The aim of the present study was to assess the persistence of clenbuterol residues in retinal tissue of pigs after repeated administration in a growth-promoting dose, using enzyme-linked immunosorbent assay (ELISA) as a screening method for quantitative determination. A growth-promoting dose of clenbuterol (20 µg/kg body mass per day) was administered orally to the experimental group \((n = 6)\) for 21 days, whereas control animals \((n = 3)\) were left untreated. Clenbuterol-treated pigs were randomly sacrificed \((n = 3)\) on days 0 and 45 of treatment discontinuation, and clenbuterol residues were determined in retinal tissue dissected from the eye. ELISA was found to be acceptable for quantitative determination of clenbuterol in retinal samples because previous method validation yielded mean recovery values of 84.3–96.5% with variation coefficients < 14%. The mean (± SD) retinal clenbuterol concentration was 1874 ± 114 ng/g immediately upon clenbuterol withdrawal (day 0) and 73 ± 4 ng/g on the last day post-withdrawal (day 45). Study results pointed to a very high potential of clenbuterol accumulation in retinal tissue and marked persistence of clenbuterol residues upon anabolic dose administration, suggesting retinal tissue to be a very useful matrix for effective control of residual clenbuterol in food-producing pigs.

**Material and Methods**

**Chemicals and apparatus**

A Ridascreen clenbuterol kit for ELISA was 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, 100, 300, 900, 2700, and 8100 ng/L), peroxidase-conjugated clenbuterol, anti-clenbuterol antibody, substrate/chromogen solution, stop reagent,
conjugate and antibody dilution buffer, and washing buffer. Clenbuterol hydrochloride from Sigma Aldrich Chemie GmbH (Steinheim, Germany) was used for sample fortification. All other chemicals used in the analysis were of analytical grade. Solid-phase extraction (SPE) clean up was done using Bond Elut-C18 columns (Varian, Santa Clara, CA). ELISA was performed by using an ELx800TM microplate reader and ELx50 washer (BioTek Instruments, Winooski, VT). Clenbuterol concentration in the samples was calculated using RIDA® SOFT Win Program, R-Biopharm (Darmstadt, Germany). Statistical data analysis was performed by use of the Statistica Ver. 6.1 software (StatSoft, Tulsa, OK).

Animals and sampling procedure

The experiment was performed on nine male pigs (six treated and three controls) cross-bred between Swedish Landrace and Zegers, aged 7 months, body mass 95–100 kg, farm-bred, and kept under the same hygienic conditions. The pigs were given 10 µg/kg body mass clenbuterol in a capsule with pure clenbuterol admixed to feed twice daily for 21 days. On days 0 and 45 of clenbuterol discontinuation, the pigs were randomly sacrificed (in groups of 3), and eyeballs of treated and control animals were collected and stored at −25°C until analysis for clenbuterol residues. The experimental protocol was designed according to the Act on Animal Welfare, as stated in the Official Gazette of the Republic of Croatia No. 135/2006 (16).

Sample preparation and extraction procedure

The eyeballs collected at slaughter were placed in clean Petri dishes. All connective tissue was removed from the eye, the eyeball was everted, and retinal tissue was scraped off by use of scalpel. For analysis of clenbuterol, 300 mg of retinal tissue was homogenized with a 10 mL distilled water using an Ultra-Turax device. Upon homogenization, the sample was divided into six sub-samples for further analysis. Then, 1 g of the homogenate was transferred to 10-mL centrifuge tube, and 0.6 mL 1 M HCl and 0.4 mL methanol (100%) were added and mixed vigorously for 30 s. Two milliliters of chloroform was added, mixed again for 30 s, and then centrifuged at 3000 rpm for 10 min at 4°C. One milliliter of the upper layer was transferred to a vial, and 0.3 mL 1 M NaOH and 3 mL of KH2PO4 (0.2 M, pH 3.0) were added and mixed. The SPE columns were first rinsed with 3 mL of methanol (100%), followed by the addition of 2 mL of 50 mM KH2PO4 buffer (pH 3), and then the complete sample volume (4.3 mL) was applied. The columns were washed with 2 mL of 50 mM KH2PO4 (pH 3.0). Air was pressed through the column to remove all liquid, and the sample was eluted with 1 mL methanol (100%). The eluate was evaporated to dryness, the residues were reconstituted with redistilled water, and the solution was used for ELISA. The validation parameters were determined using fortified blank pig retina samples.

Analysis of clenbuterol

Competitive ELISA was performed as described in package

Table 1. Evaluation of Validation Procedure for Retinal Tissue Samples Spiked at 2, 5, 10, and 20 ng/g Using Enzyme-Linked Immunosorbent Assay (ELISA)

<table>
<thead>
<tr>
<th>Validation Parameter</th>
<th>n*</th>
<th>Spiked Concentration (ng/g)</th>
<th>Clenbuterol Determined (ng/g)</th>
<th>Mean Recovery (%)</th>
<th>Coefficient of Variation† (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery</td>
<td>6</td>
<td>2</td>
<td>1.93</td>
<td>96.5</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
<td>4.72</td>
<td>94.4</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10</td>
<td>9.38</td>
<td>93.8</td>
<td>4.8</td>
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<tr>
<td></td>
<td>6</td>
<td>20</td>
<td>18.04</td>
<td>90.2</td>
<td>6.1</td>
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<tr>
<td>Repeatability</td>
<td>18</td>
<td>2</td>
<td>1.92</td>
<td>96.0</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>5</td>
<td>4.61</td>
<td>92.2</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>10</td>
<td>9.01</td>
<td>90.1</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>20</td>
<td>17.48</td>
<td>87.4</td>
<td>8.5</td>
</tr>
<tr>
<td>Within-laboratory reproducibility</td>
<td>18</td>
<td>2</td>
<td>1.89</td>
<td>94.5</td>
<td>13.8</td>
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<td>18</td>
<td>5</td>
<td>4.62</td>
<td>92.4</td>
<td>10.8</td>
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<td>18</td>
<td>10</td>
<td>8.84</td>
<td>85.6</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>20</td>
<td>17.88</td>
<td>84.3</td>
<td>11.9</td>
</tr>
</tbody>
</table>

* Number of fortified replicates.
† CV = (SD/mean) x 100%.
IgG were inserted in the microwell holder for the standards and samples to be analyzed in duplicate. To the microwells, 100 µL of diluted antibody solution was added and the plate was incubated at 2–8°C overnight. The next morning, the wells were emptied completely and washed 3 times with 250 µL of washing buffer. Then, 20 µL of clenbuterol standards (0, 100, 300, 900, 2700, and 8100 ng/L) and prepared samples were added to microwells. Diluted enzyme conjugate (peroxidase conjugated clenbuterol) (100 µL) was added to each microwell; the microwells were mixed gently and incubated for 1 h at room temperature. After washing, 100 µL of the substrate/chromogen solution (urea peroxide/tetramethylbenzidine) was added to all wells, and they were incubated in the dark for 30 min at room temperature. The reaction was stopped by adding 100 µL of stop reagent (1 M sulfuric acid), and absorbance was measured on a microplate reader at 450 nm.

Results and Discussion

Validation of analytical procedure

The estimated limits of detection (LOD) and quantification (LOQ), which are based on the mean value of 10 determinations of blank retina samples plus 3- and 10-fold standard deviation, were 0.5 and 0.8 ng/g, respectively. A typical ELISA standard curve for clenbuterol analyses in retina is presented in Figure 1. The results of method recovery (n = 54), repeatability (n = 54), and within-laboratory reproducibility (n = 54) are presented in Table I. Method validation resulted in mean recoveries ranging from 90.2 to 96.5%, repeatability ranging from 87.4 to 96.0%, and within-laboratory reproducibility ranging from 84.3 to 94.5%, with coefficients of variation (CV) of 5.4–7.2%, 7.2–9.5%, and 10.5–13.8%, respectively. The recovery levels decreased with the increasing levels of spiking, which could be explained by the fact that the increased systemic levels of clenbuterol bound more tightly to the retinal tissue. Validation results demonstrated the method of sample preparation and determination of clenbuterol in retinal tissue using ELISA as a screening method for quantitative determination to be efficient and comparable with manufacturer and literature data (14,17). Finally, clenbuterol concentrations in the retina of treated animals were calculated by taking average recoveries into account.

Clenbuterol concentration in retinal tissue

The measured mean clenbuterol concentrations (±SD) on days 0 and 45 of withdrawal in retinal tissue of treated animals are shown in Table II. On the first day of clenbuterol withdrawal after 21-day treatment (day 0), the mean clenbuterol concentration of 1874 ± 114 ng/g was recorded in retinal tissue samples of randomly sacrificed animals (n = 3). On the last day (day 45) of withdrawal, the mean clenbuterol concentration of clenbuterol was 73 ± 4 ng/g, significantly (p < 0.05) exceeding the method limit of detection (0.5 ng/g). These results showed clenbuterol residues to persist in retinal tissue for a long period of time following cessation of animal treatment with the anabolic dose of the agent. Although providing a very small amount of sample for analysis (about 0.6 g retina for both eyes together), the speed and simplicity of the analysis are advantages of retinal tissue, particularly when compared with other matrices investigated in our previous studies. The use of ELISA as a screening method proved appropriate and the use of a confirmation method would only be required for non-compliant samples.

Pharmacokinetics studies showed the retina to accumulate up to 35-fold levels of β-agonists found in other tissues, confirming the eye to be a very good target organ to estimate residual clenbuterol. The high level of residual clenbuterol in retinal tissue is attributed to the high clenbuterol binding affinity for melanin found in the pigmented segment of the eye (18–20). The binding of clenbuterol to pigmented tissue melanin is explained by the action of electrostatic forces that develop between the positive-charged clenbuterol molecule and negative groups of melanin polymers as well as by Van der Waals’ forces on the clenbuterol aromatic ring conjugates and melanin aromatic indol nucleus. Furthermore, some studies suggest that the interaction between clenbuterol and melanin may also be based on charge transfer from clenbuterol as a potent electron donor to melanin (14). Other authors report on clenbuterol residues to persist in retinal tissue for a long period of time after cessation of animal treatment, accumulating in retinal epithelium and persisting there at a 20- to 90-fold rate recorded in the liver as an edible matrix (6). In calf studies, clenbuterol residues were demonstrated to persist in ocular tissue for more than 56 days (7). The use of this matrix enabled detection of clenbuterol abuse at 140 days of treatment cessation (21). Gowik et al. (14) reported an even higher level of residual clenbuterol (2039 ± 10 µg/kg) in pigs exposed to anabolic dose of clenbuterol as compared to our results. Previous studies in porcine ocular tissue as a matrix also showed high levels of clenbuterol residues to persist in this tissue for the longest period of time. Joseph et al. (22) reported that clenbuterol residues of approximately 2000

Table II. Mean (±SD) Clenbuterol Concentrations (ng/g) in Retinal Tissue of Pigs on Days after Withdrawal

<table>
<thead>
<tr>
<th>Days after Withdrawal</th>
<th>Animal</th>
<th>Number of Replicates</th>
<th>Concentration of Clenbuterol (ng/g)</th>
<th>Standard Deviation (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>6</td>
<td>2062</td>
<td>124</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>1815</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>1745</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>4</td>
<td>6</td>
<td>68</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>73</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>79</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
µg/kg can be found in retina. The high rate of accumulation and slow elimination make it a very appropriate matrix in the control of clenbuterol abuse as an anabolic (13,14,23).

Clenbuterol residues persist in retinal tissue for a significantly longer period of time than in other potential matrix, thus enabling clenbuterol abuse to detect at a longer time of treatment discontinuation, this matrix is very useful in the control of clenbuterol abuse in food-producing animals and applicable in official residue controls using ELISA method for its determination.

Conclusions

Our study results pointed to retina as a significant matrix for the control of clenbuterol abuse during fattening in food-producing pigs, based on the marked accumulation and prolonged persistence of clenbuterol residues in this tissue after its discontinuation. Also, the ELISA method described can be used in monitoring for clenbuterol abuse as an anabolic in meat production, with a confirmation method only required in case of non-compliant sample.

Acknowledgments

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References