Mac Vet Rev 2013; 36 (1): 19–23

Available online at
www.macvetrev.mk

Original Scientific Article

UDC: 639.3:577.175.6.083.3

VALIDATION OF SCREENING METHOD FOR DETERMINATION OF METHYLTESTOSTERONE IN FISH

Uzunov Risto[1], Hajrulai-Musliu Zehra[1], Stojanovska-Dimzoska Biljana[1], Dimitrieska-Stojkovic Elizabeta[1], Todorovic Aleksandra[1], Stojkovski Velimir[2]

[1]Food Institute, Faculty of Veterinary Medicine, Skopje, Republic of Macedonia
[2]Institute for Reproduction and Biomedicine, Faculty of Veterinary Medicine-Skopje, Republic of Macedonia

Received 9 March 2013; Received in revised form 28 June 2013; Accepted 5 July 2013

ABSTRACT

Anabolic androgenic steroids are synthetic derivatives of testosterone, which is the primary male sex hormone. These anabolic agents are used to increase the weight gain, to improve the food efficiency, storing proteins 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 the validation of screening ELISA method for determination of methyltestoterone anabolic steroid in fish. The validation process was carried out according to Commission Decision 2002/657/EC criteria. The detection limit for methyltestosterone was 140.95 ng/kg and the detection capability was 564.43 ng/kg. The overall recoveries and the coefficients of variation (CV) were in the range of 82.4%-97.4% and 1.5%-6.9%, respectively, a working range between 50 to 4050 ng/kg, and the regression equation of the final inhibition curve was: y= -0,1741x + 1,5082, R^2 = 0.9927. Because of the good recovery and precision, and satisfactory detection capability, this method is applicable in official control laboratories as a rapid screening method for determination of methyltestosterone in fish.

Key words: methyltestosterone, fish, ELISA, anabolic steroids, validation.

INTRODUCTION

Methyltestosterone is a synthetically produced anabolic and androgenic steroid hormone (1, 2). Anabolic steroids are potentially useful compounds in aquaculture due to their ability to increase weight gains and muscle deposition of treated fish. Methyltestosterone promotes both muscle growth and the development of male sexual characters (1, 2). The increased growth rate in fishes, through the administration of androgenic hormone has been reported from many authors (3). The enhanced growth rate, obtained in *Cyprinus carpio* using 17α-Methyltestosterone revealed that this hormone would induce faster growth by acting probably in three different ways and they are activation on secretion of other androgenic anabolic hormones, increased food conversion and direct effect of 17α-Methyltestosterone on the gene expression in the muscle cells (3, 4). The increase in body weight gain may attribute to that androgenic steroids enhance the release of growth hormone from the pituitary somatotrops of the fish and/or induce the feed digestion and absorption rate causing increase in body weight (5). Moreover the higher level (60 mg/kg of feed) of 17-alpha methyltestosterone produced some testicular degeneration (1). After they are used in fish, a portion is discharged into the water environment by excretion and the rest remain in the animal's body. These compounds may be transfered into water, foods and food products if not well controlled and much evidence has been documented indicating that exposure to synthetic chemicals at low levels may lead to potential risk to human and wildlife health. For this reason the European Economic Community (EEC)

Corresponding author: Uzunov Risto, DVM

e-mail address: risteuzunov@gmail.com
Present address: Food institute, Faculty of Veterinary medicine-Skopje, "Ss. Cyril and Methodius" University,
Lazar Pop- Trajkov 5-7, 1000 Skopje, R. Macedonia
tel: +389 2 3240 745; fax: +389 2 3114 619

prohibited the use of anabolic compounds as growth accelerators in the food animals. The Minimum required performance limit for methyltestosterone in muscle is 1 µg/kg (6). In Republic of Macedonia, the use of anabolic hormones as growth promoters has been made illegal also. The aim of this study was to validate of the screening ELISA method for determination of methyltestosterone in fish muscle.

MATERIAL AND METHODS

For validation of this screening ELISA method for determination of methyltestosterone anabolic steroid in fish we used blank fish muscle from untreated fish (*Species Carp, Cyprinus Carpio*).

Reagents. Most of the reagents that were used are contained in the RIDASCREEN Methyltestosterone test kit (Art. No. R3603) from R-Biopharm AG, Darmstadt, Germany. Kit contents was: Microtiter plate with 96 wells (12 strips with 8 removable wells each) coated with capture antibodies, 6 standard solutions: (0 ng/kg, 50 ng/kg, 150 ng/kg, 450 ng/kg, 1350 ng/kg, 4050 ng/kg methyltestosterone in 40% methanol ready to use), conjugate (peroxidase conjugated methyltestosterone, concentrate), anti-methyltestosterone antibody (concentrate), substrate/chromogen solution who contains tetramethyl-benzidine, stop solution which contained 1 N sulfuric acid, conjugate and antibody dilution buffer. Methanol (code I649035230) and tertiary butyl methyl ether (code K38520849) were of analytical grade and purchased from Merck. 20mM Phosphate buffer (PBS), pH 7.2, was prepared by mixing 0.55 g Sodium dihydrogen phosphate hydrate (NaH_2PO_4 x H_2O (code A689146545, Merck)) with 2.85 g Disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O (code FI639486105, Merck)) and 9 g Sodium chloride (NaCl (code K36586304638, Merck)) and was filled up to 1000 ml distilled water. 67 mM PBS buffer, pH 7.2, was prepared by mixing 1.8 g Sodium dihydrogen phosphate hydrate (NaH_2PO_4 x H_2O) with 9.61 g Disodium hydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O) and 9 g Sodium chloride (NaCl) and was filled up to 1000 ml with distilled water (7).

For fortified samples and calculation of recovery for this method we used external standard methyltestosterone from Fluka (code: 46444). From this standard we prepared standard solutions of methyltestosterone in methanol with concentration of 20 µg/kg and with this concentration of standard we fortified the blank sample on three levels: 1000, 1500 and 2000 ng/kg.

Extraction procedure. Fat was removed from muscle and the muscle was grinded. Ten grams from grounded muscle was homogenized with 10 ml of 67 mM PBS buffer by mixer (Typ T25 B, serial number 895944, IKA® Werke GmbH & Co.KG, Staufen, Germany) 5 min. Two gram from homogenized sample was mixed with 5 ml Tertiary butyl methyl ether in a centrifugal screw vial and shaken carefully for 30 - 60 min and then samples were centrifuged for 10 min at 3000 rpm on 10 - 15ºC. The supernatant (ether layer) was transferred to another centrifugal vial and extraction procedure was repeated with 5 ml Tertiary butyl methyl ether. Then the samples were evaporated to dryiness and dissolved in 1 ml methanol/water (80:20; v:v). The methanolic solution was diluted with 2 ml of 20mM PBS buffer and applied to a RIDA C_{18} column 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 20 mM 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 diluted slowly by injection of 1 ml methanol/water (80:20; v:v) (flow rate: 15 drops/min) and then sample was diluted with distilled water 1:2. In the test 50 µl of the samples per well was used (7).

Validation procedure. The limit of detection (LOD) of the assay was defined as the concentration corresponding to the mean signal of 20 blank fish muscle samples plus 3 times of standard deviation of the mean. Blank fish muscle samples were obtained from untreated fish. The accuracy was evaluated by determining the recovery of spiked fish muscle samples on three concentrations of methyltestosterone external standards (1000, 1500 and 2000 ng/kg). Precision was expressed as the CV (Coefficient of variation) (%) of the calculated standards and sample concentrations. Detection capabilities (CCβ) were required to be at or lower than the MRPL (8). CCβ were evaluated by analyzing of 20 spiked fish muscle samples at 0.5 times MRPL (1000 ng/kg for methyltestosterone in muscle) for methyltestosterone and calculated in accordance with European Commission Decision 2002/657/EC (7).

Test procedure. RIDASCREEN® Methyltestosterone ELISA kits (R-Biopharm AG, Darmstad, Germany) were used for validation of Methyltestosterone in fish muscle. All reagents in the kit had to be brought to room temperature (20 - 25 °C) before use. Standards used for methyltestosterone were containing 0 ng/kg, 50 ng/kg, 150 ng/kg, 450 ng/kg, 1350 ng/kg, 4050 ng/kg methyltestosterone in 40% methanol.

50 µl of each standard solution or prepared sample were added and after that 50 µl of the diluted enzyme conjugate and 50 µl of anti-methyltestosterone antibody solution to each well were added. The solution in the microplate was carefully mixed by shaking of the plate manually. The plate was then incubated at 4 °C for 16 hours. The liquid was poured out of the wells and after the complete removal of liquid, all wells were filled with distilled water. After rinsing, the water was also discarded; the washing was repeated two more times. Then, 50 µl of substrate and 50 µl of chromogen were added, and after mixing thoroughly and incubating for 30 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 of the spectrophotometer (BIO RAD model 680) (7).

RESULTS

In this study a commercial ELISA kit was used for validation of methyltestosterone in fish muscle. The ELISA technique showed very high correlation between methyltestosterone concentration (ng/kg) and maximum absorbance (%).

The correlation between the absorbance ratio and methyltestosterone concentration was evaluated over the range from 50 to 4050 ng/kg. Linear regression analysis showed good correlation, with R^2 values 0.9927, (y= -0,1741x + 1,5082), where y was relative absorbance (%) and x was methyltestosterone concentration in ng/kg (Fig. 1).

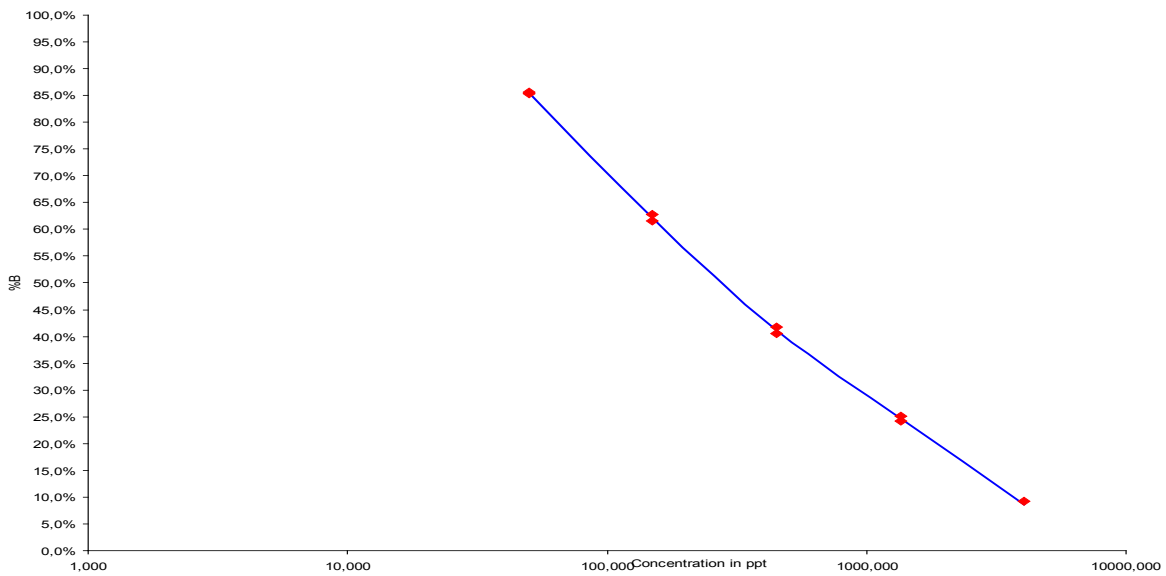

Figure 1. Linearity of the screening ELISA method for determination of methyltestosterone in fish muscle

Results for the precision of the method are presented in Table 1. The precision (Coefficient of variation (CV) %) in methyltestosterone standards ranged from 0.2% to 11.5%. The precision (CV %) in spiked fish muscle sample ranged from 2.5% to 6.8%.

Table 1. Precision of the screening ELISA method for determination of methyltestosterone in fish muscle

	Concentration (ng/kg)	CV (%)
methyltestosterone standards	0 50 150 450 1350 4050	2.5 0.2 1.4 2.1 2.8 11.5
CV % for spiked sample	1000 1500 2000	6.8 2.5 3.1

The accuracy was expressed as the recovery (%) of the estimated concentration. For the three target concentration (1000, 1500 and 2000 ng/kg) the recoveries in fish muscle sample were 97.4%, 82.4% and 85.1% respectively and they are presented in Table3. From Table 2 we can see that value of recovery is highest in the first case when the level of fortified sample is lowest.

Table 2. Accuracy of the screening ELISA method for determination of methyltestosterone in fish muscle (recovery %)

Fish muscle samples n (number of replicates)	Methyltestosterone		Recovery %
	Added (ng/kg)	Found (ng/kg)	
n = 8	1000	974	97.4
n = 8	1500	1236	82.4
n = 8	2000	1702	85.1

Detection limit for methyltestosterone was found to be 140.95 ng/kg. The detection capability (CCβ) for methyltestosterone was 564.43 ng/kg, less than MRPL level of 1000 ng/kg (6).

DISCUSSION

Raw fish and fish 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 (9, 10). Androgen excess leads to the development of insulin resistance during both hyperglycemic and euglycemic hyperinsulinemia and these findings provide direct evidence for a relationship between hyperandrogenemia and insulin resistance, and its associated risk factors for cardiovascular disease (11). Moreover, after administration high dose of methyltestosteron in humans causes negative mood as irritability, mood swings, violent feelings, and hostility, then cognitive impairment as distractibility, forgetfulness, and confusion (12). Other negative effects on the humans are increased risk of injury, increased blood pressure, gastrointestinal complications, benign and malignant liver tumours, Peliosis hepatis (blood-filled cysts), virilisation, clitoral hypertrophy, deepened voice, painful breast lumps in women, gynaecomastia and testicular atrophy in men, abnormalities of sperm count, motility and morphology, sterility, benign prostatic hypertrophy cutaneous striae (13). Because of negative effects the European Economic Community (EEC) prohibited the use of anabolic compounds as growth accelerators in food animals and fishes (14). In the presented study, the ELISA

method was used to achieve the unambiguous identification of methyltestosterone in fish muscle. This method was validated in accordance to the criteria of Commission Decision 2002/657/EC. Due to the simplicity, rapidness, and cost-effectiveness of the method and its good recovery and precision it is applicable in the official control laboratories as a screening method. But in the case when the target analyte is clearly identified above CCβ the sample is considered as non compliant and the results must be confirmed with confirmation method on GC/MS, LC/MS or with another confirmatory method (14, 15).

REFERENCES

1. Khalil, W. K. B., Hasheesh, W. S., Marie, M. A. S., Abbas H. H., Zahran E. A. (2005). Assessment the impact of 17α-methyltestosterone hormone on growth, hormone concentration, molecular and histopathological changes in muscles and testis of Nile tilapia; Oreochromis niloticus. Life Sci J, 8: 329-343.
2. Schanzer, W. (1996). Metabolism of anabolic androgenic steroids. Clin Chem, 42: 1001-1020.
3. K. P. Lone, A. J. Matty. (1981). The effect of feeding androgenic hormones on the proteolytic activity of the alimentary canal of carp *Cyprinus carpio* L. J Fish Biol, 18 (3): 353–358.
4. Felix, S. (1989). Effect of 17 a methyltsetosterone on the growth ornamental fish, Xiphophorus maculates. Indian J. Fish, 36 (3): 263-265.
5. Yamazaki, F. (1976). Application of hormones in fish culture. J. Fish. Res, 33: 948-958.
6. CRL Guidance paper. (2007). CRLs view on state of the art analytical methods for National residue control plans, 3-7.
7. R-Biopharm AG, Darmstadt, Germany: RIDASCREEN® 17 methyltestosterone, Enzyme immunoassay for the quantitative analysis of 17 methyltestosterone, R3601.
8. Commission of the European Communities: European Commission Decision of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results (2002/657/EC), Off J Eur Comm, L 221: 8-36.
9. Hoffman, B. (1996). Problems of residue and health risks of anabolic agents with hormone like activities. Proceedings of the Scientific Conference on Growth Promotion in Meat Production, European Comm. Dir. Gen. VI. Agric., Official Pub. European Comm., Brussels; 271-296.
10. Nazli, B., Colak, H., Aydin A, Hampikyan, H. (2005). The Presence of Some Anabolic Residues in Meat and Meat Products Sold in Istanbul. Turk J Vet Anim Sci, 29: 691-699.
11. Diamond, P., M., Grainger, D., Diamond, C.,M., Sherwin, S., R., DeFronzo, A., R. (1998). Effects of Methyltestosterone on Insulin Secretion and Sensitivity In Women. JCEM, 83 (12): 4420.
12. Su, T., P., Pagliaro, M., Schmidt, P., J., Pickar, D., Wolkowitz, O., Rubinow, D., R. (1993). Neuropsychiatric effects of anabolic steroids in male normal volunteers. JAMA, 269 (21): 2760-2764.
13. Rashid, H., Ormerod, S., Day, E. (2007). Anabolic androgenic steroids: what the psychiatrist needs to know. Adv. Phys, 13: 203-211.
14. Commission of the European Communities: European Commission Decision of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results (2002/657/EC), Off J Eur Comm, L 221: 8-36.
15. Commission of the European Communities: European Commission Decision of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animalproducts and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC (2002/657/EC), Off J Eur Comm, L 125: 1-28.