macedonia with ELISA methods.

MATERIALS AND METHODS

Meat samples were collected from March 2011 to March 2012. The samples were kept frozen until use. We analyzed a total of 82 cattle meat samples.

Reagents. Most of the reagents that we used were contained in the RIDASCREEN Trenbolone test kit from R-Biopharm AG, Darmstadt, Germany. Kit contain: Microtiter plate with 96 wells (12 strips with 8 removable wells each) coated with capture antibodies, 6 standard solutions, 0 ppt (zero standard), 25 ppt, 50 ppt, 100 ppt, 200 ppt and 400 ppt trenbolon in 40 % methanol, conjugate (peroxidase conjugated trenbolone), anti-trenbolone antibody, substrate (containing urea peroxide, chromogen (containing tetramethylbenzidine), stop solution (containing 1 N sulfuric acid), conjugate and antibody dilution buffer.

Methanol and tertiary butyl methyl ether were of analytical grade and purchased from Merck. PBS (Phosphate buffer solution) 67 mM, pH 7.2, was prepared

by mixing 1.79 g sodium dihydrogen phosphate hydrate (NaH_2PO_4 x H_2O) with 9.61 g disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O) and 8.7 g sodium chloride (NaCl) and filling up to 1000 ml disstilled water. 20 mM PBS buffer, pH 7.2, was prepared by mixing 0.55 g sodium dihydrogen phosphate hydrate (NaH_2PO_4 x H_2O) with 2.85 g disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O) and 9 g sodium chloride (NaCl) and filling up to 1000 ml disstilled water. From fortified samples and calculation of recovery we used external standard trenbolone (Sigma- Aldrich).

Apparatus. Microtiter, plate spectrophotometer (BIO RAD model 680) (450 nm), evaporator, mixer, shaker, vortex, centrifuge, balance, RIDA® C18 column, micropipette (20 µl, 50 µl, 100 µl, 200-1000µl).

Extraction procedure. Fat and connective tissue were removed from the muscle and 10g of the ground muscle was homogenized with 10mL of 67mM PBS buffer by mixer for 5min. 2g of homogenized sample were mixed with 5mL of tertiary butyl methyl ether (TBME) in a centrifugal screw cap vial and shaken vigorously by vortex for 30-60min. The contents were centrifuged at 3000rpm for 10min. The supernatant was kept and the extraction with TBME was repeated. The supernatants were combined and evaporated then the dried extract was dissolved in 1mL of 80% methanol. The methanolic solution was diluted with 2mL of 20mM PBS-buffer and applied to a RIDA C18 column (solid phase extraction column with C18 end-capped sorbent of an average particle size of 50µm**)** in the following manner:

- Column was rinsed by flowing of 3mL methanol (100%); Column was equilibrated by injection of 2mL PBS – Buffer; 3mL of sample was loaded on column; Column was rinsed by injection of 2mL methanol (40%); Column was dried by pressing nitrogen through it for 3min; Sample was eluted slowly by injection of 1mL methanol (80 %). An aliquot of the eluate was diluted with water (1+1, v/v), then 20µL per well of resulting solution was used in the test.

Test procedure. Ridascreen ELISA kit was obtained from R Biopharm GmbH, Germany. Trenbolone standard solution used for the calibration curve were at levels of 0, 25, 50, 100, 200, and 400 ppt trenbolone in 40 % methanol,, whereas the antibody used had cross reactivity with other related compounds, as indicated by the manufacturer's literature and shown in Table 1. The standard and samples were analyzed in duplicate. To the marked microwells, 50µL of the diluted enzyme conjugate (peroxidase conjugated testosterone) was added. Then 20µL of standards or samples were added. After that 50µL of the diluted antibody solution was added and after mixing gently by rocking the plate manually, the contents were incubated at room temperature for 2h. The liquid poured out of the wells and after removal of liquid completely, all wells were filled with distilled water (250µL). After rinsing, the water was also discarded; washing was repeated two more times. Then, 50µL of substrate (urea peroxide) and 50µL of chromogen (tetramethylbenzidine) were added. After mixing thoroughly and incubating for 30min at room temperature and dark, 100µL of stop solution (1M sulphuric acid) was added. After mixing, the absorbance was read at 450nm. Colour was stable for 60min. Data were analyzed using a special software RIDAWIN ELISA (R-Biopharm, Darmstadt, Germany). The mean absorbance values obtained for the standards and the samples divided by the absorbance value of the first standard (zero standard) and multiplied by 100 was the % absorbance. The zero standard was thus made equal to 100 % and the absorbance values were quoted in percentages.

Table 1. *Cross reactivity of trenbolone antibody with various compounds*

Compound	Cross reactivity (%)
17β-Trenbolone	100
Trendione	100
17α-trenbolone	82
19 nortestosterone	0,06
Testosterone	<0,01
Estradiol	<0,01
Zeranol	<0,01
DES	<0,01
Chloramphenicol	<0,01

Method validation. The limit of detection (LOD) was obtained by spiking with 0, 5 times from MRPL which 1 ppb is according the guidance letter from Community Reference Laboratories' (7 December 2007). The method recovery was determined at three level by spiking meat samples with 0,5; 1 and 1,5 times from MRPL level. For determination of repeatability, the same steps were repeated on two occasions in the same analytical conditions. Detection capabilities (CCβ) was evaluated by analyzing 20 spiked samples at 0,5 MRPL level. A typical ELISA standard curve is presented in Figure 1. Final trenbolone concentrations in meat were calculated by taking the average recoveries into account.

RESULTS AND DISCUSSION

The calculation of the gained results was made by RIDAWIN Software. For construction of the calibration curve the mean of the absorbance values obtained for the standards was divided by the absorbance value of the first standard (zero standards) and multiplied by 100. The absorption is inversely proportional to the concentration of trenbolone. As can be seen in Fig.1, the trenbolone calibration curve was found to be virtually linear in the 20 to 400 ppt.

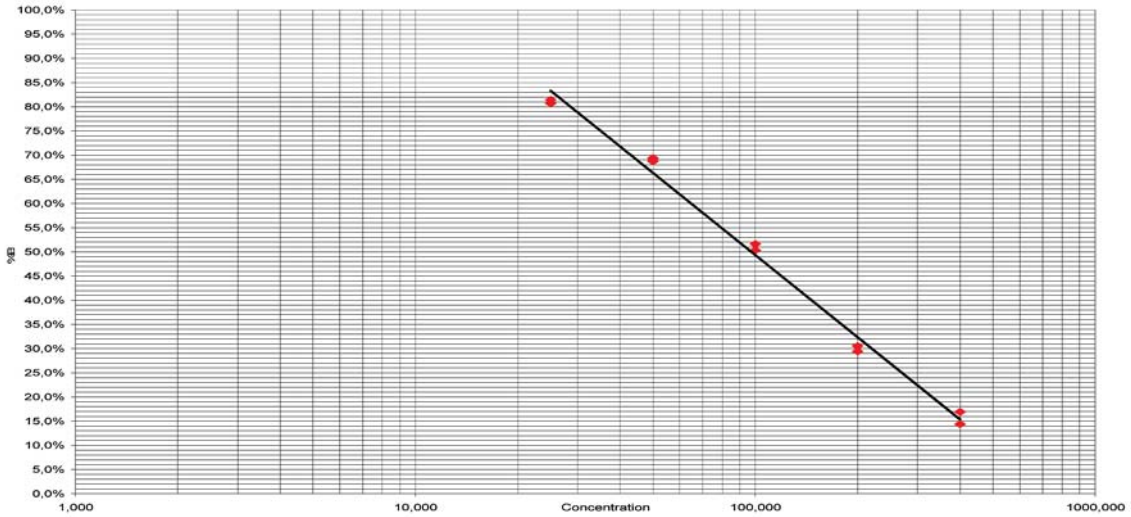

Figure.1 *Linearity of calibration curve for trenbolone standards (25-400 ppt)*

The estimated LOD for meat samples for trenbolone was 91.5 ppt. The CCβ for meat samples for trenbolone was 492.9 ppt. In Fig.2 the correlation between the absorbance ratio and trenbolone concentration was evaluated over the range 0 – 400 ppt, R^2=0.9928.

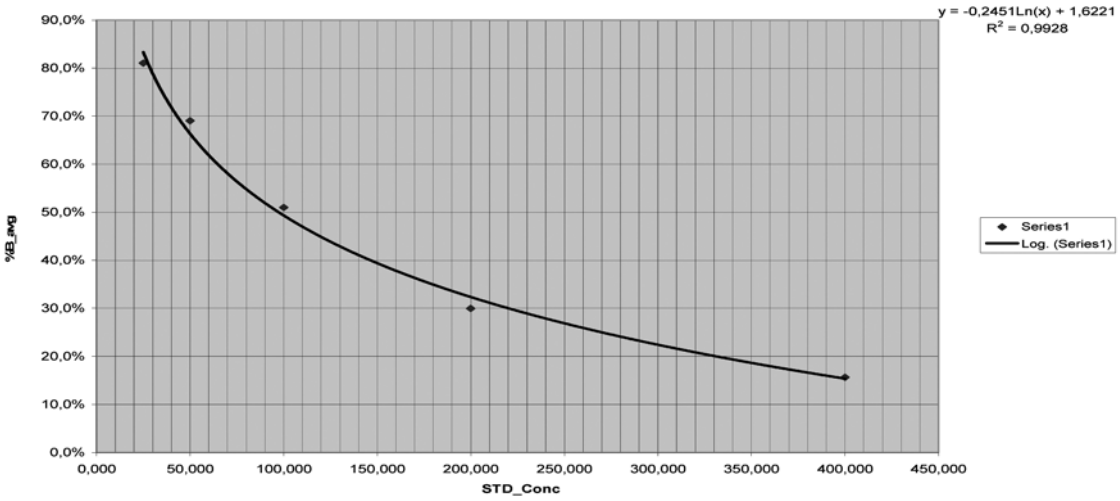

Figure.2. *Calibration curve for trenbolone standards (0-400 ppt)*

The precision of the method was calculated by measured CV%. The precision dates are shown in Table 2.

Table 2. *Precision of the method (% CV)*

	Concentration (ppt)	CV%
Standards	0.0	3.0
	25.0	0.6
	50.0	1.8
	100.0	1.5
	200.0	0.7
	400.0	4.7
CV for fortified samples	500.0	8.2
	1000.0	6.5
	1500.0	4.1

The results of method recovery (n=18) and repeatability (n=54) are presented in table 3.

Table 3. *Recovery and repeatability*

Validation parameter	No. of replicates	Spiked concentration ng/kg	Determinated concentration ng/kg	Mean recovery %	Coefficient of variation %
Recovery	6	500	418.6	83.7	8.2
	6	1000	962,1	96.2	6.5
	6	1500	1494,8	99.6	4.1
Repeatability	18	500	442.4	88.4	10.2
	18	1000	911.4	91.1	3.6
	18	1500	1432.7	95.5	4.7

Validation of the method used in on trenbolone determination resulted in the mean recovery of 83.7%-99.6% and repeatability of 88.4%-95,5% with coefficient of variation (CV) of 4.1%-8.2% and 3.6%-10.2%.

The analyses of the meat samples showed concentrations from 66.7 ppt to 258.8 ppt (mean concentration of trenbolone was 152.2 ppt).

Our test results are of importance as they give information about the use of trenbolone preparations in national animal husbandry and in the food industry. The European Economic Community (EEC) banned the use of anabolic compounds as growth accelerators in food animals while the United States Food and Drug Administration (USFDA) permitted the limited use of some hormones with natural origins (such as estradiol and testosterone) and some synthetic hormones such as trenbolone in animal husbandry (1-4). The permitted limit values for trenbolone is 1 ppb in muscle (CRL GUIDANCE PAPER (7 December 2007)). A survey carried out in R. Macedonia from March 2011 to March 2012 demonstrated that the incidence of residues of trenbolone in tissues of slaughter animals is not a problem, which means that trenbolone gave no evidence of illegal use of these hormones in R. Macedonia. The National Residues Control Plan guarantees fulfilment of the requirements which are of importance to health of both humans and animals as well as marketing of animals, food and other products of animal origin.

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ОПРЕДЕЛУВАЊЕ НА ТРЕНБОЛОН ВО МЕСО ОД ГОВЕДА СО ELISA МЕТОД

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АПСТРАКТ

Во последниве години хромоните и сипстанците слични на хормони се користат во одгледувањето на добитокот за добивање на високо приносни карактеристики за пократок временски период.

Овие анаболици се употребуваат за зголемување на масата, подобрување на екфектот на искористување на храната, депонирање на протеините и намалување на масното ткиво. Но во зависност од примената на анаболиците во добиточната храна, нивни резидуи кои може да се јават во месото и производите од месо претставуваат ризици за здравјето на луѓето. Оваа студија беше спроведена за детектирање и квантификување на нивоата на резидуи од тренболон (синтетички аналог на тестостеронот) во примероци од месо од Република Македонија. Примероците од говедско месо беа собирани во текот на 12 месеци, а беа доставувани од овластените ветеринарни инспектори. Вкупно 82 примерока говедско месо беа анализирани за утврдување на нивото на тренболон со Ензимски врзан имуносорбентен метод. Лимитот на детекција при определувањето беше 91,5 ppt. Вкупниот аналитички принос и коефициент на варијанца (CV) беше во опсегот од 83,7-99,6 % и 4,1-8,2 %, соодветно, со работен опсег од 25 до 400 ppt. Равенка на регресија на конечната инхибиција беше : y= -0,2451x + 1,6221, R^2 = 0,9928. Вкупната експериментално определена вредност на тренболонот во месо беше 152,2 ppt. Оваа вредност укажува дека нема нелегална употреба на хормони во Република Македонија, но овие резултати не ја исклучуваат можноста за злоупотреба на овие потенцијално штетни супстанции во иднина. Заради тоа, неопходно е да се спроведува постојана контрола на овие хормони како мерка за контрола на квалитетот на храната и безбедност за здравјето.

Клучни зборови: тренболон, ELISA, валидација, резидуи, говедско месо.