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The Use of Enzyme Linked Immun-Sorbant Assay (ELISA) for Preliminary Screening of Medroxyprogesterone Acetate (MPA) and 17β-estradiol Hormones in Marketed Slaughtered Chicken

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Abstract

Administration of hormones to broiler chickens for performance-enhancing purposes may lead to deposit of residues in their carcasses. This could be a potential human health hazard upon exposure to these residues. Therefore, this study was carried out on ninety randomly collected fresh broiler samples to detect medroxyprogesterone acetate (MPA) and 17β- estradiol hormonal residues in muscle and fat of broiler carcasses by using Enzyme Linked Immun-Sorbant Assay (ELISA). The mean value of 17β-estradiol hormonal residue in muscle and fat of broiler carcasses were 0.090±0.005 and 0.182±0.010 ppb respectively. Sixty eight (75%) of muscle samples and thirty eight (42%) of fat samples were within the physiological limit (<0.05ppb). 17β-estradiol hormonal residue were within the permissible limit (1ppb) in all examined samples of muscle and fat. Medroxyprogesterone acetate residue (MPA) could not be detected in all examined samples. The public health significance and recommendation for the use of hormones were discussed.

Key words: Medroxyprogesterone acetate (MPA)-17β-estradiol hormones residue– ELISA- chicken.

Introduction

Broilers are some of the most economical meat protein sources available to consumers. Currently, chicken prices remain a bargain for the nutritional value, and this has held true for the last 40 years. The ability to efficiently use foodstuffs with minimal time to market size is the primary reason chickens lead as primary meat sources. Often this efficiency is misinterpreted as unsafe because broilers are given hormones in order to achieve the growth rate with so little feed.

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 may cause risks to human health (**Asiya and Akzira, 2016).**

Hormones produced by the bodies of humans and animals are called endogenous or natural hormones. Compounds chemically synthesized to mimic the effect of natural hormones are called synthetic or xenobiotic hormones. Hormones

are vital in normal development, maturation and physiological functioning of many vital organs and processes in the body **(Platter *et al.*, 2003).**

Medroxyprogesterone acetate (MPA) is a synthetic hormone that belongs to the gestagen class. It often substitutes as a prophylactic in human medicine and is widely used in the treatment of hormone-dependent tumors. Among veterinary drugs, MPA is considered a hormone that can speed up fattening **(Bernard, 1971).**

17β-estradiol is a natural occurring sex hormone but is also available as commercial growth promoters. It is also known as a steroid or estrogen sex hormone, and the primary female sex hormone. It is is important in the regulation of the estrous and menstrual female reproductive cycles. 17β-estradiol is essential for the development and maintenance of female reproductive tissues but it also has important effects in many other tissues including bone. While estrogen levels in male are lower compared to female, estrogens have essential functions in men as well **(Ryan, 1982).**

The immunological methods are based on the interaction antigen–antibody which is very specific for a particular residue. The most usual technique consists in the enzyme linked-immunosorbent assay (ELISA) and the detection system is usually based on enzyme-labeled reagents. There are different formats for antigen quantification like the double antibody or sandwich ELISA tests and direct competitive ELISA tests. Radioimmunoassay (RIA) is based on the measurement of the radioactivity of the immunological complex **(Samarajeewa *et al.*, 1991).**

The administration of health-risk related substances such as growth promoting agents and hormones is a recurring problem in animal production where these compounds are often used to increase the productivity and to reduce breeding costs **(Toffolatti *et al.*, 2006)**.

Uses of hormones in broiler chicken farms for performance-enhancing purposes may lead to deposit of residues in their carcasses, particularly when the birds are slaughtered without the observance of withdrawal period of the hormones**.** Ignorance of observation of withdrawal period leads to a serious threat to human health upon exposure to these residues. Therefore, residues monitoring are required in detecting anabolic hormones for the safety of consumers **(Donoghue and Hairston, 2000).** . A hormone withdrawal time of 20 days before marketing of treated chickens is recommended **(Roushdy *et al.*, 1992)**.

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 of any substances having a growth promoting action to farm animals. 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).**

In order to control the hormonal residues in chicken meat and to ensure the safety of Egyptian consumers, it is imperative that a monitoring system be put in place to address the concerns. The objective of the present study was to develop a method of determining medroxyprogesterone acetate and 17β-estradiol residues in chicken meat and fat by using enzyme-linked immunosorbent assay (ELISA) and to assess the present status of the levels of these hormones in Egyptian's chicken industry.

Materials and Methods

2.1 Samples:

Ninety samples of broiler chicken carcasses were collected from different markets localities. The samples were collected separately and sent to the laboratory for detection of medroxyprogesterone acetate (MPA) and 17β-estradiol hormones on both meat and fat of each carcass.

2.2 Determination of medroxyprogesterone acetate (MPA) residue by ELISA according to Chifang *et al.* (2006):

2.2.1 Intended use:

RIDASCREEN® Acetylgestagene is a competitive enzyme immunoassay for the quantitative analysis of medroxyprogesterone acetate (MPA) in animal tissues. Medroxyprogesterone acetate (MPA), 17α-acetoxyprogesterone, megestrol acetate and chlormadinone acetate belong to the group of acetylgestagenes. Acetylgestagenes are known to increase the efficiency of animal production and have been used in illegal cocktails for fattening. However, these substances are not permitted as resources for animal fattening.

2.2.2 Test Principle:

The microtiter wells are coated with capture antibodies directed against antiacetylgestagene antibodies. Acetylgestagene standards or sample solutions, MPA enzyme conjugate and anti-acetylgestagene antibodies are added. Free and MPA enzyme conjugate compete for the acetylgestagene antibody binding sites (competitive enzyme immunoassay). At the same time, the anti-acetylgestagene antibodies are also bound by the immobilized capture antibodies. Any unbound enzyme conjugate is then removed in a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethyl benzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colorless chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm. The absorption is inversely proportional to the acetylgestagene concentration in the sample.

2.2.3. Assessment of the specificity of antibody of the Acetylgestagene test :

The specificity of the RIDASCREEN® Acetylgestagene test was determined by analyzing the cross-reactivities to corresponding substances in buffer system.

Medroxyprogesterone acetate..... ...100 %

17α-Acetoxyprogesterone ..... 126 %
Megestrol acetate ..... 50 %
Chlormadinone acetate.....50 %
Melengestrol acetate ..... 0.6 %
Cross reaction of all other substances ..... ≤ 0.002 %

2.2.4 Preparation of samples:

1- Grinding the sample (meat or fat).

2-Weighing 1 g of grinded sample into a 20 ml vial for extraction and add 15 ml petroleum ether and shake softly in a water bath at 40 °C for extraction overnight.

3- Centrifugation the extract at 2000 rpm for 15 minutes. (Freeze the sample before centrifugation).

4- Decanting the petroleum ether supernatant into a new vial and evaporate at 60 °C in a water bath.

5- Redisolving the residue in 2 ml methanol and vortex for 20 sec (close vial tightly).

6- Freezing for 45 minutes.

7-Centrifugation at 2000 rpm for 5 minutes and decant the supernatant into a new vial.

8-Diluting the methanolic supernatant with 5 ml distilled water and purify by RIDA® C18 column (Art. No.: R2002) as follows:

Flow rate: 1 drop per second.

-Rinsingthe column twice, each time with 1 ml methanol (100 %)

- Equilibrate the column twice, each time with 1 ml methanol/20 mM tris-HCl, pH 8.5 (20:80, v/v)

- Applying diluted sample (approx. 7 ml)

- Rinsing the column twice, each time with 1 ml methanol/20 mM tris-HCl, pH 8.5 (20:80, v/v)

- Rinsing the column twice, each time with 1 ml methanol/ distilled water (40:60, v/v)

- Removing fluid residues by evaporation at 60 °C.

- Eluting sample with 1 ml methanol/distilled water (80:20, v/v) into a new vial

- Collecting residues by applying positive pressure.

- Diluting the eluent 1 + 1 with distilled water to get a methanol/distilled water solution (40:60, v/v)

2.3 Determination of 17β-estradiol residue:

2.3.1. Intended use:

RIDASCREEN® 17β-estradiol is a competitive enzyme immunoassay for the quantitative analysis of 17β-estradiol in tissues.

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2.3.2. Test principle:

The basis of the test is the antigen-antibody reaction. The microtiter wells are coated with capture antibodies directed against anti-17β-estradiol antibodies. Standards or sample solution, 17β-estradiol enzyme conjugate and anti-17β-estradiol antibodies are added. Free and enzyme conjugated 17β-estradiol compete for the antibody binding sites (competitive enzyme immunoassay). At the same time, the anti-17β-estradiol antibodies are also bound by the immobilized capture antibodies. Any unbound enzyme conjugate is then removed in a washing step. Enzyme substrate and chromogen are added to the wells and incubated. Bound enzyme conjugate converts the chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm. The absorption is inversely proportional to the 17β-estradiol concentration in the sample.

2.3.3. Assessment of the specificity of antibody of the 17β-estradiol test:

The specificity of the RIDASCREEN® 17β-estradiol test was determined by analyzing the cross-creativities to corresponding substances in buffer system.

17ß-Estradiol.....100 %
Estradiol-3-benzoate.....50 %
17α-Estradiol..... 0.9 %
Estron.....0.7 %
Trenbolone.....1.0 %
19-Nortestosterone.....0.5 %
Testosterone ..... < 0.25 %
Zeranol..... < 0.25 %
DES..... < 0.25 %
Estriol ..... < 0.1 %

2.3.4. Preparation of samples:

1. Grinding the sample (meat or fat).
2. Homogenizing 10 gram of ground sample with 10 ml of 67 mM PBS buffer* by shaking for 5 minutes.
3. Mixing 2 gram of the homogenized sample with 5 ml of tertbutyl methyl ether in a centrifugal screw cap vial and shake vigorously for 30 - 60 minute.
4. Centrifugation for 10 minutes at 3000 rpm.
5. Transfer the supernatant to another centrifugal screw cap vial
6. Repeating the extraction procedure with another 5 ml of tertbutyl methylether
7. Evaporating the combined ether layers to dryness and dissolve with 1 ml of methanol (80 %)

8. Diluting the methanolic solution with 2 ml of 20 mM PBS buffer** and further clean with RIDA® C18 column (Art. No. R2002) as follow: flow rate: 1 drop / sec.

- Rinsing column with 3 ml methanol
- Equilibrate column with 2 ml of 20 mM PBS buffer
- Applying sample (= 3 ml)
- Rinsing column with 2 ml of methanol (40 %)
- Pressing out residues of rinse-solution, dry column for 3 min.
- Eluting sample slowly (15 drops / min) with 1 ml methanol (80 %)

* 67 mM PBS buffer, pH 7.2: dissolve 1.8 g NaH_2PO_4 + 9.61 g Na_2HPO_4 + 9 g NaCl in 1000 ml distilled water.

** 20 mM PBS buffer, pH 7.2: dissolve 0.55 g NaH_2PO_4 x H2O + 2.85 g Na_2HPO_4 + 9 g NaCl, in 1000 ml distilled water.

2.4 Test procedure:

The test procedures for acetylgestagene and 17β-estradiol were done according to the chart enclosed in the kits of RIDA® and RIDASCREEN® is registered trademarks of R-Biopharm AG. Manufacturer: R-Biopharm AG, Darmstadt, Germany. R-Biopharm AG is ISO 9001 certified. The detection limits for acetylgestagene and 17β-estradiol were 0.3 and 0.02 ppb respectively.

Calculation: % absorbance = $\frac{\text{OD of (standard or sample)}}{\text{OD of } (Zero\ standard)} \times 100$

The zero standard is thus made equal to 100 % and the absorbance values are quoted in percentages. The values calculated for the standards are entered in a system of coordinates on a separate semilogarithmic graph paper against the MPA and 17β-estradiol concentration µg/kg (ppb). (Fig 1, 2).

Results and Discussion

Table (1) illustrates the mean value of 17β-estadiol and Medroxyprogesterone acetate residues in each of meat and fat of examined chicken carcasses. The mean value in chicken meat was 0.090 ±0.005 µg/kg with minimum and maximum value of ND and 0.199 µg/kg respectively.While in chicken fat the mean value was 0.182±0.010 µg/kg with minimum and maximum value of ND and 0.68 µg/kg. These finding values were higher than the acceptable daily intake (ADI) value for estradiol of 0.05 µg/kg body weight /day as assessed by **JECFA (1999).** Lower results were obtained by **Doyle (2000)** who found 17β-estradiol residues in chicken meat was ranged from <0.03-0.02 ppb and **Hala (2009)** who found that 17β-estradiol residues in chicken meat and fat were 0.031±0.005 and 0.046±0.006 ppb, respectively. **Abbas *et al.* (2006); Fahmy (1998); Abu-Taleb (2003) and El-shorbagy (1997)** could not detect hormonal residues in their examined samples. On contrary, higher results were recorded by **Sadek (1992)** and **Kadim *et al.* (2010)** who found 17β-estradiol

residues in chicken muscle in a concentration of 0.54± 0.48 and 0.704 µg/kg (ppb), respectively.

Table (2) showed the number of samples contain 17β-estradiol residue within physiological level <0.05 ppb in muscle and fat samples which were 68 (75%) and 38 (42%), while **El-Neklawy (1989) and Doyle (2000)** found 46, 45 samples within the physiological level, respectively. No samples were exceed the permissible limits (1ppb) stated by **Gracey (1986).**

Medroxyprogesterone acetate residue could not be detected in all examined chicken samples.

The presence of natural steroid hormones in chicken meat may be attributed to that the natural steroid hormones are secreted by the gonads and adrenals. The complex feedback mechanisms control the level of secretion. These compounds are transported in blood plasma bound to specific proteins. The hormone interacts with proteins and activates the receptor which consists in a change of its conformation, resulting in changing in each of protein synthesis and the phenotype of the cell. This substitutes the hypothesis reported by **Rico *et al.* (1981).**

The illegal use of compounds exhibiting sex hormone like activities as anabolic agents in farm animals entails a special risk to public health. The ingestion of anabolic agent residues present in meat poultry (0.206-1.462 µl estradiol) by women cause an increase in progesterone hormone level 97.6±33.9 nmol/litre in comparison with the normal level 7.6-81.0. However, FSH, LH, prolactin, estradiol and testosterone were nearly within the normal level. Irregularity of menstrual cycle was detected in 12 cases out of 46 women **(Ismail *et al.*, 2008).**

The health concerns associated with hormonal compounds used as growth promotions are their carcinogenic and endocrine-disrupting potentials **(Henderson and Feigelson, 2000).**

Conclusion

From the present data it could be concluded that the presence of hormone residues in examined chicken carcasses were below the permissible limit which may be attributed either to legislation and laws, which prohibit the use of anabolic agents as growth promoters registered to their harmful effect on health of consumers, or may be due to increase of moisture content in treated chickens with hormones.

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Table (1): 17β-estradiol and Medroxyprogesterone acetate (MPA) residues (ppb) in examined chicken meat and fat samples (n= 90).

17β-estradiol						Acceptable daily intake (ADI)	Medroxyprogesterone acetate						Acceptable daily intake (ADI)
Meat			Fat				Meat			Fat			
Min	Max	Mean ±SE	Min	Max	Mean ±SE	0.05 ppb/kg bw/day	Min	Max	Mean ±SE	Min	Max	Mean ±SE	0.03 ppb /kg bw/day
ND	0.199	0.090 ±0.005	ND	0.68	0.182 ±0.01		ND	ND	ND	ND	ND	ND	

ND=Not Detected

Table (2): Incidence of 17β-estradiol residue in the samples:

Type of samples	No. of sample	Within physiological Level<0.05ppb		Above physiological level			
				Within permissible limit <1 ppb		Above permissible limit > 1ppb	
		No. of samples	%	No. of samples	%	No. of samples	%
Muscle	90	68	75	90	100	0	0
Fat	90	38	42	90	100	0	0

References

Abbas, H.E.; Tag El-Din, H. A. and Sultan ,H. M.(2006): Determination of some hormones ,antibiotics and heavy metals residues in poultry meat of different species. Egypt. J. Appl. Sci., 21: 400-415

Abu-Taleb, S. M. (2003) : Determination of hormone residues in poultry meat products. Ph. D. Thesis, Faculty of Vet. Med. Zagazig Univ.

Asiya, S. and Akzira, O. (2016): The Effect of Hormones on the Quality of Poultry Meat. Int. J. Curr. Res. Biosci. Plant Biol., 3(1): 113-121.

Bernard F.E. (**1971**): The preparation of antigenic hapten-carrier conjugate: A survey. Principles, Methods, 70: 85-103.

Chifang, P.; Chuanlai, X.; Zhengyu, J.; Xiaogang, C. and Liying, W. (2006): Determination of Anabolic Steroid Residues (Medroxyprogesterone Acetate) in Pork by ELISA and Comparison with Liquid Chromatography Tandem Mass Spectrometry. J. of Food Sci., 71: 44-50.

Donoghue, D. J. and Hairston, H. (2000): Food safety implication: certain antibiotics and hormones may rapidly contaminate egg albumen during the process of its formation. British Poultry Sci., 41:174–177.

Doyle, E. (2000): Human safety of hormone implants used to promote growth in cattle. A Review of the Scientific Literature**.** Food Research Institute, University of Wisconsin Madison. WI 53706.

EEC (European Economic Community) (1989): Commission of the European communities, commission Directive of 14 November, 1989. Laying dawn the reference method and the list of national reference laboratories for detecting residues (89/106/EEC).Official Journal of European Communities, L 315:39-40.

El-Neklawy, E.M. (1989) : Determination of hormone residues in different kinds of meat. Ph. D., Thesis , Fac. of Vet. Med., Cairo Univ.

El-Shorbagy, E.M. (1997): Hormonal residues in poultry meat and effect of some methods of preservation on it. Ph. D. Thesis, Faculty of Vet. Med., Zagazig Univ.

Fahmy , M. ,Z. (1998) : Detection of hormone residues in imported and local and meat chickens. M. V. Sc. Thesis, Faculty of Vet. Med. Zagazig Univ.

Gracey, J.F. (1986): Chemical residues in meat. Meat hygiene 8th ed. pp 517. Bailliere Tindall, London.

Hala, A. M.I. (2009): Hormonal residues in chicken carcasses. Ph. D., Thesis, Fac. of Vet. Med., Beni – Suef Univ.

Henderson, B. E. and Feigelson, H .S. (2000): Hormonal carcinogenesis. Carcinogenesis. Oxford J., 21(3): 427-433.

Ismail, H. M.; Salam, H. N.; Essawy, A. E. and Sadek, I. A. (2008): Hormonal residues in meat and poultry and its relation to menstrual cycle. Intern. J. of Biol. and Biotechnol., 1 : 101- 103.

JECFA (1999): Residues of some veterinary drugs in animals and food. Fifty second meeting of the Joint FAO/WHO Expert Committee on Food Additives. Rome, 2-11 February. FAO Food and Nutrition paper 41/12.

Kadim, I.T.; Mahgoub, O. ; AL-Marzooqi, W.; AL-Maqbaly, R.; Annamali, K. and Khalaf, S.K. (2010): Enzyme-Linked Immunosorbent Assay for screening antibiotic and hormone residues in broiler chicken meat in the Sultanate Of Oman. J. of Muscle Foods, 21: 243–254.

Platter, W.J.; Tatum, J.D.; Belk, K.E.; Scanga, J.A. and Smith, G.C. (2003): Effects of repetitive use of hormonal implants on beef carcass quality, tenderness, and consumer ratings of beef palatability. J. Animal Sci., 81: 984-996.

Rico, A. G., Burgot-Sacaze , V.; Braun, J.P.and Benard , P.(1981): Metabolism of endogenous anabolic agents in cattle Anabolic agents in beef and veal production, CEC Workshop, Brussels.

Roushdy, A. S.; El-Neklawy, E.M. and El-Bayomy, A.M. (1992): Estradiol residues in chicken tissues influence of freezing and cooking an hormone levels in chickens.
Fleischwirtschaft, 72 : 63-64.

Ryan, K. J (1982): Biochemistry of aromatase: significance to female reproductive physiology. Cancer Res. 42 (8 Suppl.): 3342s–3344s.

Sadek, I. (1992): Hormonal residues in food personal communication. Ph.D.,Thesis, Fac. of Sc. Alex. Univ.

Samarajeewa, U.; Wei, C. I.; Huang, T. S. and Marshall, M. R. (1991): Application of immunoassay in the food industry. Crit. Rev. Food Sci. Nutr., 29: 403–434.

Toffolatti, L.; Rosa, G. L.; Patarnello, T.; Romualdi C.; Merlanti, R.; Montesissa, C.; Poppi, L.; Castagnaro, M. and Bargelloni, L. (2006): Expression analysis of androgen responsive genes in the prostate of veal calves treated with anabolic hormones. Domestic Animal Endocrinology, 30: 38–55.