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A Comparison between Some Methods Used for Extraction and Estimation of Testosterone Hormone in Meat

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Abstract

The increased public concern and the launch of legislative strategies within the framework of method validation and risk evaluation induced the need for the development of highly sensitive and specific analytical methods for the determination of steroid hormones in edible matrices. In this study, three extraction techniques of testosterone residues are tested, the manufacturer procedure described by the ELISA kits for testosterone in tissues, the method stated by Umberger et al. (1958) without or with cleanup.

Extraction followed by estimation of testosterone residue by ELISA kits either for tissue or serum. Fifty imported meat samples were collected randomly from markets; each was subjected to six tested treatments. The cost of chemicals, materials and kits of each treatment was computed to be compared economically. The obtained results indicated that, there is a significant testosterone residue increase in the extraction technique stated by Umberger et al., (1958). Also, clean up procedure has no significant effect. Moreover, there is no significant difference between commercial ELISA kits for tissues and that for serum. The most expensive treatment was that described by manufacturer of r-Biopharm kit. On the other hand the cheapest treatment was the extraction according to Umberger et al., (1958) followed by detecting testosterone residue using commercial kit for estimation of testosterone in serum. Taking into consideration both significance and economical points of view it is recommended to use Umberger et al., (1958) for extraction of meat testosterone and commercial ELISA kit for serum to detect testosterone residue in the extract.
Keywords: ELISA, Meat, Serum, Testosterone.

Introduction

It needs to be stressed that the improper use of both legal and illegal formulations of steroid hormones may lead to residues in edible matrices (Daxenberger et al., 2001).The increased public concern and the launch of legislative strategies within the framework of method validation and risk evaluation induced the need for the development of highly sensitive and specific analytical methods for the determination of steroid hormones in edible matrices. Any analytical procedure has three (or four) major steps: Extraction of the analyte from the matrix, Pre-purification, Quantitation & Quality assurance (QA) (Makin et al., 2010).

Because of the low levels of hormones (µg/kg to ng/kg) in the tissues of animal origin foods and the complexity of tissues of bio-sample matrices, the analysis of hormones and hormone-like substances is a challenging task. This implies that an effective sample preparation process and sensitive analytical instruments are necessary to achieve the optimal sensitivity, selectivity and specificity. There are many proposed sample-preparation procedures for bio-samples .The Commission of the European Communities has presented a routine sample-preparation procedure with clean extract for determination (Heitzman, 1994). This procedure takes a long time and requires solvents with a volume of more than 900 ml, which is not friendly to the operator and the environment. Other procedures use multi-step solid phase extraction to isolate different types of steroid hormones, which can be time-consuming and tedious (Hartmann and Steinhart, 1997; Marchand et al., 2000; Impens et al., 2002). Immuno-affinity, molecularly imprint and tetrapeptide solid extraction phase columns may be promising for complex bio-samples (Heitzman 1994; Ye et al., 2001; Tozzi et al., 2002) their application scope is limited to several compounds and does not cover all hormones and hormone-like substances. In addition, the capability of column materials is limited.

In immunoassays steroids were extracted from biological matrices may not always behave in the same way as standard steroids used for the standard curve (Jawad et al., 1981). Although many of these techniques are more than 20 years old, they should not be ignored and may still have applications to particular problems of today, especially when combined with more modern technology (Makin et al., 2002; Huang and Yuan, 2007). In the past, immunological techniques like radio immunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) were used for screening of a

limited number of steroid hormones depending on the antibodies used. Nowadays, multi-residue screening methods are developed based on immunochemical techniques. Scippo et al. (2002) developed a multi-analyte detection assay for the detection of compounds with estrogenic, gestagenic, androgenic or glucocorticoidic activity using recombinant receptors, this technique allowed the detection of steroid hormones at the action limit of 2μgkg^{-1}.Routine application for steroid hormones in edible matrices of animal origin is, based on available literature, to date sparse. It also has to be stressed that binding assays represent potential screening methods but need to be confirmed by chromatographic separation methods such as gas or liquid chromatography coupled to mass spectrometric detection. In the case of natural hormones, background concentrations of these compounds have to be taken into account (Scippo et al., 2002).

In this study, three extraction techniques of meat testosterone are tested, the manufacturer procedure described by the ELISA kits for testosterone in tissues, the method stated by Umberger et al., (1958) without and with clean up and followed by estimation of testosterone by ELISA kit either for tissues or serum.

Material and Methods

Fifty imported meat samples were collected randomly from markets, each was subjected to six treatments (three methods of extraction followed by two different hormone evaluation kits for each) to estimate testosterone residue levels.

(a)Extraction by manufacturer method of Bio-pharm kit and hormone estimation by Biopharm kit for tissues

Extraction was carried out by using commercial ELISA kits for different animal tissues (Art. No. DRG1559) obtained from r- Biopharm AG, Germany. Kits were supplied with reagents for the enzyme immunoassay including standards and specific coated micro- titer plates. The sample extraction and testosterone detection were performed based on the manufacturer procedure described by the ELISA kits. The sensitivity range of testosterone assay was 0.05-0.09 ng/ml as described by the manufacturer (control group).

(b) Extraction by manufacturer method of Bio-pharm and hormone estimation by BioCheck kit for serum

The previous extract was detected by commercial kit for estimation of testosterone in serum" BioCheck, Inc.323 Vintage Park Drive. Foster City, catalog number: BC-1115" .The minimum detectable concentration of testosterone ELISA assay is estimated to be 0.05 ng\ml as described by the manufacturer.

(c) Extraction by Umberger et al., (1958) method of hormone estimation by Biopharm kit for tissues

Extraction was carried out according to Umberger et al., (1958)-cited by(Code of Federal Regulations Title 21 Food and Drugs parts 120-129, 1968) with modifications carried out by Lab of hormones ,Chemistry Department ,Animal Health Research Institute, Agriculture Research Centre, Egypt . Briefly, testosterone hormone was extracted from 50 g homogenized meat by 150 ml ethyl alcohol with 50 g siliceous earth overnight followed by hydrolysis of extractive alcohol with 25 ml diluted hydrochloric acid (2/3 volume was evaporated), this solution is next extracted with chloroform in two steps using separating funnel ,first 300 ml distilled water and 70 ml chloroform, the second 100 ml distilled water and 50 ml chloroform .The chloroform extract was washed two times with 10% sodium carbonate to remove strongly acidic substances ,the chloroform extractive of hormone was then extracted two times with 1 % sodium hydroxide. This extract was filtered through Whattman No. 1 filter paper, then the chloroform was evaporated and the residue was dissolved in 10 ml absolute ethyl alcohol and filtered before preservation for hormone estimation. The estimation of testosterone in the final extract was carried out by the reagents that included with r-Biopharm ELISA kit for tissues.

(d) *Extraction by Umberger et al., (1958) method and hormone estimation by BioCheck kit for serum*

Testosterone residues in previous extract was estimated by BioCheck commercial kit for estimation of testosterone in serum.

(e) Extraction by Umberger et al., (1958) +C_{18} Clean up and hormone estimation by Biopharm kit for tissues

The final extract of the Umberger method was purified by means of Bakerbond solid phase extraction SPE Octadecyl (C18) according to Shackleton and Whitney (1980) the final purified extract was estimated by r-Biopharm ELISA kit for tissues.

(f) *Extraction by Umberger et al., (1958) + C_{18}Cleanupand hormone estimation by BioCheck kit for serum.*

Testosterone residues in the previous cleaned up extract was estimated by BioCheck kit for serum.

Statistical analysis

Data obtained were statistically analyzed using analysis of variance (ANOVA) using F- test according to SPSS-18 (2009).

Economical Study

The cost of chemicals, materials and kits of each treatment was computed in Egyptian Pound according to purchase price at the time of the experiment, equipment and personnel cost were neglected. The treatments were compared economically.

Results

Regardless of type of kit that used for testosterone residue in meat extract the represented data in table (1) revealed that there is a significant testosterone residue increase in the extraction technique described by Umberger et al. (1958) .Also, clean up procedure has no significant effect.

Table 1. Effect of extraction technique on meat testosterone concentration

Extraction technique	Testosterone concentration (ng/g)
Manufacturer method of Bio-pharm kit	0.29±0.04^{b}
Umberger *et al.*, (1958) method	0.45±0.03^{a}
Umberger *et al.*, (1958)+C_{18} Clean up	0.40±0.03^{a}

Values (mean±SE) of 100 samples within the same column with different superscripts are significantly different (P< 0.01).

There is no significant difference between commercial ELISA kits for different animal tissues and commercial kit for estimation of testosterone in serum regardless of the effect of extraction technique (table, 2).

Table 2. Effect of ELISA kit type on meat testosterone concentration

Type of ELISA kit	Testosterone concentration (ng/g)
r- Biopharm for tissues	0.38±0.21^{a}
BioCheck for serum	0.37±0.14^{a}

Values (mean±SE) of 150 samples within the same column with different superscripts are significantly different (P< 0.01).

Statistical analysis of testosterone residues in meat obtained by all tested treatments (table, 3) revealed that using extraction by Umberger et al., (1958) method & hormone estimation by Biopharm kit for tissues is significantly increased than using BioPharm kit for both extraction and estimation. On the other hand this treatment has not differed significantly with both that which extracted by manufacturer method of Bio-pharm& hormone estimation by BioCheck kit for serum and that which extracted by Umberger et al., (1958) followed by C_{18} Cleanup &hormone estimation by Biopharm kit for tissues. Clean up has no significant effect on the extracted testosterone residues.

Table 3. Effect of extraction technique and Type of ELISA kit on testosterone residue in meat

Treatment	Testosterone concentration (ng/g)
Extraction by manufacturer method of Bio-pharm kit & hormone estimation by Biopharm kit for tissues.	0.25 ± 0.02^{c}
Extraction by manufacturer method of Bio-pharm & hormone estimation by BioCheck kit for serum.	0.32 ± 0.07^{bc}
Extraction by Umberger et al., (1958) method &hormone estimation by Biopharm kit for tissues.	0.49 ± 0.03^{a}
Extraction by Umberger et al., (1958) method & hormone estimation by BioCheck kit for serum.	0.41 ± 0.05^{ab}
Extraction by Umberger et al., (1958) +C_{18} Cleanup &hormone estimation by Biopharm kit for tissues.	0.37 ± 0.04^{abc}
Extraction by Umberger et al., (1958) +C_{18} Cleanup &hormone estimation by BioCheck kit for serum.	0.42 ± 0.02^{ab}

Values (mean±SE) of 50 samples within the same column with different superscripts are significantly different ($P< 0.01$).

Economical study (table 4) cleared that the most expensive treatment was that described by manufacturer of r-Biopharm kit for extraction and detection of hormone by the same kit followed by using extraction according Umberger et al., (1958) +C_{18} Cleanup &hormone estimation by Biopharm kit .On the other hand the cheapest treatment was the extraction according to Umberger et al., (1958) &detecting testosterone residue using commercial kit for estimation of testosterone in serum.

Table 4. The cost of different treatment

Treatment	Extraction	Clean Up	Kit	Total Cost
Extraction by manufacturer method of Bio-pharm kit & hormone estimation by Biopharm kit for tissues.	21	-	39	60
Extraction by manufacturer method of Bio-pharm & hormone estimation by BioCheck kit for serum.	21	-	8	29
Extraction by Umberger et al., (1958) method & hormone estimation by Biopharm kit for tissues.	3	-	39	42
Extraction by Umberger et al., (1958) method & hormone estimation by BioCheck kit for serum.	3	-	8	11
Extraction by Umberger et al., (1958) + C_{18}Clean up & hormone estimation by Biopharm kit for tissues.	3	17	39	59
Extraction by Umberger et al., (1958) + C_{18}Clean up & hormone estimation by BioCheck kit for serum.	3	17	8	22

Values are expressed as the cost in Egyptian Pound according to purchase price at the time of the experiment.

Discussion

The level of hormone detected in the Umberger method (table 1) increased significantly than that detected in extraction technique described by Bio- pharm kit that may be due to overnight extraction with ethyl alcohol and using chloroform which considered as trans-esterification agent in Umberger et al., (1958).Contents of steroids with and without trans-esterification were compared where the higher contents in trans-esterificated samples indicate the occurrence of fatty acid esters (Schmidt and Steinhart, 2002). This may provide a rational interpretation for the obtained results. There is no significant difference between commercial ELISA kits for different animal tissues and commercial kit for estimation of testosterone in serum regardless of the effect of extraction technique (table

2). This is a reasonable result as both of ELISA kit preparations have the same reagents considering enzymes, antisera and substrate. This finding is in consistent with China Testosterone ELISA test kit manufacturer REAGEN LLC (United States) .It produced testosterone ELISA test kit that provides a competitive enzyme immunoassay for the quantitative analysis of testosterone in fish, shrimp, meat, plasma and urine. Comparing the whole results in table (3) in relation to the obtained economic data (table 4) leads to recommend Extraction by Umberger et al., (1958) method and hormone estimation by BioCheck kit for serum.

Conclusion and Recommendations

Taking into consideration both significance and economical points of view it is recommended to use Umberger et al., (1958) method for extraction of meat testosterone and commercial ELISA kit for serum to detect testosterone residue in the extract. Authors recommended more studies on testosterone extraction methods from meat and performing recovery test .Also comparing and confirming ELISA technique with liquid chromatography tandem mass spectrometry.

References

Code of federal regulations, title 21 food and drugs parts120-129, 1968. .S. government printing office Washington, D.C.

Daxenberger, A., Ibarreta, D. and Meyer, H.H.D., 2001. Possible health impact of animal oestrogen in food Hum. Rep. Update, 7, 340-355.

Hartmann, S. and Steinhart, H., 1997.Simultaneous determination of anabolic and catabolic steroid hormones in meat by gas chromatography-mass spectrometry. J. Chromatogr. B, Biomed Science Appl., 704, 108–117.

Heitzman, R.J., 1994. Veterinary Drug Residues: Residues in Food Producing Animal and Their Products: Reference Materials and Methods, Cambridge University Publishing, Cambridge, UK.

Huang, X. and Yuan, D., 2007. Preparation of stir bars for sorptive extraction based on monolithic material. J. Chromatogr. *A.*, 1154, 152–157.

Impens,S ., De Wasch, K ., Cornelis, M. and De Brabander, H.F., 2002. Analysis on residues of oestrogens, gestagens and androgens in kidney fat and meat with gas chromatography –tandem mass spectrometry. J. Chromatogr. A, 970, 235–247.

Jawad, M.J., Wilson, E.A. and Rayburn, F., 1981.Effect of serum lipids on radio-immunoassays of unconjugated estriol. in serum. *Clin. Chem.*, 27, 280–285.

Makin, H.L., Trafford, D.J. and Taylor, N.F., 2002. Mass fragmentography of cortisol and cortisone: preliminary studies on the development of a reference method. Collect. Czech. Chem. Comm., 67,1–9.

Makin, H. L.J., Honour, J. W., Shackleton, C. H.L. and Griffiths, W. J., 2010. Steroid Analysis: General Methods for the Extraction, Purification, and Measurement of Steroids by Chromatography and Mass Spectrometry. 2nd ed., 163-282.

Marchand, P., Le Bizec, B., Gade, C., Monteau, F. and Andr´e, F., 2000. Ultra trace detection of a wide range of anabolic steroids in meat by gas chromatography coupled to mass spectrometry. Chromatogr J. A., 867, 219–233.

Schmidt, G. and Steinhart, H., 2002. Impact of extraction solvents on steroid contents determined in beef. Food Chemistry, 76, 83-88.

Scippo, M.L., Van De Weerdt, C., Willemsen, C., Franc¸ois, J.M., Rentier-Delrue, et al., 2002. Detection of illegal growth promoters in biological samples using receptor binding assays. Anal. Chim. Acta, 473, 135-141.

Shackleton, C.H.L. and Whitney, J.O., 1980. Use of Sep-Pak cartridges for urinary steroid extractions: evaluation of the method for use prior to gas chromatographic analysis. Clin. Chim. Acta., 107, 231–243.

SPSS -18, 2009.Statistical package for social science. Spss for windows release standard version copyright Spss Inc. one-way ANOVA test.

Tozzi, C., Anfossi, L., Giraudi, G., Giovannoli, C., Baggiani, C. andVanni, A., 2002. Chromatographic characterization of an estrogen-binding affinity column containing tetrapeptides selected by a combinatorial-binding approach. J. Chromatogr. A, 966, 71–79.

Umberger, E.J., Gass, G.H. and Curtis, S., 1958. Design of a biological assay method for the detection and estimation of estrogenic residues of the edible tissues of the domestic animals treated with estrogens. Endocrinology .63, 806-815.

Ye, L., Yu, Y.H. and Mosbach, K., 2001.Towards the development of molecularly imprinted artificial receptors for the screening of estrogenic chemicals. Analyst, 126, 760–765.

مقارنة بين الطرق المستخدمة فى استخلاص و تقدير هرمون التيستيستيرون فى اللحوم

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زيادة الاهتمام العام و اطلاق الاستراتيجيات التشريعية فى اطار التحقق من صحة الاسلوب وتقييم المخاطر ادى الى الحاجة لتطوير طرق تحليلية حساسة و متخصصة لبقايا الهرمونات الاستيرودية فى الانسجة المأكولة.فى هذه الدراسة تم تجميع خمسين عينة لحوم من الاسواق و تم تقسيمها عشوائيا الى ستة مجموعات لاختبار ثلاثة تقنيات لاستخلاص بقايا هرمون التيستيستيرون من اللحوم,و هم الطريقة المرفقة لمشخصات التيستيستيرون فى الانسجة بالاليزا المنتجة من شركة بيوفارم و طريقة مأخوذة عن (Umbergeret al., 1958) و نفس الطريقة بتنقية المستخلص باستخدام اعمدة الفصل الصلب (C18).

تم تقدير بقايا الهرمون فى كل من المستخلصات الثلاثة بواسطة مشخصات الاليزا لتقدير التيستيستيرون فى الانسجة "بيوفارم"و بواسطة مشخصات الاليزا لتقدير التيستيستيرون فى المصل البشرى . وتم حساب تكلفة كل من المجموعات الست المختبرة للتقييم و المقارنة اقتصاديا.

ولقد اثبتت النتائج المتحصل عليها ان هناك زيادة معنوية فى بقايا هرمون التيستيستيرون المستخلصة بطريقة (Umberger et al., 1958) عن الطرق الاخرى وكذلك عدم وجود اختلافات معنوية عند استخدام التنقية باعمدة الفصل. بالاضافة الى ذلك فانه لم تكن هناك فروق معنوية بين المشخصات الخاصة بالانسجة وتلك الخاصة بالمصل,علاوة على ذلك فان استخدام طريقة الاستخلاص المرفقة بمشخصات الانسجة واستخدام المشخصات ذاتها فى تقدير بقايا التيستيستيرون هى الاعلى فى التكلفة الاقتصادية.

من النتائج السابقة و بالأخذ فى الاعتبار الفروق المعنوية و التكلفة الاقتصادية يمكن النصح باستخدام طريقة(Umberger et al., 1958) فى الاستخلاص ومشخصات التيستيستيرون الخاصة بالمصل فى تقدير بقايا التيستيستيرون فى أنسجة اللحوم.