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VALIDATION METHODS FOR THE DETERMINATION OF β AGONISTS RESIDUES IN FEED

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ABSTRACT

Veterinary drugs are widely used in modern animal husbandry. Their usage is not only for therapeutic and prophylactic purposes but also for better breeding efficiency. A part of veterinary drugs are used as growth promoters in form of specified compounds or mixtures of compounds. Therefore, among other compounds, steroids and other substances that have similar pharmacological activity are used to improve efficiency of protein conversion. As a result of enhanced protein conversion the growth of animals is faster which lead to earlier slaughter. The aim of this study was to detect levels of β – agonist residues since they are used as additives in the feed. By using ELISA method level of β – agonist was determinate. Total of 49 feed samples were screened for the presence of β–agonist as a part of national monitoring residue plan. The feed samples were collected and delivered by the authorised veterinary inspectors within period of 1 year. The validation process was carried out according to Commission Decision 2002/657/EC criteria. Limit of detection (LOD) for β–agonist in feed was determined to be 0.63 ng/ml. The recovery was between 71.8% and 77.2%, a working range between 0.3 to 25.0 ng/ml. The regression equation of the final inhibition curve was: y=- 0.1143·Ln(x)+0.5906, R^2=0.9904. Additionally detection capability (CCβ) was 5.46 ng/ml. The levels of β – agonist residues were below the international allowable levels set by the Macedonian Residue Control Plan and the European Union. According to this study for β – agonist, feed in Republic of Macedonia is free and safe for animal consumption. However, it is necessary further monitoring of these chemicals as a food quality control measure. Shown data in this study will be helpful for screening of β – agonist residues and regulations on its illegal use in feed.
Keywords: *β – agonist, feed, ELISA, validation*

INTRODUCTION

β2-agonists or β2-adrenergic agonists have been illegally used to improve production performance and carcass condition of livestock. Pharmacologically, these compounds have been found to exert a repartitioning activity causing an increase in muscle accretion and decrease in fat deposition [7]. From many literary data we can say that beta agonists are transmitted in meat and meat products and present a risk for public health [6,7, 8]. Given orally or parenterally, β adrenergic agonists consistently decrease carcass fat and increase carcass

protein accretion in poultry, pigs, sheep and cattle [8]. Skeletal muscle protein accretion rate was increased by 130% during the first week of feeding the β - adrenergic agonist cimaterol to rapidly growing rats [6]. Although the chronic response was transient [6], skeletal muscle mass and protein content were increased by 20 to 30% after 3 week to 12 week treatment intervals [2,10,11]. Mexico and South Africa had approved use of β-agonists, including the feed additives zilpaterol hydrochloride and ractopamine hydrochloride, more than 10 year ago to improve feedlot performance. In 2003, ractopamine hydrochloride was approved for use in cattle in the United States, and zilpaterol hydrochloride was just approved in 2006 for increased rate of weight gain, improved feed efficiency, and increased carcass leanness in cattle fed in confinement for slaughter during the last 20 to 40 day on feed. In Mexico, consumption of viscera from animals fed with clenbuterol has caused acute toxicity in consumers, indicating an abuse in the use of this product, and therefore this β2-agonist was removed from the market [1]. However, the residues of β2- agonists may present health risk to public health [7].

The European Economic Community (EEC) banned the use of β-agonists and anabolic compounds as growth accelerators in feed while the United States Food and Drug Administration (USFDA) permitted limited use of some hormones with natural origin (such as oestradiol and testosterone) and some synthetic hormones such as trenbolone in animal husbandry [3,4]. The permitted limit values for all β-agonists are 50 ng/ml in feed [5]. The use of β-agonists as growth promoters is illegal in Republic of Macedonia, too. The Macedonian regulatory agencies implemented a monitoring surveillance program that uses only preliminary methods for determination of β-agonists. The examinations were carried out in the Faculty of veterinary medicine, Food Institute in Macedonia in five specialized veterinary diagnostic laboratories according to the requirements of the European Community.

MATERIAL AND METHODS

Sample collection: A total of 49 feed samples were screened for the presence of β–agonist as part of national monitoring residue plan. The samples were collected within period of 1 year as they were delivered by the authorised veterinary inspectors.

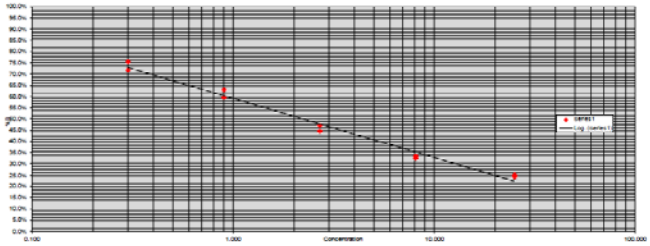
Enzyme-linked immunosorbent assay (ELISA): The concentrations of β-agonist in feed samples were determined using a commercial β-agonist ELISA kit (provided by R-biopharm Darmstadt, Germany). Each kit contained a microtiter plate with 96 wells coated with antibodies to rabbit IgG, clenbuterol standard solutions (0, 0.3, 0.9, 2.7, 8.1 and 25.0 ng/ml), peroxidase-conjugated clenbuterol, anti-clenbuterol antibody, substrate/chromogen solution, stop reagent, conjugate and antibody dilution buffer, and washing buffer. The extraction and clean-up procedures were those described by the ELISA kit manufacturer. The feed samples were minced and 1 g of ground feed was transferred into a suitable container, then 10 ml of 1 M HCl and 90 ml of distilled water were added and mixed vigorously for 15 min. Than samples were

centrifuged for 10 min at 4000 rpm on room temperature (20 - 25°C). The supernatant was transferred into a new vial and pH was checked and adjusted with 1 M NaOH (pH 8). Than samples were centrifuged for 10 min at 4000 rpm on room temperature (20 - 25 °C) and 20 µl of the supernatant was used per well in the assay. Data were analyzed using a special software RIDAWIN ELISA (R-Biopharm, Darmstadt, Germany).
Test procedure: All reagents in the kit had to be brought to room temperature (20 - 25 °C) before use. Standard used for β-agonist contain 0, 0.3, 0.9, 2.7, 8.1 and 25.0 ng/ml clenbuterol in 10% aqueous solution. We added 100 µl of diluted antibody to each well, mixed gently by shaking the plate manually and incubated for 15 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, after that 100 µl of the diluted enzyme conjugate was added. The solution in the microplate was carefully mixed by shaking the plate manually. Plate was incubated for 30 min at room temperature (20 - 25°C). The liquid was poured out of the wells and after complete removal of the 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 (tetramethylbenzidine) were added, and after mixing thoroughly and incubating for 15 min at room temperature and dark, 100 µl of stop solution (0.5 M H_2SO_4) was added. After mixing, the absorbance was read at 450 nm.
Method validation: The limit of detection (LOD) was obtained by analyzing 20 blank feed samples. The method recovery was determined at three levels by spiking feed samples (1, 2 and 5 ng/ml). For determination of repeatability, the same steps were repeated on two occasions in the same analytical conditions. Detection capabilities (CCβ) was evaluated by analyzing 20 spiked samples lover than MRPL level. The robustness of the method was determined with analyzing of spiking feed samples (1, 2 and 5 ng/ml) on two levels of pH (pH 8 and pH 5). In the latter case to determine the robustness had changed the procedure of extraction. Samples were dissolved in 10 ml 1M HCl and 90 ml distilled water in the first case and 1 ml of 1 M HCl and 9 ml of distilled water in the second case.

RESULTS

The calculation of the gained results was made by RIDAWIN Software. For construction of the calibration curve the mean of the absorbance values obtained for the 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 β-agonist. The obtained calibration curves for the ELISA method in the range 0.3-25.0 ng/ml in linear and exponential form are presented on Figure 1. The curve equation $y=-0.1143 \cdot Ln(x)+0.5906$, where y was relative absorbance (%) and x was clenbuterol concentration in ng/ml, was

utilized for determining β-agonist concentration in feed samples, obtaining high regression coefficient (R^2=0.9904).



Graph 1. Linearity of calibration curve for β-agonist standards

The results of method recovery (n=18) and repeatability (n=54) are presented in table 1.

Table 1. Recovery and repeatability of the method

Validation parameter	No. of replicates	Spiked concentration ng/ml	Determinated concentration ng/ml	Mean recovery %	Coefficient of variation %
Recovery	6	1	0.718	71.8	0.42
	6	2	1.552	77.6	2.75
	6	5	3.860	77.2	1.18
Repeatability	18	1	0.801	80.1	4.37
	18	2	1.483	74.1	1.60
	18	5	3.972	79.4	3.04

Validation of the method used in β-agonist determination resulted in the mean recovery of 71.8%-77.6% and repeatability of 74.1%-80.1% with coefficient of variation (CV) of 0.42%-2.75% and 1.60%-4.37% respectively.
The results of method robustness are presented in table 2.
At pH 5 and pH 8 recoveries were from 53.5 to 60.8% and from 71.8 to 77.6% respectively. Extraction with 90 ml distilled water and 10 ml of 1 M HCl in our case gave better results than extraction that provides producers, where extraction is with 1 ml 1 M HCl and 9 ml distilled water. Recovery was from 71.8 to 77.6% with first extraction. With second extraction recovery was from 54.0 to 65.8%. Therefore created a modification of the method by the manufacturer and we use extraction with 90 ml distilled water and 10 ml of 1 M HCl. The estimated LOD for feed samples for β-agonist was 0.63 ng/ml. The CCβ for feed samples for β-agonist was 5.46 ng/ml.

Table 2. Robustness of the method

Validation parameter Robustness	No. of replicates	Spiked concentra-tion ng/ml	Determinated concentration ng/ml	Mean recovery %
pH 8	6	1	0.718	71.8
	6	2	1.552	77.6
	6	5	3.860	77.2
pH 5	6	1	0.608	60.8
	6	2	1.070	53.5
	6	5	2.907	58.1
Extraction with 10 ml HCl+90 ml distill. water	6	1	0.718	71.8
	6	2	1.552	77.6
	6	5	3.860	77.2
Extraction with 1 ml HCl+9 ml distill. water	6	1	0.658	65.8
	6	2	1.225	61.3
	6	5	2.698	54.0

Forty nine feed samples were tested applying the validated screening method. Eight of them (16.33 %) were with concentration less than LOD (< 0.63. ng/ml) and other samples were with concentration from 0.68 to 4.35 ng/ml. Feed samples with concentration above CCβ were not determined.

CONCLUSIONS

The European Economic Community (EEC) banned the use of β-agonist compounds as growth accelerators in feed. Elisa method was used as screening method for analyzing β-agonist in feed. ELISA method is 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 would be useful for correct test result [9]. For this reason our test results are of importance as they give information about the use of β-agonist preparations in national animal husbandry and in the feed industry. A survey carried out in R. Macedonia demonstrated that the incidence of residues of β-agonist in feed is not a problem, which means that β-agonist gave no evidence of illegal use of them in R. Macedonia. The National Residues Control Plan guarantees fulfilment of the requirements which are of importance to health of both humans and animals as well as marketing of animals, feed and products of animal origin.

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