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Determination of residual ractopamine concentrations by enzyme immunoassay in treated pig's tissues on days after withdrawal

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ABSTRACT

The objective of this study was to measure residual ractopamine concentrations in tissues of pigs as experimental animals after treatment with dietary ractopamine for 28 consecutive days. Ractopamine was administered orally to the experimental group (n = 9) in a dose of 0.1 mg/kg body mass *per* day, whereas control animals (n = 3) were left untreated. Treated pigs (60 kg) were sacrificed on days 1, 3 and 8 of treatment discontinuation and residues were determined in kidney, liver, muscle, brain and heart tissues using previously validated enzyme-linked immunosorbent assay (ELISA) as a quantitative screening method. Validation showed good mean recoveries (approx. 70–90%) with acceptable inter- and intra-day relative standard deviations (RSD < 13%), demonstrating the method efficiency in determination of ractopamine tissue concentrations. The highest ractopamine concentration on day 1 (24 h) after the last exposure was recorded in the kidney (12.49 ± 7.96 ng/g), followed by the liver (7.21 ± 2.73 ng/g), heart (1.26 ± 0.12 ng/g) and brain (0.63 ± 0.05 ng/g); at this time of withdrawal, residues were not detected in the muscle. Ractopamine was depleted rapidly from all study tissues, with mostly no detectable residues on day 8 of withdrawal.

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1. Introduction

Ractopamine is a phenethanolamine β-adrenergic agonist, which has a repartitioning effect. If used in meat production, it promotes muscle growth of treated animals by increasing nitrogen retention (Anderson et al., 1989), protein synthesis (Bergen et al., 1987) and lipolysis, while suppressing lipogenesis (Bergen et al., 1987; Merkel et al., 1987; Peterla & Scanes, 1990). At the same time, ractopamine also increases the rate of weight gain and feed conversion (Mitchell et al., 1990; Veenhuizen et al., 1987), and may be illegally used in Europe to enhance growth rates in livestock species. Ractopamine was approved by the U.S. Food and Drug Administration for use in finishing swine in 1999 and is now cleared in 21 other countries (Anderson et al., 2009), whereas in European Union the use of ractopamine like other β-agonists is completely banned (Council Directive 96/22/EC). Performance improvements associated with ractopamine feeding in pigs are affected by several factors, including but not limited to nutrient concentrations of the diet, dietary ractopamine concentration and duration of feeding (Apple et al., 2007; Moody et al., 2000). Literature data show that ractopamine feed concentrations of 10 to 20 mg/kg of feed and feeding duration of 28 to 34 days result in significant improvements in carcass characteristics (Armstrong et al., 2004; Watkins et al., 1990; Williams et al., 1994). Despite the proven beneficial effects of β-agonists on animal performance, there are a number of well documented cases where the illegal use of such compounds resulted in human food poisoning (Martinez-Navarro, 1990; Pulce et al., 1991). As all these incidents without exception were caused by clenbuterol toxicity the European Union has placed ban upon the use of all β-agonists, thus requiring strict monitoring for the illegal use of this and other β-agonists.

Therefore, control and monitoring programs mandated by government have necessitated the development of assays for ractopamine determination and depletion. In the literature, there are reports on ractopamine determination in various matrices using different techniques (Shelver & Smith, 2003; Smith et al., 1993; Thompson et al., 2008; Turberg et al., 1995). Studies suggest the use of LC/MS/MS systems or an immunoassay as probably the best methods to improve sensitivity in determination of β-adrenergic agonists, while retaining excellent selectivity (Smith & Shelver, 2002). As the analysis of ractopamine at all stages of production is important for monitoring illegal use, development of simple, rapid and inexpensive methodologies in different matrices is required.

In the present study, residual concentrations and depletion of ractopamine were assessed in pig liver, kidney, muscle, heart and brain tissues. The aim of the study was to determine and compare ractopamine residual concentrations in inner organs on different days of withdrawal using validated enzyme-linked immunosorbent assay (ELISA) as a screening method for quantitative determination of low ractopamine residue levels.

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2. Materials and methods

2.1. Chemicals and apparatus

A Ridascreen ractopamine kit for ELISA were provided by R-Biopharm (Darmstadt, Germany). Each kit contains a microtiter plate with 96 wells coated with antibodies to rabbit IgG, ractopamine standard solution concentrations of 0, 100, 300, 900, 2700 and 8100 ng/mL, peroxidase conjugated ractopamine, anti-ractopamine antibody, substrate/chromogen (tetramethylbenzidine), stop reagent (1 M sulphuric acid), sample dilution buffer and washing buffer (10 mM phosphate buffer, pH = 7.4). Ractopamine hydrochloride standard (for animal treatment and method validation) was provided by Sigma-Aldrich-Chemie (Steinheim, Germany). Standard solutions for validation were prepared as aqueous stock and working solution in concentrations of 10000 ng/mL and 30 ng/mL, stored at +4 °C until analysis and used for sample fortification, respectively. All other chemicals used in the analysis were of analytical grade. ELISA was performed by use of ChemWell (Awareness Technology Inc., USA). Statistical data analysis was performed by use of the Statistica Ver. 6.1 software (StatSoft Inc. 1984–2003, USA) with statistical significance set at the level of 95% ($P = 0.05$).

2.2. Animals and sampling procedure

The experiment was carried out in 12 castrate male pigs, Zegers hybrid type, aged 90 days, body mass 60 kg, farm-bred, and kept under the same zoohygienic conditions. Animals were divided into two groups: 9 pigs treated with ractopamine and 3 control animals. Treated animals were oraly administered 0.1 mg/kg body mass ractopamine hydrochloride *per* day adjusted throughout the study (average 6.5 mg *per* day) for 28 days in the form of a pure chemical capsule admixed to feed. Feed ration was restricted to a constant daily amount. Three animals served as a control group and were left untreated. On days 1 (24 h), 3 and 8 of ractopamine discontinuation, the treated pigs were randomly sacrificed (in groups of 3) and liver, kidney, muscle (m. semitendinosus), heart and brain samples were collected. Control animals were sacrificed on day 1 of experimental group treatment withdrawal. The samples were stored at −20 °C until analysis for residual ractopamine. The experimental protocol was designed according to the Act on Animal Welfare No.135, 2006.

2.3. Sample preparation and extraction procedure

Separated liver, kidney, muscle, brain and heart tissues were minced and ground to a fine mass with Ultraturax, then analysed in duplicate. For validation, blank tissues sampled from control animals were fortified with ractopamine working standard solution (30 ng/mL) and analysed as follows. Three grams of the homogenized samples were transferred into a centrifugal screw cap vial, 8 mL of acetonitrile and 1 mL of ethyl acetate were added, and mixed well. It was shaken for 30 min and then centrifuged at 4000 rpm for 10 min at 20 °C. Four mL of the supernatant were transferred to a new vial and evaporated to dryness at 60 °C. The residues were dissolved in 2 mL n-hexane, then 3 mL of sample dilution buffer were added, vortexed for 30 s and centrifuged at 4000 rpm for 10 min at 20 °C. The lower phase was removed carefully and 20 μL of the supernatant were used in the assay.

2.4. Analysis of ractopamine

Competitive ELISA was performed as described in package insert provided by the manufacturer. Microtiter strips coated with sheep antibodies directed against anti-ractopamine were inserted in the microwell holder for the standards and samples to be analyzed in duplicate. Using device, 100 μL of diluted antibody solution was added to the microwells, mixed gently and the plate was incubated at room temperature for 15 min. The wells were emptied completely and washed 3 times with 250 μL of washing buffer. Then, 20 μL of ractopamine standards (0, 100, 300, 900, 2700 and 8100 ng/L) and prepared samples were added to microwells. To each microwell, 100 μL of diluted enzyme conjugate were added, mixed gently and incubated for 60 min at room temperature. After washing, 100 μL of the substrate/chromogen solution were added to all wells and incubated in the dark for 15 min at room temperature. The reaction was stopped by adding 100 μL of stop reagent and absorbance was measured on a microplate reader at 450 nm.

2.5. Method validation

The limit of detection (LOD) and limit of quantitation (LOQ) were obtained by adding 3 and 10 times standard deviation of 10 analysed blank samples to the mean blank value, respectively. The method recovery was determined at three different levels by spiking control tissues (kidney, liver, muscle, brain and heart) sampled from control group of animals with working solution of the ractopamine standard to yield concentrations equivalent to 2, 5, 10 and 20 ng/g (six replicates *per* concentration level *per* day). Concentrations were calculated from six-point calibration curves. For determination of intermediate precision, the same steps were repeated on two other occasions with the same analysis conditions. Final concentration results were calculated by taking the average recoveries into account and expressed as mean value (ng/g) ± SD.

3. Results and discussion

3.1. Validation of analytical procedure

The estimated LOD and LOQ were 0.5 ng/g and 0.7 ng/g for liver, kidney and heart; 0.4 ng/g and 0.5 ng/g for muscle; and 0.3 ng/g and 0.4 ng/g for brain, respectively. Several authors propose screening methods for ractopamine determination using EIA or ELISA (Elliot et al., 1998; Haasnoot et al., 1994; Shelver & Smith, 2003) with detection limits in the 1–50 ng/g range. The method used in our study proved capable to determine ractopamine residues well below the Minimum Required Performance Levels (MRPLs) of 1 μg/kg (Gaudin et al., 2007).

A typical ELISA standard curve for ractopamine analysis is presented in Fig. 1. The results of method recovery (n = 24) and intermediate precision (n = 72) for each analysed matrix are shown in Table 1. Method validation resulted in mean recoveries ranging from 69.8% to 93.1% and intermediate precision ranging from 67.6% to 91.8%, with coefficients of variation (CV) of 4.9%–8.8% and 7.3%–12.3%, respectively. The lower recovery values at higher concentration levels (10 and 20 ng/g) could be explained by the additional eluate dilution (higher dilution factor) needed to be in the range of

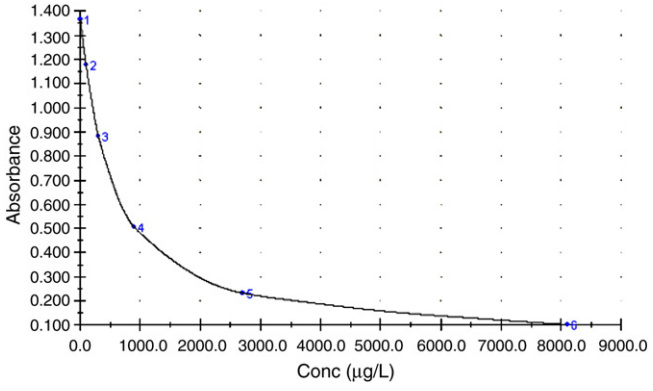

Fig. 1. ELISA standard curve for ractopamine.

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