PRELIMINARY SCREENING FOR THE LEVELS OF TESTOSTERONE HORMONE IN THE MARKET MEAT IN TEHRAN

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Abstract- Many xenobiotic and natural compounds such as testosterone have been used and sometime misused to improve the growth of cattle and other livestock animals. In order to control the testosterone hormone residues in meat and to ensure the safety of Iranian consumers, a monitoring system must be put in place to address the concerns. The present study was undertaken to detect and quantify the levels of testosterone residue in the market meat. Cattle meat samples were collected randomly from the market in Tehran. A total of 120 samples of cattle meat were analyzed for the level of testosterone by Enzyme-linked immuno sorbent assay (ELISA) method. The average experimental value of testosterone in cattle meat was 810.9 ng/kg. The average value of cattle meat testosterone was significantly upper than FDA (Food and Drug Administration) allowable level but was in agreement with the values proposed by JESFA (Joint Expert Committee on Food Additives). So it seems that the present status of this anabolic hormone in market meat is not at risk but there is need to routinely monitor this chemical as a food quality control measure.

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INTRODUCTION

In the old ages, products of animal origin harvested by hunting were considered as noble food contributing to strength, health, longevity and the well being of man (1). With advances in science, many xenobiotic and natural compounds (steroids hormones and β-agonists) have been used and sometime misused to improve the growth of cattle, sheep and other livestock animals (2). 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 (3). The prohibition covers both the use of these hormones for domestic production and import of meat from animals treated with hormones for growth promotion (4). Unlike the EC, some countries permit the use of the hormones in cattle as anabolic agents (3). The United States Food and Drug Administration (FDA), developed scientific and rational system to assure human safety form both naturally occurring and synthetically derived hormones and β-agonists used for meat production and allowed natural steroids to be used in veterinary medicines (2). In Iran, the use of

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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 (5). 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 (2). The method of choice should be accurate, sensitive, specific and precise, with minimum false negative and false positive results. Recently, enzyme-linked immunosorbent assays (ELISA) have been established as screening methods (6-9).

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 testosterone hormone in meat and to assess the present status of the levels of anabolic in Iranian's meat industry.

MATERIALS AND METHODS

Meat

Cattle meat samples were obtained randomly form the market in Tehran, from May 2004 to April 2005. We analyzed a total of 120 samples of cow's meat. Meat samples were kept frozen until use.

Reagents

Most of the reagents used were contained in the RIDASCREEN test kit. Methanol, tertiary butyl methyl ether (TBME) were of analytical grade and purchased from Merck. PBS buffer 20 mM (pH=7.2) was prepared by mixing 0.55 g sodium dihydrogen phosphate hydrate with 2.85 g disodium hydrogen phosphate-2-hydrate and 9 g sodium chloride and filling up to 1000 ml whit distilled water.

PBS buffer 67 mM (pH=7.2) was prepared by 7.8 g $NaH_2PO_4 \times H_2O$+9.61 g $Na_2HPO_4 \times 2H_2O$+9 g NaCl fill up to 1000 ml with distilled water. Testosterone standard solutions used for the calibration curve were at levels of 0, 50, 200, 800, 3200 and 12800 ng/lit and all included in the ELISA test kit.

Apparatus

Microtiter plate spectrophotometer (450 nm), centrifuge, RIDA C_{18} column, mixer and shaker were used.

Extraction of muscle

Fat and connective tissue were removed from the muscle and 10 g of the ground muscle was homogenized with 10 ml of 67 mM PBS buffer by mixer for 5 min. Two g of homogenized sample were mixed with 5 ml of tertiary butyl methyl ether (TBME) in a centrifugal screw cap vial and shaken vigorously by vortex for 30-60 min.

The contents were centrifuged at 3000 rpm for 10 min. The supernatant was kept and the extraction with TBME was repeated. The supernatants were combined and evaporated, then the dried extract was dissolved in 1 ml of 80% methanol. The methanolic solution was diluted with 2 ml of 20 mM PBS-buffer and applied to a RIDA C_{18} column (solid phase extraction column with C18 end-capped sorbent of an average particle size of 50 μm**)** in the following manner: 1) column was rinsed by flowing of 3 ml methanol (100%), 2) column was equilibrated by injection of 2 ml PBS–Buffer (20 mn), 3) 3 ml of sample was loaded on column, 4) column was rinsed by injection of 2 ml methanol (40%), 5) column was dried by pressing nitrogen through it for 3 min, 6) sample was eluted slowly by injection of 1 ml methanol (80%)

The further procedure for testosterone analysis was: an aliquot of the eluate was reduced to dryness and redissolved in 10% methanol. Then 20 μl per well of resulting solution was used in the test.

Test procedure

RIDASCREEN ELISA kits were obtained form R-Bio-pharm GmbH, Germany.

The standards used for testosterone contained 0, 50, 200, 800, 3200 and 12800 pg/ml^{-1} testosterone 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.

Table 1. Cross reactivity of testosterone antibody with various compounds.

Compound	Cross reactivity
Testosterone	100.0
17β-nor testosterone	10.0
17β-Trenbolone	1.0
17β-Estradiol	<0.1
Cortisol	<0.1
Corticosterone	<0.1
progesterone	<0.1

*Data are given as percent.

The standard and sample were analyzed in duplicate. To the marked microwells, 50 µl of the diluted enzyme conjugate (peroxidase conjugated testosterone) was added. Then 20 µl of standards or samples were added and after that 50 µl of the diluted antibody solution was added, and after mixing gently by rocking the plate manually, the contents, were incubated at room temperature for 2 h. The liquid poured out of the wells and after removal of liquid completely, all wells were filled with distilled water (250 µl). After rinsing, the water was also discarded; washing was repeated two more times. Then, 50 µl of substrate (urea peroxide) and 50 µl of chromogen (tetramethylbenzidine) were added, and after mixing thoroughly and incubating for 30 min at room temperature and dark, 100 µl of stop solution (1 M sulfuric acid) was added. After mixing, the absorbance was read at 450 nm. Color was stable for 60 min.

Evaluation of testosterone

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 testosterone. As can be seen in Fig. 1, the testosterone calibration curve was found to be virtually linear in the 50-3200 ng/kg range. The detection limit for testosterone was found to be 50 ng/kg.

Statistical analysis

For data statistical analysis, and comparison the average value of the sample testosterone concentration with the allowable value of FDA, one sample *t* test was used. The data analyzed using SPSS software version 11. A *P* value less than 0.05 was considered significant.

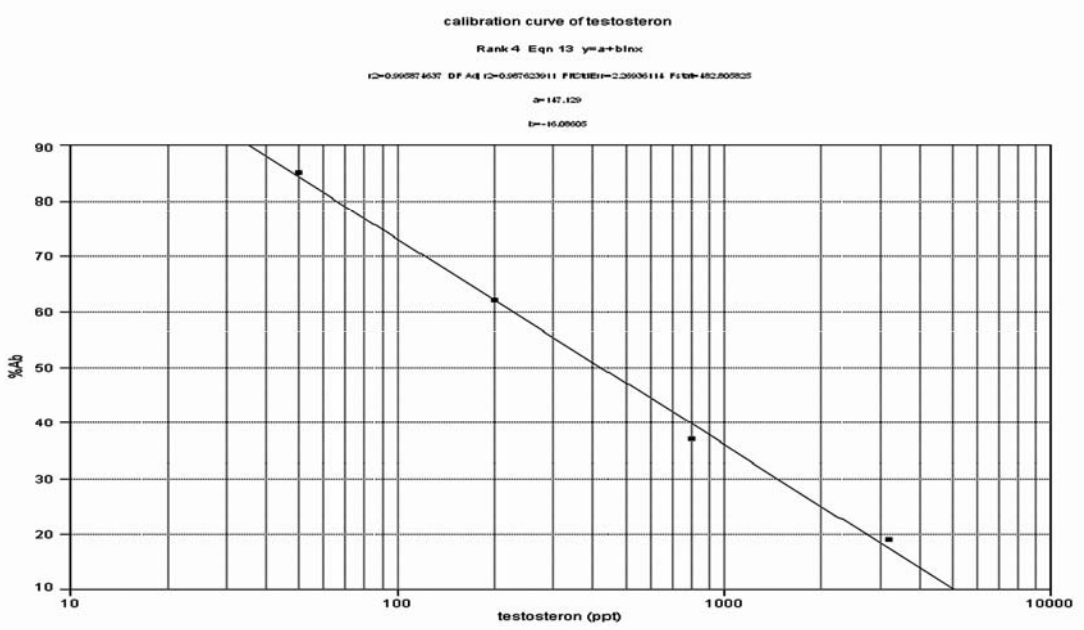

Fig. 1. Linearity of calibration curve for testosterone standards (50-3200 ng/kg). Running head: screening for testosterone levels in meat.

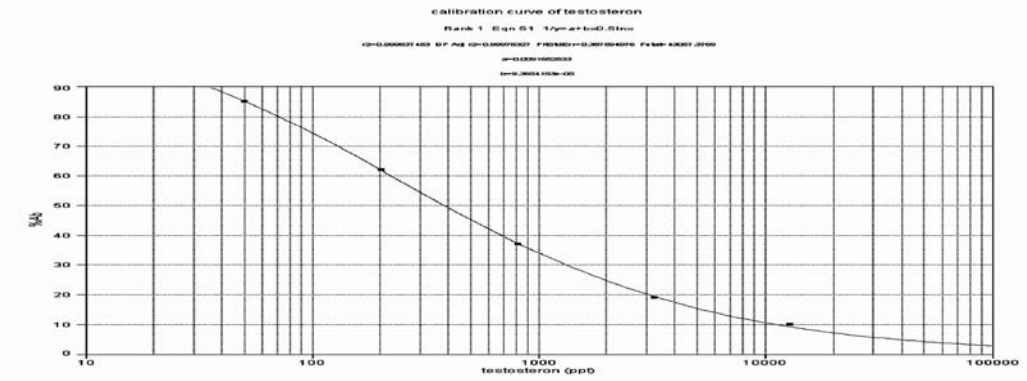

Fig. 2. Calibration curve for testosterone standards (0-12800 ng/kg).

RESULTS

Method validation

- Calibration curve

As can be seen in Fig. 2, the correlation between the absorbance ratios and testosterone concentration was evaluated over the range 0-12800 ng/kg (r^2=0.9958, a=147.129, b=-16.086).

-Accuracy

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%.

-Precision

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

Sample screening

Analyze of the market meat samples (n=120) showed a mean ± SD testosterone concentration of 810.9 ± 804.97 ppt (min 89.2 ppt, max 3983.8 ppt).

Table 2. Precision (%CV)

	Concentration (ng/kg)	CV%
Standards	12800	3.2
	3200	3.8
	800	4.3
	200	5
	50	6.9
Sample	2300	9.2

DISCUSSION

Testosterone is a hormone naturally present in humans at different concentrations depending on age, sex, diet, exercise, and stage in the reproductive cycle. The problem is then to discriminate between physiological concentration and elevated hormone levels due to the administration of natural anabolic. Even diets high in fat, calories and animal protein and low in fiber have been associated with higher testosterone level (10, 11). Testosterone can cause cervical-uterine tumors in female rats and prostate cancer in males (12). Some epidemiological studies have also found a link between prostate cancer and higher testosterone level in males. Testosterone was found to bind covalently to rat liver DNA. Testosterone exhibited a weak transforming effect on Syrian hamster embryo cells (13, 14).

Currently, testosterone is approved for implant in cattle in the U.S.A. But these implants have been officially prohibited in Europe since 1989. For the naturally occurring hormone testosterone, FDA has set the following allowable incremental increases in hormone levels above those normally present: 640, 260, 190 and 130 ng/kg for muscle, liver, kidney and fat, respectively (15). However, JECFA (Joint Expert Committee on Food Additives) has not set acceptable residue level for testosterone, because this committee emphasizes on good husbandry practice (GHP) and concludes that residues from use according to GHP are unlikely to pose a hazard to human health (16, 17). Acceptable daily intake (ADI) maxima of testosterone were established by

JECFA at 2 μg/kg body weigh (bw), based on reported no-or lowest observed effect levels in humans of 1.7 mg testosterone/kg bw (16).

In this study the mean concentration of cattle meat testosterone, 810.9 ng/kg, has a significant difference with the FDA allowable level, 640 ng/kg, but the daily intake of testosterone by meat will not be upper than JECFA ADI. So it seems that the present status of this anabolic hormone in market meat in Tehran is not at risk. However, the number of samples included in this survey was relatively small compared to the total sold in the market, and these results do not exclude the possibility of misuse of this anabolic hormone in future and significant increase exposure of humans, particularly children, to testosterone which may adversely affect health. There is, therefore, need to routinely monitor this chemical as a food quality control measure.

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Conflict of interests

We have no conflict of interests.

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