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Research Article

The Presence of Some Anabolic Residues in Meat and Meat Products Sold in İstanbul*

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Abstract: The aim of this study was to detect some anabolic (zeranol, trenbolone, diethylstilbestrol, clenbuterol) residues in meat and meat products marketed in Istanbul. In the study, a total of 60 samples (30 fresh meat and 30 meat products) were obtained from different markets and used as test materials. The anabolic residues in the sample extracts were detected by ELISA. Initially, recovery tests were performed and then residues of one or more anabolics were analyzed in all samples. Among anabolizing residues studied, zeranol was detected in all samples (100%), trenbolone was detected in 48 samples (80%) and diethylstilbestrol was detected in 21 samples (35%) whereas no clenbuterol residues were detected in any of the samples.

The results indicated that the level of zeranol and diethylstilbestrol in considerable part of samples found to be more than the acceptable limits. Therefore, meat and meat products with excessive levels of anabolics might be harmful for the consumer. In conclusion, the use of anabolic agents in animal husbandry in Turkey must be strictly controlled.

Key Words: Anabolic, residues, meat, meat products, ELISA

İstanbul'da Satışa Sunulan Et ve Et Ürünlerinde Bazı Anabolizan Madde Kalıntılarının Varlığı Üzerine Bir Çalışma

Özet: Bu çalışmanın amacı, İstanbul'da satışa sunulan et ve et ürünlerinde bazı anabolizan madde kalıntılarını (zeranol, trenbolon, dietilstilbestrol, klenbuterol) tespit etmektir. Çalışmada 30 adet et ve 30 adet et ürünü olmak üzere toplam 60 örnek farklı marketlerden toplanarak test materyali olarak kullanıldı. Örneklerdeki anabolik kalıntılar ELISA yöntemi ile tespit edildi. İlk önce geri kazanım testleri yapıldı. Daha sonra örneklerdeki bir veya daha fazla anabolik kalıntılar analiz edildi. Örneklerin tamamında (% 100) zeranol, 48 adedinde (% 80) trenbolon, 21 adedinde (% 35) dietilstilbestrol bulunurken numunelerin hiçbirinde klenbuterol varlığına rastlanmadı.

Sonuç olarak; incelenen örneklerin önemli bir kısmında zeranol ve dietilstilbestrol kalıntı miktarları bildirilen kabul edilebilir limitleri aştığı saptandı. Dolayısıyla satışa sunulan et ve et ürünlerinin tüketici sağlığı açısından zararlı olabileceği kanısına varıldı. Bu nedenle Türkiye'de hayvan yetiştirciliğinde anabolik ajanların kullanımı, ciddi olarak kontrol altına alınmalıdır.

Anahtar Sözcükler: Anabolik, kalıntı, et, et ürünleri, ELISA

Introduction

In recent years, hormones and hormone-like compounds have been frequently used in vegetable production 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 (1-3). 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 (4). As a result, many countries restrict or prohibit the use of anabolic compounds in livestock production (5-7).

Zeranol (α-zearalanol) is a nonsteroidal, oestrogenic mycotoxin produced by several Fusarium species (8). It is

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used in livestock for increasing the rate of weight gain, improving the feed efficiency, and for high quality carcass (9). In cattle, zeranol is discharged 65 days after implantation with a rate of 96.3% and zeranol level decreases in all organs and tissues below 2 ppb (µg/kg) (10). Zeranol residues should not exceed 0.05 ppb in daily human food, 2 ppb in cattle muscle and 10 ppb in cattle liver (11,12).

Trenbolone acetate (TBA), a kind of 19-nortestesteron, is a synthetic steroid with anabolic properties (12-14). TBA 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 (15,16). The permitted limit values for trenbolone are 2 ppb in muscle and 10 ppb in liver (11,12).

Diethylstilbestrol (DES) is a synthetic estrogenic compound with carcinogenic and anabolic effects (3). Its most important effect is to improve the growth rate by increasing the quantity of digestible feed in livestock. As diethylstilbestrol is a carcinogenic compound, its use has been banned in animal production in European Union countries (12,17).

Clenbuterol is a well-known pharmaceutical product that belongs to the b-agonists (18-20). β-agonists are chiefly used for the treatment of asthma in humans and animals (21,22). In addition, they are used as growth prometers in livestock (23). The most widely used β-agonist is clenbuterol (21,22,24). The anabolic effect can be seen when clenbuterol is administered at dosages in excess of 5-10 fold the recommended therapeutic dose in meat-producing animals (> 1 µg/kg of body weight per day) (25). Rose et al. (22) declared the maximum residue level as 0.5 ppb in all edible tissues but because of its harmful effect on humans, it has not been approved for the use in livestock for growth promotion in the USA (26) or in European Union (24,25).

In Turkey, the use of anabolic compounds began in 1970s with agricultural greenhouse products and spread out in animal husbandry due to their effects in increasing the rate of weight gain, meat quality, and improving the feed efficiency (8). When the anabolic compounds (either natural or synthetic) are not discharged or neutralized by the organism, and residues occur. These residues in meat and meat products may be consumed by humans and cause health problems.

There are some regulations for detection of anabolic residues in imported meat and meat products in Turkey. However, there is a lack of control in Turkish meat industry based on these regulations. This undesirable situation may cause severe health problems. Therefore, in this study, it was aimed to detect anabolizing residues in meat and meat products frequently consumed in Istanbul.

Materials and Methods

In this study, 30 meat samples (10 minced meat samples, 10 front quarter samples, 10 rear quarter samples) and 30 processed meat samples (10 fermented sausage samples, 10 cooked salami samples and 10 frankfurter samples,), altogether 60 random samples of various products, were obtained from different markets in Istanbul and used as test material.

Extraction procedure: Extraction procedure was applied to test materials for each anabolic agent as described previously (7,27-29).

Isolation of Zeranol: One g of ground sample was homogenized with 2 ml of distilled water and 10 ml diethyl ether was added. The resulting mixture was shaken vigorously and centrifuged for 10 minutes (3000 g, 15 °C) and decanted the ether layer to a centrifuge tube. The aqueous phase was frozen. Five ml of diethyl ether was added and extraction was repeated as above. Ether extracts were pooled and evaporated by drying (at 60 °C under a weak nitrogen flow). Dried residue was dissolved in 1 ml of chloroform. Three ml of 1 M sodium hydroxide was added. It was centrifuged for 10 minutes (2000 g, 15 °C). The sodium hydroxide extract was pipetted into a vial containing 250 ml of 90% acetic acid. Five ml diethyl ether was added into the vial. It was centrifuged for 10 minutes (2000 g, 15 °C) and frozen as described above. The ether phase was decanted into a glass vial and evaporated until drying. The dried extract was dissolved in 0.5 ml of sample dilution buffer and 50 µl was used per well in the assay.

Isolation of Trenbolone: Ten g of ground sample was homogenized with 10 ml of 76 mM phosphate buffer (pH 7.2). Eight g of the homogenate was extracted with 8 ml of tert.butylmethylether and centrifuged for 10 minutes (3000 g, 15 °C). Supernatant was kept and the extraction was repeated with 8 ml of tert.butylmethylether. The extracts were combined and evaporated for drying. Dried residue was dissolved in 1

ml of 80% methanol. The methanolic solution was washed with 2 ml of petroleum ether. Residual petroleum ether was removed completely by heating the sample in a water bath for a short time (1-2 minutes). The methanolic solution was diluted with 3 ml of distilled water. The solution was purified by means of C18 columns (RIDA, Art No: R2002). Twenty µl of eluate was used per well in the assay.

Isolation of Diethylstilbestrol*:* Five g of ground sample was homogenized with 10 ml of 67 mM phospate buffer (pH 7.2). The homogenate equivalent to 3 g of the sample was extracted with 8 ml of tertiary butylmethylether, after being thoroughly shaken. The contents were centrifuged at 3000 g for 10 minutes. The residue was extracted again with tertiary butylmethylether. The supernatants were mixed and dried, and the residue was dissolved in 1 ml of 70% methanol. After this, 3 ml of petroleum ether was added and the contents were thoroughly mixed. The mixture was centrifuged and the hydrocarbon layer was discarded. The methanolic solution was dried, and then dissolved in 1 ml of dichloromethane and extracted once with 3 ml of 1 M NaOH. The extract was neutralized with 300 ml of 6 M phosphoric acid, and the mixture was loaded on a C18 column (RIDA Cat. No: R2002) for elution. Twenty µl of eluate was used per well in the assay.

Isolation of Clenbuterol: Five g of well minced sample was homogenized with 25 ml 50 mM tris-buffer (pH 8.5) by shaking for 30 minutes. Fifteen ml of n-heptane was added and the mixture was shaken for 5 minutes. It was centrifuged for 5 minutes (4000 g or at a higher speed, 10-15 °C). Upper heptane layer was removed and extraction was repeated with another 15 ml of heptane as described above. A 0.5 ml portion of half concentrated HCl was added to the aqueous meat homogenate and the mixture was shaken for 1 hour. Six g of the meat homogenate (correspondent to 1.0 g tissue) was transferred into a centrifuge vial. It was centrifuged for 15 minutes (4000 g or at a higher speed, 10-15 °C) and then the supernatant was transferred to another centrifugeable vial. 300 µl of 1 M NaOH was added and the vial was mixed for 15 minutes, 4 ml of 0.5 M KH_2PO_4 buffer (pH 3) was added and mixture was stored at 4 °C for at least 1.5 hours or overnight. It was centrifuged for 15 minutes (4000 g or at a higher speed, 10-15 °C). Supernatant (should be almost clear) was purified by means of C18 columns (RIDA Cat. No: R2002). Twenty µl of eluate was used per well in the assay.

Enzyme Immuno Assay: The ELISA instrument (Bio-tek Instr. ELX 800 reader, Bio-tek Instr. ELX 50 washer) and the Ridascreen ELISA test kits (R3301, R2601, R2701, R1701) were obtained from r-Biopharm (Darmstadt, Germany) and used for detecting the residues in sample extracts. These tests were always performed twice.

Recovery Analysis: Recovery applications were performed on 64 meat and meat product samples. Recoveries from meat and meat product samples were at 1.0-3.0 ppb with zeranol, 0.075-0.3 ppb with trenbolone, 0.2-1.0 ppb with diethylstilbestrol, 0.1-2.0 ppb with clenbuterol. The same extraction procedures were performed on these samples and screened by ELISA test. Finally, the coefficient of variation (CV) and the mean value of anabolics that were added to these samples were estimated.

Statistical Analysis: ANOVA (30) was used to analyze data and Duncan test (31) was used for comparing the differences among the groups of fresh meat and meat products.

Results

Recovery tests were performed on meat and meat product samples. ELISA method was validated by estimating of the recovery value and coefficient of variation (CV). The results of our recovery studies are summarized in Tables 1 and 2. The average recovery values and coefficient of variations in meat samples were detected as 73.67% (CV=2.77) for zeranol, 72.99% (CV=0.47) for trenbolone, 68.64% (CV=0.7) for diethylstilbestrol, and 77.43% (CV=2.97) for clenbuterol (Table 1).

In meat products, the mean recovery value of zeranol was 70.16% (CV=7.37) in fermented sausage, 70.25% (CV=6.35) in cooked salami, and 72.19% (CV=9.3) in frankfurter. For trenbolone, it was 71.10% (CV=1.15) in fermented sausage, 71.56% (CV=1.22) in cooked salami, and 73.20% (CV=2.02) in frankfurter; and for diethylstilbestrol 66.63% (CV=1.75) in fermented sausage, 67.19% (CV=1.77) in cooked salami and 69.22% (CV=3.45) in frankfurter. The mean recovery value of clenbuterol was 74.98% (CV=4.17) in fermented sausage, 75.80% (CV=5.9) in cooked salami, and 77.08% (CV=2.92) in frankfurter (Table 2).

Table 1. Recovery values and coefficient of variations of anabolic agents in meat samples.

Added µg/kg	Zeranol		Trenbolone		DES		Clenbuterol	
	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV
0.075			70.26	0.4				
0.1			71.90	0.4			76.40	0.6
0.2			75.70	0.9	68.95	0.3		
0.3			74.10	0.2				
0.5					69.16	0.8	77.34	3.4
0.75					68.49	1.3		
1.0	75.01	3.9			67.96	0.4	78.94	2.8
1.5	73.68	1.8						
2.0	73.52	0.0					77.06	5.1
3.0	72.47	5.4						
Average	73.67	2.77	72.99	0.47	68.64	0.7	77.43	2.97

CV : Coefficient of variations
DES : Diethylstilbestrol

Among anabolizing agent residues analyzed totally in 60 meat and meat product samples, zeranol was detected in 60 samples (100%), trenbolone was detected in 48 samples (80%), and diethylstilbestrol was detected in 21 samples (35%), whereas no clenbuterol residues were detected in any of the samples (Tables 3, 4 and 5).

Zeranol residues detected in samples amounted to 0.01-0.50 ppb in 27 of 60 samples (45% of the total test samples), to 0.51-1.0 ppb in 10 samples (16.7%), 1.01-2.00 ppb in 14 samples (23.3%), and more than 2.0 ppb in 9 samples (15%) (Table 3).

Trenbolone was detected in 48 of 60 samples (80% of the total samples). In 48 samples in which trenbolone residues were detected, 27 samples were fresh meats and 21 samples were processed meat products (Table 4).

Diethylstilbestrol residues were detected in 21 of 60 samples (35% of the total) whereas 18 of them were fresh meat preparations and 3 of them were meat product samples (Table 5).

Statistical analysis are given in Table 6. According to the results, the difference in levels of zeranol was not found significant between fresh meats and meat products ($P > 0.05$), similar to the results of trenbelon and clenbuterol ($P > 0.05$), whereas the diethylstilbestrol levels was significant between fresh meat samples ($P < 0.05$) and not significant between meat product samples ($P > 0.05$).

Among the meat product samples, the difference between the highest level of diethylstilbestrol residue detected in front quarter meat samples and the levels detected in minced meat and rear quarter meat samples was significant ($P < 0.05$). Similarly, the difference between the highest level of zeranol residue detected in minced meat and hind-quarter meat samples and the levels of other anabolizing agent residues was found to be significant ($P < 0.05$). The difference between the highest level of zeranol in all meat samples and the levels of other anabolizing agents were considered significant ($P < 0.05$).

During the general evaluation of meat and meat product samples, only the differences among the diethylstilbesterol residue levels were significant ($P < 0.05$) whereas the differences among other anabolizing agent levels were considered insignificant ($P > 0.05$). The highest level of diethylstilbestrol was detected in front quarter meat samples. The difference between the trenbolone and clenbuterol levels was considered insignificant ($P > 0.05$) while the difference between these two groups and others was significant ($P < 0.05$).

Table 2. Recovery values and coefficient of variations of anabolic agents in meat product samples.

	Added µg/kg	Zeranol		Trenbolone		DES		Clenbuterol	
		Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV	Recovery (%)	CV
Fermented sausage	0.075			68.93	1.4				
	0.1			70.20	0.6			75.10	1.1
	0.2			72.45	2.4	66.70	1.6		
	0.3			72.83	0.2				
	0.5					68.04	3.4	75.18	1.2
	0.75					66.85	0.0		
	1.0	71.85	5.7			64.92	2.0	76.37	10.8
	1.5	70.96	7.4						
	2.0	69.99	14.1					73.29	3.6
	3.0	68.96	2.3						
	Average	70.16	7.37	71.10	1.15	66.63	1.75	74.98	4.17
Cooked Salami	0.075			69.73	1.4				
	0.1			71.20	1.9			75.90	3.2
	0.2			72.80	1.1	65.60	0.9		
	0.3			72.50	0.5				
	0.5					69.02	3.3	75.80	3.8
	0.75					66.31	1.1		
	1.0	70.68	8.9			67.85	1.8	76.67	12.4
	1.5	69.48	2.8						
	2.0	69.99	9.1					74.85	4.2
	3.0	70.86	4.6						
	Average	70.25	6.35	71.56	1.22	67.19	1.77	75.80	5.9
Frankfurter	0.075			71.07	1.5				
	0.1			72.60	1.0			76.10	1.3
	0.2			74.95	2.9	68.85	1.6		
	0.3			74.20	2.7				
	0.5					69.50	3.3	77.92	2.1
	0.75					68.80	1.7		
	1.0	73.05	8.2			69.75	7.2	77.29	1.6
	1.5	70.96	7.4						
	2.0	71.73	9.2					77.03	6.7
	3.0	70.86	12.5						
	Average	72.19	9.3	73.20	2.02	69.22	3.45	77.08	2.92

CV : Coefficient of variations
DES : Diethylstilbestrol

Table 3. Numbers of meat and meat products containing zeranol.

Detected level (mg/kg)	Minced meat	Front Quarter Beef Carcass	Rear Quarter Beef Carcass	Fermented sausage	Cooked Salami	Frankfurter	Total	%
0.0	(-)	(-)	(-)	(-)	(-)	(-)	0	0
0.01-0.50	6	6	4	5	4	2	27	45.0
0.51-1.00	1	3	3	1	(-)	2	10	16.7
1.01-2.00	1	1	2	2	4	4	14	23.3
> 2.00	2	(-)	1	2	2	2	9	15.0
TOTAL	10	10	10	10	10	10	60	100

Table 4. Numbers of meat and meat products containing trenbolone.

Detected level (mg/kg)	Minced meat	Front Quarter Beef Carcass	Rear Quarter Beef Carcass	Fermented sausage	Cooked Salami	Frankfurter	Total	%
0.0	1	(-)	2	3	3	3	12	20.0
0.01-0.10	4	(-)	2	1	2	3	12	20.0
0.11-0.50	5	10	6	6	4	4	35	58.3
0.51-1.0	(-)	(-)	(-)	(-)	1	(-)	1	1.7
> 1.0	(-)	(-)	(-)	(-)	(-)	(-)	0	0
TOTAL	10	10	10	10	10	10	60	100

Table 5. Numbers of meat and meat products containing Diethylstilbestrol.

Detected level (mg/kg)	Minced meat	Front Quarter Beef Carcass	Rear Quarter Beef Carcass	Fermented sausage	Cooked Salami	Frankfurter	Total	%
0.0	6	1	5	10	9	8	39	65.0
0.01-0.20	2	1	1	(-)	1	2	7	11.7
0.21-0.50	2	(-)	1	(-)	(-)	(-)	3	5.0
0.51-1.0	(-)	1	1	(-)	(-)	(-)	2	3.3
> 1.0	(-)	7	2	(-)	(-)	(-)	9	15.0
TOTAL	10	10	10	10	10	10	60	100

Discussion

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 (32). 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 eustradiol and testosterone) and some synthetic hormones (such as zeranol and trenbolone) in animal husbandry (3,12).

The World Health Organization declared that zeranol residues should not exceed 0.05 ppb in daily human food,

Table 6. The evaluation of the average values of the anabolic residues of the meat and meat product samples from statistical aspects.

Samples	Anabolic	N	Zeranol		Trenbolone		DES		Clenbuterol	
			$\bar{X}$	$S\bar{x}$	$\bar{X}$	$S\bar{x}$	$\bar{X}$	$S\bar{x}$	$\bar{X}$	$S\bar{x}$
Meat	Minced meat	10	0.94a A	0.403	0.10a B	0.017	0.09b B	0.039	0.00a B	0.000
	Front Quarter	10	0.59a B	0.161	0.15a C	0.009	0.99a A	0.168	0.00a C	0.000
	Rear Quarter	10	1.04a A	0.337	0.11a B	0.022	0.32b B	0.180	0.00a B	0.000
	Total	30	0.85ns	0.180	0.12ns	0.010	0.47*	0.107	00ns	0.000
Meat Products	Fermented sausage	10	1.18a A	0.353	0.11a B	0.026	0.00a B	0.000	0.00a B	0.000
	Cooked Salami	10	1.52a A	0.429	0.15a B	0.072	0.02a B	0.019	0.00a B	0.000
	Frankfurter	10	1.32a A	0.279	0.09a B	0.022	0.03a B	0.018	0.00a B	0.000
	Total	30	1.34ns	0.202	0.11ns	0.027	0.02*	0.009	00ns	0.000
General Total		60	1.10	0.138	0.12	0.014	0.24	0.061	0.00	0.000

a,b,c : The differences between the groups which have different letters under the same subgroup columns are statistically significant ($P < 0.05$).

A,B,C: The differences between the groups which have different letters under the same group rows are statistically significant ($P < 0.05$).

ns : $P > 0.05$

* : The difference of hormone residue level in meat and meat products are statistically important ($P < 0.05$).

2 ppb in cattle muscle and 10 ppb in cattle liver (11). As shown in Table 3, higher values were obtained in the present study than permitted levels of European Commission's standards. This indicates that acceptable tolerance limits mentioned above should be immediately taken into account for usage of zeranol in animal husbandry and its control in meat and meat products in Turkey. On the other hand, zeranol use was banned by the European Community in 1985 (33). The fact that the tolerance limit stated by the World Health Organization, for zeranol residues was exceeded in 9 samples in the present study, and the said amount was likely to be the equivalent of daily human food in 14 samples indicated a total of 23 samples to become risky from the viewpoint of consumer health.

Trenbolone was detected in 48 of 60 samples (80% of the total) (Table 4). Laitem et al. (14) reported that, 2 or 3 months after implantation, residues in carcasses were still parts per billion. The permitted limit value for trenbolone amount is 2 ppb for muscle and 10 ppb for liver (11). The amount of trenbolone did not exceed the tolerance limit in any of the samples and reached to 0.77 ppb in only one sample. This result was found to be positive for consumer health.

As diethylstilbestrol is a carcinogenic and is not metabolized by the organism, it is banned in animal husbandry and not permitted to be present in food stuffs (17). It's a fact that 21 meat and meat product samples were found to be positive. However, as diethylstilbestrol residues were detected only in (35% of the total test samples) and amounted to low values such as 0.01-1.0 ppb in 12 samples (85% of the total test samples) and exceeded 1 ppb in only 9 samples (15% of the total), it may be said that diethylstilbestrol is not widely used in Turkish animal husbandry (Table 5).

On the other hand, diethylstilbestrol residues amounted to 0.01-0.20 ppb in 4, to 0.21-0.50 ppb in 3, to 0.51-1.0 ppb in 2 and more than 1.0 ppb in 9 of the fresh meat samples while only 3 of the meat product samples were found to contain low diethylstilbestrol level to 0.01-0.20 ppb. This shows that fresh meat samples may be harmful for consumer health.

In the European Community countries and the USA, the use of clenbuterol is not permitted in animal husbandry since it is harmful to health (22,24-26). The

absence of clenbuterol residues in the samples in this study is considered positive for consumer health.

ELISA is a rapid and practical method for residue detection in food products and is recommended by EU. It is mentioned that conducting recovery tests before the study will be useful for a correct test result. For this reason our test results are of importance as they give some information about the use of hormone preparations in national animal husbandry and in the food industry.

This study shows that the anabolic agents present in all 60 samples at a different level. This indicates the importance of analyzing the residue level of the anabolic agents in different meat and meat products, since the anabolic agents may pose a potential risk to public health. Therefore, further studies are necessary for residual analysis of anabolic agents and hazard analysis for humans. However, although there seem to be some differences between the levels of anabolic residues detected in meat and meat product, these findings are insufficient to claim which residues pose a potential risk for a particular meat and meat product. In conclusion, it is difficult to evaluate the present situation in Turkey for satisfactory preventive measures, usage of anabolic agents and their existence in meat and meat products. Further investigations are necessary to clarify these points.

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