

Determination of Clenbuterol in Pig Liver Following Prolonged Administration of a Growth-Promoting Dose

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Summary

Liver concentrations of clenbuterol, a β_2 -adrenergic agonist, were measured by enzyme-linked immunosorbent assay (ELISA) in 12 female pigs on days 0, 7, 14 and 21 after prolonged administration of a growth-promoting dose of clenbuterol. The analytical procedure showed good recovery (>80 %), while intra-assay results showed acceptable variation in individual measurements for all samples to which 0.5, 2 or 5 ng/g clenbuterol were added (RSD <10 %). The assay detection limit was 0.13 ng/g and quantification limit 0.16 ng/g. The results indicated that >80 % clenbuterol depletion in pig liver occurred during the first 7 days after cessation of administration, however, more than 14 days were required for the clenbuterol concentration to fall below the maximal residual level of 0.5 ng/g. On day 21 of clenbuterol discontinuation, residues of the drug were detectable in pig liver (0.22 ng/g), suggesting that clenbuterol residues persist in liver as an edible tissue and may induce a risk for consumer health.

Key words: β_2 -adrenergic agonist, residual clenbuterol, anabolic agent, enzyme immunoassay, pig liver

Introduction

Clenbuterol (4-amino- α -[*t*-butylaminomethyl]-3,5-dichlorobenzyl alcohol hydrochloride) is a β_2 -adrenergic agonist (BAA), a synthetic derivative of adrenaline and noradrenaline, hormones secreted by the adrenal medulla. Clenbuterol is used in human and veterinary medicine for its broncholytic and tocolytic action. In 5- to 10-fold therapeutic doses, clenbuterol acts as a repartitioning agent to improve the performance of food-producing animals. Such a growth-promoting dose of clenbuterol influences animal growth and carcass composition by increased muscle mass and decreased fat mass, presumably through a direct effect of BAA on skeletal muscle and adipose tissue, indirect effect on many other tissues, or a combination of both (1). Because of these effects, clenbuterol has been illegally used

in food-producing animals. Cases of consumer intoxication due to consumption of meat and liver contaminated with residual clenbuterol have been described in the literature (2–5). Many countries have imposed total ban on the use of clenbuterol in food-producing animals, and the legislation within the European Union demands that the assays developed for the detection of illegal use of clenbuterol detect residues in liver tissue to <0.5 ng/g (defined as Maximal Residue Level – MRL). As compared with urine and bile, retina and liver are preferred matrices for clenbuterol abuse monitoring because of residue persistence in these tissues. Thus, previous reports showed that higher accumulation of clenbuterol was found in the retina as a non-edible tissue than in the liver of cattle (6–8), because of the high level of spe-

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cific β -adrenergic receptors in the eye. Data on the persistence of clenbuterol residues in the liver, as an edible tissue of ruminants (6,9–11), poultry (12) and pigs (13) have been reported. However, little data refer to the study of clenbuterol residue persistence in the liver of pigs. Therefore, the aim of this study was to assess the level of residual clenbuterol in the liver of female pigs, in order to study its tissue persistence after cessation of prolonged administration in a growth-promoting dose, using an enzyme immunoassay ELISA in quantitative determination of clenbuterol.

Materials and Methods

Chemicals

A Ridascreen clenbuterol kit for enzyme-linked immunosorbent assay (ELISA) was provided by R-Biopharm (Darmstadt, Germany). Each test kit contains a microtiter plate with 96 wells coated with antibodies to rabbit IgG; standard solutions (0.0 ppb; 0.100 ppb; 0.300 ppb; 0.900 ppb; 2.700 ppb and 8.100 ppb); peroxidase conjugated clenbuterol; anti-clenbuterol antibody; substrate, chromogen; stop reagent; conjugate dilution buffer and washing buffer. Clenbuterol hydrochloride from Sigma-Aldrich-Chemie (Steinheim, Germany) was used for fortification of samples. All other chemicals used in the analysis were of analytical grade.

Animals and sampling procedure

The experiments were carried out in 12 female pigs of a known breed (a cross-breed between Dutch and Swedish Landrace, and Large Yorkshire) aged 6–7 months, body mass 80–100 kg, farm-bred and kept under the same hygienic conditions. The pigs were given 5 μ g clenbuterol/kg body mass as an intravenous injection into the ear vein twice a day for 21 days. After the cessation of administration, on days 1, 7, 14 and 21, three pigs from the experimental group were randomly sacrificed. Animals were slaughtered by stunning (captive-bolt pistol) and complete exsanguination under surveillance at an authorized abattoir was performed. Samples of pig liver were collected and frozen until the analysis for residual clenbuterol. The experimental protocol was designed according to the Act on Animal Welfare, as stated in the Official Gazette of the Republic of Croatia (14).

Preparation of samples and solid-phase C 18 purification

A portion of liver was homogenized using an Ultraturax homogenizer (DI 25 basic, Ika-Werke, Staufen, Germany), 5 g of homogenate were mixed with 25 mL 50 mM HCl by shaking for 1.5 h. The amount of 6 g of the homogenate was centrifuged, the supernatant was collected in a tube containing 300 μ L of 1 M NaOH, mixed for 15 min, 400 μ L 0.5 M KH_2PO_4 were added and stored at 4 °C overnight. The mixture was centrifuged at 2750 g for 15 min, the supernatant was purified by RP-18 cartridges (Supelco, Bellefonte, USA) and clenbuterol was eluted with methanol as described elsewhere (6). The eluent was collected by vacuum and the solvent was evaporated under nitrogen stream. Dried residue

was redissolved in 400 μ L of water, and 20 μ L per well were analyzed by ELISA following the manufacturer's instructions. Distilled water was used for further dilution of highly contaminated samples.

Fortification

Liver samples of untreated pigs already tested as negative for clenbuterol by ELISA (<0.13 ng/g) were used in fortification studies. Fortification was carried out by pipetting 25 μ L of methanolic solutions containing clenbuterol concentrations of 300, 120 and 30 ng/mL in the liver homogenate, resulting in fortification levels of 5, 2 and 0.5 ng/g, respectively. The fortified samples were used for calculation of recovery.

Procedure for immunoassay

Residues of clenbuterol in pig liver were determined by a competitive enzyme immunoassay (ELISA). Immunoassays were performed as described in the package inserts provided by the manufacturer. Briefly, 100 μ L of diluted anti-clenbuterol antibody solution were added to antibody-coated wells and incubated at 2–8 °C for 15 h overnight. After washing, 100 μ L of diluted enzyme conjugate and 20 μ L of standard or prepared sample, after preparation and solid-phase purification on C-18 columns as described above, were added to the bottom of each well and incubated for 1 h at room temperature. After washing, 50 μ L of substrate (urea peroxide) and 50 μ L of chromogen (tetramethylbenzidine) were added to each well and incubated in the dark for 30 min at room temperature. The reaction was stopped by adding 100 μ L of stop reagent (1 M sulfuric acid). Absorbance was measured at 450 nm. All samples and standards were run in duplicate.

Results

ELISA standard curves for clenbuterol (amount: 6; replicates: 2; conc.: 0.0; 0.100; 0.300; 0.900; 2.700 and 8.100 ppb) performed by RIDA® SOFT Win PC program, R-Biopharm GmbH, Darmstadt, Germany, are shown in Fig. 1. The intra-assay variation for clenbuterol determination in pig liver by ELISA is given in Table 1. The de-

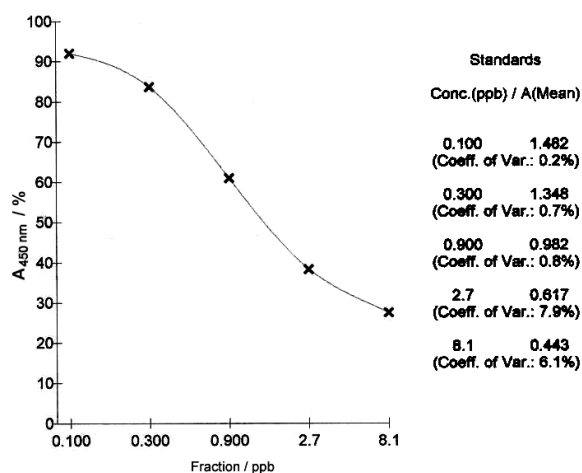


Fig. 1. ELISA standard curve for clenbuterol

tection limit of the assay was estimated to be 0.13 ng/g (determined mean concentration of 10 blank liver samples collected from untreated pigs + 3 times standard deviation), and the quantification limit (determined mean concentration of 10 blank liver samples collected from untreated pigs + 6 times standard deviation) was 0.16 ng/g. Results of the clenbuterol concentration measurement in pig liver homogenates after cessation of animal exposure to clenbuterol are shown in Fig. 2.

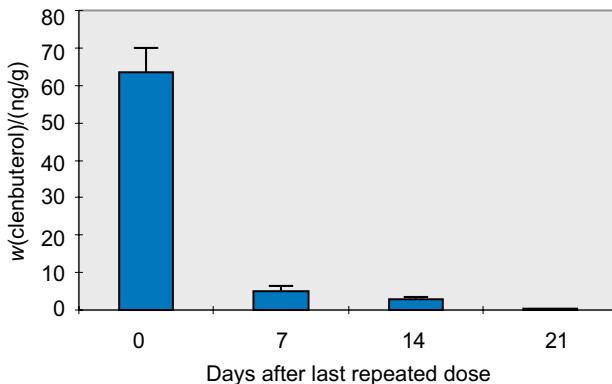


Fig. 2. Mean (\pm SE) clenbuterol concentration (ng/g) in pig^a liver after the last repeated dose^b

^aFemale pigs (12 cross-breed between Dutch and Swedish Landrace, and Large Yorkshire, $n=3$, on days 1, 7, 14 and 21) aged 6–7 months, body mass 80–100 kg

^bFemale pigs were given 5 μ g/kg body mass of clenbuterol twice a day intravenously for 21 days

Table 1. Intra-assay variation of control pig liver fortified with clenbuterol

Clenbuterol w (added)/ (ng/g)	n	Mean concentration w (found)/(ng/g)	Relative standard deviation RSD/%	Recovery R/%
0.5	5	0.44 \pm 0.04	9.09	88.0
2.0	5	1.80 \pm 0.17	9.44	90.0
5.0	5	4.40 \pm 0.41	9.32	88.0

Discussion

Previous studies evaluated the usefulness of commercial ELISA kits as a screening method for BAA determination (15–17). This immunoassay has high sensitivity, allows the measurement of residue levels of <0.5 ng/g, it can handle large sample numbers and requires smaller sample size and less clean-up than other techniques, but it has a disadvantage that a cross-reactivity can occur. In case of clenbuterol, conjugation with glucuronic acid or sulfate, or both, as its metabolic pathway of biotransformation, has been reported (18). Furthermore, clenbuterol exists as a mixture of stereoisomers and various biological preparations may not contain equal ratios of stereoisomers. Recent reports have suggested that using commercially available immunoassay kits to quantitate clenbuterol might lead to cross-reactions with these conjugated metabolites and stereoisomers, and that the kit should be carefully evaluated prior

to use (19). Because of the possible cross-reaction and to avoid false-positive results, a confirmatory technique such as GC-MS (20) or LC-MS (21) is required. However, a previous study showed that re-analysis of query-positive samples by the same ELISA reduced the incidence of false-positives to 0.4 % (15). In our work, validation of the ELISA showed good recovery ($R>80$ %), while intra-assay results showed acceptable variation for individual measurements for all samples to which 0.5, 2 or 5 ng/g clenbuterol were added ($RSD<10$ %), which is consistent with previous reports (22). Our results indicated that clenbuterol residues persisted in the liver (0.22 ng/g) of experimental animals for 21 days after cessation of prolonged administration of a growth-promoting dose. Thus, more than 14 days were required for clenbuterol concentration to fall below MRL of 0.5 ng/g. This is consistent with previous reports on ruminants (9,10) and broiler chickens (12). Furthermore, >80 % of clenbuterol depletion in pig liver occurred during the first 7 days after cessation of administration (Fig. 2), which is in agreement with the data reported for calves, broiler chickens and pigs, using various analytical methods for clenbuterol determination such as enzyme immunoassay (6,12) and gas-chromatography-mass spectrometry (9,13). The data provided by the manufacturer of the kit used in our study indicated a cross-reaction of BAAs including clenbuterol (cross-reaction: 100 %) and negligible cross-reaction (<0.01 %) with its natural analogs (adrenaline and noradrenaline). Early studies with ELISA as a screening method and a combination of HPLC/ELISA as a confirmation method in determination of residual clenbuterol in liver tissues of veal calves pointed to good accordance of the two methods (6), suggesting the absence of immunoactive metabolites in liver tissue that could interfere with the clenbuterol content determined by ELISA.

Conclusion

The results of our study indicated that prolonged administration of a growth-promoting dose of clenbuterol to female pigs led to residue accumulation in the liver as an edible tissue, which may induce a pharmacotoxicologic reaction in consumers. Comparison of the results of clenbuterol concentration in pig liver obtained by ELISA with an analytical confirmation method to avoid possible cross-reaction, as recommended in recent reports, and using eye tissue as a matrix of choice for the clenbuterol abuse control in the analytical procedure provide a challenge for our future studies.

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Određivanje klenbuterola u svinjskoj jetri nakon dužeg davanja doze koja pospješuje rast

Sažetak

U jetri krmača izmjerena je koncentracija klenbuterola, β_2 -adrenergičkog agonista, 0., 7., 14. i 21. dana nakon što im se prestala davati doza klenbuterola. Za određivanje klenbuterola primijenjena je imunoenzimska metoda (ELISA). Točnost je metode veća od 80 %, dok su vrijednosti relativne standardne devijacije (RSD) manje od 10 % za svako pojedinačno mjerenje uzorka kojemu je dodano 0,5, 2 ili 5 ng/g klenbuterola. Rezultati upućuju na to da se više od 80 % klenbuterola u jetri razgrađuje tijekom prvih 7 dana nakon prestanka davanja. Potrebno je više od 14 dana da bi koncentracija klenbuterola pala ispod maksimalno dopuštene razine od 0,5 ng/g. Maseni udjel klenbuterola u jetri (0,22 ng/g), izmjerena 21. dana nakon prestanka davanja, još je uvijek na razini koja se može odrediti, što pokazuje perzistentnost ostataka klenbuterola u jetri kao jestivom tkivu i može predstavljati rizik za zdravlje potrošača.