

## HUMAN HEALTH AND TRENBOLONE RESIDUE IN BOVINE MEAT

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### ABSTRACT

In recent years, hormones and hormone-like compounds have been frequently used in vegetable and livestock production to obtain a high yield performance in a shorter period of time, but depending on the use of anabolics in animal feed, anabolic residues that may occur in meat and meat products would present the risks to the human health. The present study was undertaken to detect and quantify the levels of trenbolone residues (a potent synthetic analog of testosterone) in the market meat in Iran. Cattle meat samples were collected from the markets in Tehran. A total of 120 samples of cattle meat were analyzed for level of trenbolone by Enzyme-Linked Immunosorbant Assay method. The average experimental values of trenbolone in cattle meat were  $3.76 \pm 5.26$  ng/kg. This value gave no evidence for the illegal use of hormones in Tehran, but these results do not exclude the possibility of misuse of these potentially harmful chemicals in future. There is, therefore, need to routinely monitor these chemicals as a food quality and health control measure.

**Key words:** Trenbolone, human health, cattle meat, Enzyme-Linked Immunosorbant Assay

### INTRODUCTION

In recent years, hormones and hormone-like compounds have been frequently used in vegetable and livestock production to obtain a high yield performance in a shorter period of time. These anabolic agents are used for increasing the rate of weight gain, improving the feed efficiency, storing protein and decreasing fatness (Sawaya *et al.*, 1998). But depending on the use of anabolics in animal feed, anabolic residues that may occur in meat and meat products present risks to human health. As a result, many countries restrict or prohibit the use of anabolic compounds in livestock production.

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 $\beta$ -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 (Berge and Colioli, 1993).

Trenbolone was found to bind covalently to rat liver DNA. Trenbolone exhibited a weak transforming effect on Syrian hamster embryo cells (Lasne *et al.*, 1990; Tsutsi *et al.*, 1995).

Meat and meat products, which play an important role in human nutrition should be safe and should not contain any factors or substances harmful for human health. However, the anabolic agents used for various purposes in animal husbandry for slaughter tend to leave residues and thus cause some problems in consumer health (Hoffman, 1996). 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 (Sawaya *et al.*, 1998; European Commission, 1999; Nazli *et al.*, 2005).

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The permitted limit values for trenbolone are 2ppb in muscle and 10ppb in liver (Codex Alimentarius, 1997; European Commission, 1999).

In the old times, products of animal origin harvested by hunting were considered as noble food contributing to strength, health, longevity and the well being of man (Aumaitre, 1999). Since the 1st January 1989, according to Directive 88/146/EEC (The European Economic Community) replaced later by Directive 96/22 EC, the European Community (EC) prohibited the application by any means to farm animals, substances having a thyrostatic, oestrogenic or gestagenic action for growth promotion purposes (Wozniak, 2002). The prohibition covers both the use of these hormones for domestic production and import of meat from animals treated with hormones for growth promotion (EEC, 1989). Unlike the EC, a few countries permit the use of the hormones in cattle as anabolic agents (Wozniak, 2002).

The United States Food and Drug Administration (FDA), has developed a scientific and rational system to assure human safety from both naturally occurring and synthetically derived hormones and  $\beta$ -agonists used for meat production and allowed natural steroids to be used in veterinary medicines (Saeed *et al.*, 1999). In Iran, the use of hormones as growth promoters has been made illegal too. Although these compounds have been banned or limited, the concentrations of the preparations are sometimes 100 times higher than the permissible legal limits (Vanoosthuyze *et al.*, 1994).

To detect the use of legal or illegal xenobiotic drugs, laboratories are required to develop extensive monitoring programs for the drug residues in meat products (Saeed *et al.*, 1999). The method of choice should be accurate, sensitive, specific and precise, so that both false negative and false-positive results should be obtained. For this purpose routine methods are needed. Recently, Enzyme-Linked Immuno Sorbant Assays (ELISA) have been established as screening methods (Meyer *et al.*, 1991; Dursch and Meyer, 1992; Scippo *et al.*, 1993; Sawaya *et al.*, 1998).

In order to control the hormone residues in meat and to ensure the safety of Iranian consumers, it is imperative that a monitoring system be put in place to address the concerns. The present study

was undertaken to detect and quantify the residues of trenbolone hormone in meat and to assay its risk to human health and to assess the present status of the levels of anabolics in Iranian meat industry.

## MATERIALS AND METHODS

### *Meat samples*

Cattle meat samples were obtained randomly from the markets in the city of Tehran. A total of 120 samples of cow meat were gathered and kept frozen until use.

### *Reagents*

Most of the reagents used were contained in the RIDASCREEN test kit. Methanol and tertiary butyl methyl ether (TBME) were of analytical grade and purchased from Merck.

Phosphate buffer solution (PBS) 20mM (pH=7.2) was prepared by mixing 0.55g sodium dihydrogen phosphate hydrate with 2.85g disodium hydrogen phosphate-2-hydrate and 9g sodium chloride and filling up to 1000mL with distilled water. PBS buffer 67mM (pH=7.2) was prepared by 7.8g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  + 9.61g  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  + 9g NaCl fill up to 1000mL with distilled water.

Trenbolone standard solutions used for the calibration curve at levels of 0, 25, 50, 100, 200, and 400ng/L (ppt) and were all included in the ELISA test kit.

### *Apparatus*

Microtiter plate spectrophotometer (450nm), centrifuge, RIDA  $\text{C}_{18}$  column, mixer and shaker were used for the analysis.

### *Extraction of muscle*

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 C<sub>18</sub> column (solid phase extraction column with C<sub>18</sub> 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 (20mn).
- 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, then 20µL per well of resulting solution was used in the test.

#### Test procedure

Ridascreen ELISA kits were obtained from R-Bio-pharm GmbH, Germany. Trenbolone standard solution used for the calibration curve were at levels of 0, 25, 50, 100, 200, and 400ng/L(ppt) trenbolone in aqueous solution, whereas the antibody used had cross reactivities 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 sulfuric acid) was added. After mixing, the absorbance was read at 450nm. Color was stable for 60min.

Table 1: Cross reactivities of trenbolone antibody with various compounds

Compound	Cross-recativity (%)
17β-Trenbolone	100
Trendione	100
17α-trenbolone	82
nortestosterone	0.06
Testosterone	<0.01
Estradiol	<0.01
Zeranol	<0.01
DES	<0.01
Chloramphenicol	<0.01

#### Evaluation of trenbolone

For the 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 standard) and multiplied by 100 (percentage maximum absorbance). The absorption is inversely proportional to the trenbolone. The detection limit for trenbolone was found to be 25ng/kg.

#### Statistical analysis

For data statistical analysis, and comparison the average value of the sample trenbolone concentration with the allowable value of FDA, one sample T-test was used.

## RESULTS

#### Method validation

The accuracy of the method was verified by means of recovery assay. This was accomplished by an analyzing standard solution and spiked (enriched) samples; the analytical recovery was 85%.

The precision of the method was calculated by the measured %CV (n=10). The precision data are shown in Table 2.

Table 2: precision (%CV)

	Concentration (ng/kg)	%CV
standards	25	4.9
	50	6.8
	100	5.2
	200	4.5
	400	3.7
sample	25	8.7
	100	9
	400	6.4

#### Sample screening

The samples were collected from the market and

analyzed for trenbolone concentration. 20 $\mu$ L of Samples solution +20 $\mu$ L of standard 50ng/kg were added to the wells to analyse (standard addition method was used because of detection limit 25ng/kg). The results indicate that mean of concentration for cattle meat (n=120) was  $3.76 \pm 5.26$  ng/kg (min=0.03, max=20.1ng/kg).

## DISCUSSION

In this study cattle meat samples were collected from the markets in Tehran. A total of 120 samples of cattle meat were analyzed for level of trenbolone by Enzyme-Linked Immunosorbant Assay (ELISA) method. The average experimental values of trenbolone in cattle meat were  $3.76 \pm 5.26$  ng/kg. The mean concentration of cattle meat trenbolone residue,  $3.76 \pm 5.26$  ng/kg, has a significant difference ( $P < 0.001$ ) with the FDA allowable level, 50000ng/kg, and daily intake of trenbolone by meat will not be upper than JECFA ADI (FDA has set the following allowable increases in trenbolone levels: 50, 100, 150 and 200 $\mu$ g/kg for muscle, liver, kidney and fat, respectively (Doyle, 2000). But JECFA has lower acceptable residue level for trenbolone, 2 $\mu$ g/kg for muscle and 10 for liver (Joint FAO/WHO Expert Committee on Food Additives, 1999; preston, 1999). Acceptable daily intake (ADI) maxima of trenbolone were established by JECFA at 0.02 $\mu$ g/kg body weight (BW)). So it seems that the present status of this anabolic hormone in market meat in Tehran is not at risk. The number of samples included in this survey was relatively small compared to the total sold in the market, and on the other hand these results do not exclude the possibility of misuse of this anabolic hormone in future and significantly increase exposure of humans, particularly children, to trenbolone which may adversely affect health. There is, therefore, need to routinely monitor this chemical as a food quality control measure.

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