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RESEARCH ARTICLE

Use of ELISA for Preliminary Screening of 19 Nortestosterone Anabolic Steroid in Cattle Meat in Republic of Macedonia

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Summary

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. However, depending on the use of anabolic agent in animal feed, anabolic residues that may occur in meat and meat products present risks to human health. The aim of this study was to detect the levels of 19 nortestosterone residues in the market cattle meat in R. Macedonia. In this study, a total of 86 samples were obtained from different markets and used as a test material. 19 nortestosterone residues were analyzed with ELISA method. The average experimental level of 19 nortestosterone in cattle meat was 375.20 ppt. The recovery was between 79.54% and 114.39%, a working range between 50 to 3.000 ppt. The regression equation of the final inhibition curve was: $y = - 0.1453 x + 1.4057$, $R^2 = 0.9972$. The levels of 19 nortestosterone residues were below the international allowable levels set by the Macedonian Residue Control Plan and the European Union. According to the results of our study on this anabolic steroid, the obtained 86 cattle meat samples are safe for human consumption. However, it is still necessary to monitor this chemical as a food quality control measure.

Keywords: *19 nortestosterone, Cattle meat, ELISA, Residues, Public health*

Makedonya Cumhuriyeti'nde Etlerde 19 Nortestosteron Anabolik Steroidinin Ön Taramasının ELISA Kullanılarak Yapılması

Özet

Son yıllarda, hormonlar ve hormon benzeri maddeler kısa bir sürede yüksek verim performansı elde etmek için hayvancılık üretiminde kullanılmaktadır. Bu anabolik ajanlar, ağırlık kazancı artırmak, gıda verimliliğini ve protein depolamayı geliştirmek ve şişmanlığı azaltmak için kullanılmaktadır. Ancak, hayvan yemi olarak anabolik ajan kullanımına bağlı olarak, et ve et ürünlerinde oluşabilecek anabolik artıkları insan sağlığı için risk teşkil etmektedir. Bu çalışmanın amacı, Makedonya Cumhuriyeti'nde piyasadaki sığır etlerinde 19 nortestosteron kalıntı seviyelerini tespit etmektir. Bu çalışmada, farklı marketlerden toplam 86 numune elde edilmiş ve test materyali olarak kullanılmıştır. 19 nortestosteron artıkları ELISA yöntemi ile analiz edildi. 19 nortestosteronun ortalama deneysel seviyesi 375.20 ppt olarak bulundu. Düzelme 50 - 3.000 ppt çalışma aralığında %79.54 ve %114.39 arasında bulundu. Nihai inhibisyonun regrasyon eşitliği: $y = - 0.1453 x + 1.4057$, $R^2 = 0.9972$ olarak belirlendi. 19 nortestosteron kalıntı düzeyleri Makedonya Kalıntı Kontrol Planı ve Avrupa Birliği tarafından belirlenen uluslararası kabul edilebilir seviyelerin altında idi. Bu anabolik steroid üzerine olan bu çalışmanın sonuçlarına göre, elde edilen 86 sığır eti örneği insan tüketimi için güvenlidir. Ancak, gıda kalite kontrol önlemi olarak bu kimyasalın izlemesi hala gereklidir.

Anahtar sözcükler: *19 nortestosteron, Sığır eti, ELISA, Kalıntılar, Halk sağlığı*

GİRİŞ

Meat is one of the most important constituents of human diet as it provides proteins, energy, vitamins and minerals [1]. However, meat could also become a source of health hazards if it contains harmful material such as toxins, residues or chemical agents. Residues in meat may result from many sources such as animal drugs used to prevent or treat diseases

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or to promote growth, pesticides, feed and agricultural or industrial chemicals [2]. 19 Nortestosterone, also called nandrolone (NT), (17β-hydroxyestra-4-en-3-one or $C_{18}H_{26}O_2$ (*Fig. 1* - structural formulas), MW = 274.40 Da), one of the most powerful anabolic steroids, has been widely used in veterinary medicine as well as human medicine for treatment of protein deficiency diseases, osteoporosis and male contraception. 19 nortestosterone has also been employed as a growth-promoting agent to accelerate weight gain, to improve feeding efficiency in meat producing animals and as a doping agent to boost muscular strength and performance in sports and horse racing [3]. The side effects of these substances, which include increased risk of coronary heart disease and hepatic carcinogenicity, are related to their androgenic and/or anabolic properties [4]. 19 nortestosterone and its metabolites in meat also produce some other important adverse effects, such as cardiomyopathy, coronary artery disease, peliosis hepatitis, hepatic carcinogenicity, cholestasis, hypoproteinemia, adrenal atrophy, cerebral dysfunction, and emotional instability (mood swings, aggressiveness depression, psychosis addiction etc.), testicular shrinkage, sperm count and sperm motility, alterations in sperm morphology, decreased semen production, infertility, prostatic hypertrophy, prostatic carcinoma, gynecomastia, menstrual cycle disorders, masculinization, deepening of the voice, shrinkage of the breasts, male-pattern baldness and an increase in sex drive, acne, body hair and clitoris size, uterine atrophy, breast atrophy [5-8]. As their possible harmful effects result from the intake of hormone residues and their metabolites, usage of growth-promoting drugs for fattening livestock have been banned in many countries. However, illegal use of 19-nortestosterone as a growth promoter has been widely reported in many countries. Thus, it is necessary to control 19-nortestosterone's abuse [3]. As in most countries and in EU use of 19 nortestosterone is banned and no residues of these substances are allowed in meat products. Therefore, any cattle kept for export to the EU must be shown to be free of these substances [9-11]. In addition monitoring hormonal residues of growth-promoters in animal materials is essential to enforce this ban and to protect public health against the harmful effects of these substances in food products.

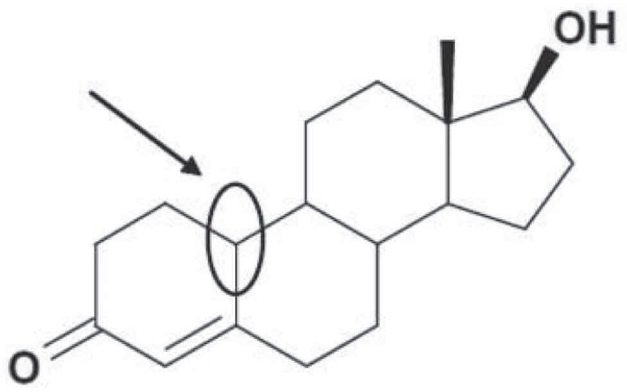

Fig 1. Structural formulas for 19 nortestosterone (nandrolone)
Şekil 1. 19 nortestosteron'un yapısal formülü

For determination of 19 nortestosterone is used immunoassay screening methods, gas chromatography coupled to mass spectrometric (GC/MS), liquid chromatography coupled to mass spectrometric (LC/MS) and other confirmatory methods. Confirmation methods are costly, time-consuming, require extensive sample preparation and highly trained personnel to operate sophisticated instruments and interpret complicated chromatograms [12,13]. The aim of this study was to monitor the use of 19 nortestosterone as anabolic substance and determination of 19 nortestosterone in cattle meat in Republic of Macedonia with screening ELISA method. ELISA method is simple, rapid, and cost-effective alternative to those traditional methods in cases where high-throughput and/or on-site screening is needed and permits analysis with little or no sample pre-treatment, thanks to the very high specificity of the bio specific reagents used (antibodies) [12-14].

MATERIAL and METHODS

Samples

A total of 86 meat samples were obtained randomly from 22 supermarkets, from 11 cities in Macedonia, from July 2010 to July 2011. The samples were kept frozen until use and the examinations were carried out according to the requirements of the European Community in five specialized veterinary diagnostic laboratories belonging to the Faculty of Veterinary Medicine, Food Institute.

Reagents

RIDASCREEN 19 nortestosteron test kit (R-Biopharm AG, Darmstadt, Germany) and its reagents were used to determine the presence and the 19 nortestosteron levels in the cattle meat. Methanol (Merck, 1060352500) and tertiary butyl methyl ether (Merck, 1018492500) used were of analytical grade. 20mM 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 1.000 ml distilled water. 19 nortestosterone external standard (Fluca Chemica 74640) we used for recovery investigations. From this standard we prepared standard solution of 19 nortestosterone in 1 ml of methanol-water (80:20, v/v) corresponding to 1.000, 1.500 and 2.000 ppt, respectively for spiking of the blank cattle meat sample on three levels.

Extraction Procedure

Fat was removed from muscle and the muscle was ground. One gram from ground muscle was transferred in the test tube (graduated conical tube 50 ml, BSM477) and then muscle was homogenized in 1 ml of 20 mM PBS buffer by mixer (Ikalabortechnik, t 25 basic) for 10 min. The homogenate was mixed with 10 ml tertiary butyl methyl ether in a centrifugal screw vial and shaken carefully for 30 min and then

samples were centrifuged for 10 min at 4.000 rpm on 15ºC. The supernatant (ether layer) was transferred to another centrifugal vial, then the samples were evaporated to dryness and dissolved in 1 ml methanol/water (80:20; v:v). The methanolic solution was diluted with 2 ml of 20 mM PBS buffer and applied to a RIDA C18 column (RIDA® R-Biopharm AG, Darmstadt, Germany, R2002) in the following manner: column was rinsed by flowing of 3 ml methanol (100%); then the column was equilibrated by injection of 2 ml of 20mM PBS buffer; a sample (3 ml) was applied on column; column was rinsed by injection of 2 ml methanol/water (40:60; v:v); column was dried by pressing N_2 trough it for 3 min; the sample was eluted slowly by injection of 1 ml methanol/water (80:20; v:v) (flow rate: 15 drops/min). Next step was evaporation of the eluted sample to dryness, at 60ºC under a weak nitrogen flow. Dried residue was dissolved in 2 ml methanol/water (10:90; v:v). In the test 20 µl of standard/sample for per well was used [15].

Validation

The limit of detection (LOD) of the assay was defined as the concentration corresponding to the mean signal of 20 blank cattle meat samples plus 3 times of standard deviation of the mean. Blank cattle meat samples were obtained from untreated cattle. The accuracy was evaluated by determining the recovery of spiked blank cattle meat samples with three concentration of 19 nortestosterone standards (1.000, 1.500 and 2.000 ppt). Precision was expressed as the CV (Coefficient of variation) (%) of the calculated standards and sample concentrations. Detection capabilities (CCβ) is required to be at or lower than the MRPL [16]. CCβ was evaluated by analyzing 20 spiked blank cattle meat samples at 0.5 times MRPL (1.000 ppt for 19 nortestosterone in muscle) [17] level for 19 nortestosterone and calculated in accordance with European Commission Decision 2002/657/EC.

Test Procedure

RIDASCREEN® 19 Nortestosterone ELISA kits (R- Biopharm AG, Darmstad, Germany) were used in order to determine the presence and levels of 19 Nortestosterone in the cattle meat samples investigated. All reagents in the kit had to be brought to room temperature (20-25°C) before use. Standard used for 19 nortestosterone contain 0, 50, 150, 500, 1.000 and 3.000 ppt 19 nortestosterone in 10% of methanol.

100 µl of diluted antibody was added to each well, mixed gently by shaking the plate manually and incubated for 30 min at room temperature (20-25°C). Liquid was poured out of the wells and after complete removal of the liquid; all wells were filled with washing buffer. Washing was repeated two more times. Then 20 µl of each standard solution or prepared sample were added and after that 100 µl of the diluted enzyme conjugate was added. The solution in the microplate was carefully mixed by shaking the plate manually. The plate was then incubated at room temperature (20-25°C) for 30 min. The liquid poured out of the wells and after the complete removal of liquid, all wells were filled with washing buffer. After rinsing, the water was also discarded; the washing was repeated two more times. Then, 100 µl of substrate/chromogen (tetramethyl-benzidine) were added, and after mixing thoroughly and incubating for 15 min at room temperature in the dark, 100 µl of stop solution (1 N sulphuric acid) was added. After mixing, the absorbance was read at 450 nm by using a spectrophotometer (BIO RAD model 680) [15].

The assay of Cross Reactivity

The standards used for 19 nortestosterone contained 0, 50, 150, 500, 1.000 and 3.000 ppt 19 nortestosterone in 10% methanol, whereas the antibody used had cross reactions with other related compounds, as indicated by the manufacturer's literature and shown in *Table 1*.

RESULTS

Calculation of the gained results was made by RIDAWIN Software. For construction of the calibration curve the mean of absorbance values obtained for six 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 19 nortestosterone. As can be seen in *Fig. 2*, the 19 nortestosteron calibration curve was found to be virtually linear between 50 and 3.000 ppt.

In *Fig. 3* the correlation between the absorbance ratio and 19 nortestosterone concentration was evaluated over the range 0-3.000 ppt. Linear regression analysis showed good correlation, with r^2 values 0.9972, ($y = - 0.1453 x + 1.4057$), where y was relative absorbance (%) and x was 19 nortestosterone concentration in ppt.

Table 1. *Cross reactivity of 19 nortestosterone antibody with various compounds*

Tablo 1. *19 nortestosterone antikorunun çeşitli bileşikler ile çapraz reaksiyonları*

Compound	Cross Reactivity
19-Nortestosterone 17β	100%
19-Nortestosterone 17α	approx. 80%
19-Norethisterone	approx. 74%
19-Norandrostendione	approx. 100%
18-Methyl-19 NT-17β	approx. 59%
17α-Ethyl-19 NT-17β	approx. 40%
15α, 16α-Methyl.-19 NT-17β-acetate	approx. 71%
Trenbolone	approx. 10%
17β-Estradiol	approx. 0.1%
Zeranol	< 0.1%
DES	< 0.1%
MPA	< 0.1%

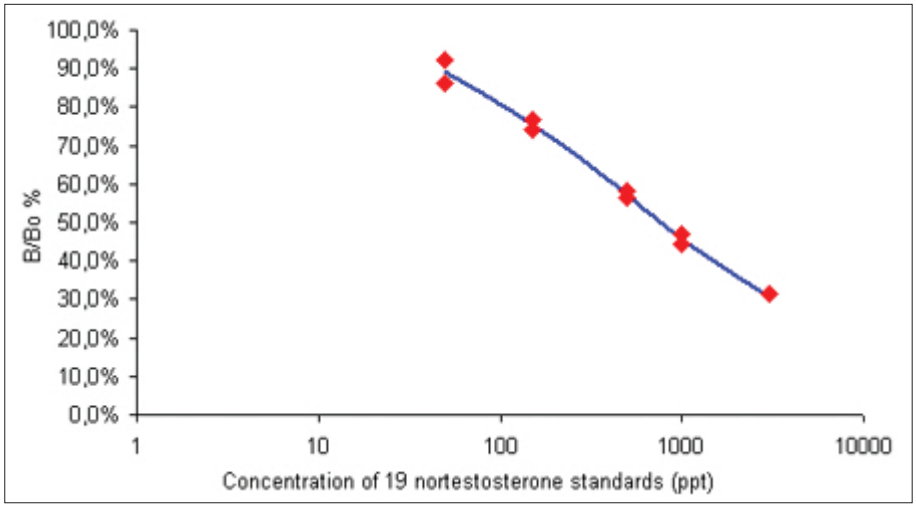

Fig 2. Linearity of calibration curve for 19 nortestosterones standards (50-3.000 ppt)

Şekil 2. 19 nortestosterone standartları için kalibrasyon eğrisinin doğrusallığı (50-3.000 ppt)

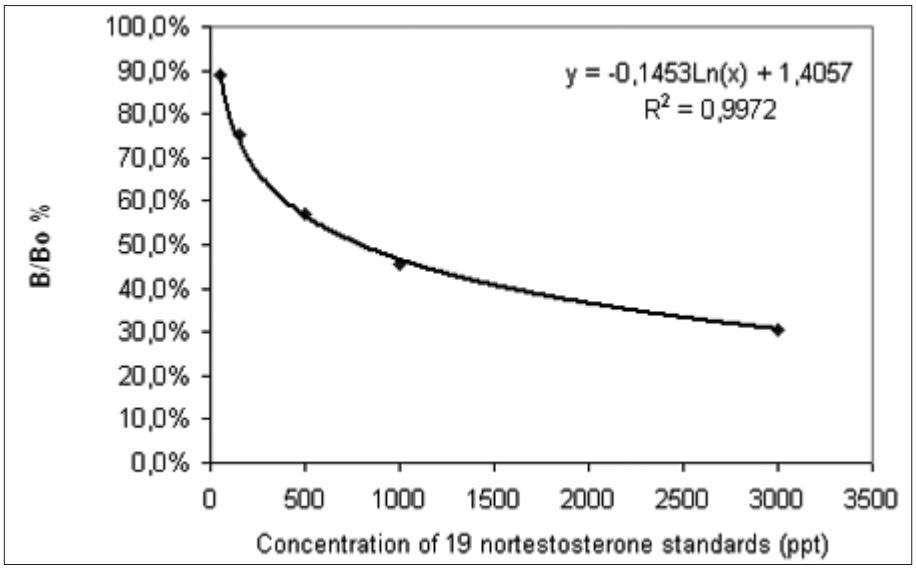

Fig 3. Calibration curve for 19 nortestosterone standards (0-3.000 ppt)

Şekil 3. 19 nortestosterone standartları için kalibrasyon eğrisi (0-3.000 ppt)

Table 2. *Precision of the method*
Tablo 2. *Metodun hassasiyeti*

Item	Concentration (ppt)	CV (%)
19 nortestosterone standards	0.0	1.6
	50.0	1.2
	150.0	1.4
	500.0	2.1
	1000.0	9.2
	3000.0	2.0
CV % for spiked sample	500.0	4.0
	1500.0	4.6
	2000.0	1.9

Results for the precision of the method are presented in *Table 2*. The precision (Coefficient of variation (CV) %) in 19 nortestosterone standards ranged from 1.2% to 9.2%. The precision (CV %) in spiked cattle meat sample ranged from 1.9% to 4.6%.

The accuracy was expressed as the recovery (%) of the estimated concentration. For the three target concentration

Table 3. *Accuracy of the method (recovery %)*
Tablo 3. *Metodun doğruluğu (geri kazanım %)*

Cattle Meat Sample n (Number of Replicates)	19 Nortestosterone		Recovery %
	Added (ppt)	Found (ppt)	
n = 6	1000	795.35	79.54
n = 6	1500	1715.84	114.39
n = 6	2000	2165.79	108.29

(1.000, 1.500, 2.000 ppt) the recoveries in cattle meat sample was 79.54%, 114.39% and 108.29% respectively and they are presented in *Table 3*.

These results are in agreement with the internationally accepted ranges for these parameters, and the standard deviations indicate that the method is sufficiently precise. Detection limit for 19 nortestosterone was found to be 124 ppt. The detection capability (CCβ) for 19 nortestosterone was 730 ppt, less than MRPL level of 1000 ppt. The analyzes of the cattle meat samples showed a values from 125.33 ppt to 625.07 ppt. In our case the calculated values of the analyzed samples are less than CCβ value.

DISCUSSION

Raw 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 tend to leave residues and this causes some problems in consumer health [18,19]. The European Economic Community (EEC) banned the use of anabolic compounds as growth accelerators in food animals [16]. In the present work, the ELISA method was used to achieve the unambiguous identification of 19 nortestosterone in cattle meat samples. This method was validated in accordance in the criteria of Commission Decision 2002/657/EC and is used in routine analyses in our laboratory. Because of the simple, rapid, and cost-effective of the method and its good recovery and precision it is applicable in official control laboratories as a screening method. In our opinion all ELISA kits aren't suitable for this purpose. For example P4 kits by Sorin and Tecna, and the T kit by Serono tended to underestimate and to give false negative results, while the P4 kit by Ovucheck and the T kit by Ridgeway Science tended to overestimate and to give false positive results [20].

In the case when the target analyte is clearly identified above CCβ the sample is considered as non compliant and we must confirm the results with confirmation method on GC/MS, LC/MS or another confirmatory method [16]. The methodologies and full procedure for confirmatory analysis require trained personnel with high expertise and they are costly in time, chemicals and equipments [21]. Identification is easier for a limited number of target analytes which are obtained with screening methods. In this study the calculated values of the analyzed samples are less than CCβ value, obtained with validation of the kit, so it seems that the present status of 19 nortestosterone in cattle meat is not at risk. But the number of samples included in this study is relatively lower compared to the total cattle meat sold in the market. These results do not exclude the possibility of misuse of 19 nortestosterone in the future. Due to the fact, it is still necessary to monitor this chemical as a food quality control measure.

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