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Original Article

Ocular bulb as a matrix of selection in detection of clenbuterol: an effective monitoring in breeding turkey

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ABSTRACT

Clenbuterol is a β_2-agonist licensed in Europe solely as a muscle relaxant in pregnant cattle, usually during calf delivery, and for tocolysis and treatment of respiratory diseases in horses. Different use in animals is considered illegal because this growth promoter can endanger human health. The aim of this work was to monitor the presence of clenbuterol in a not official and inedible matrix (where there is strong evidence of its potential accumulation), as ocular bulb, collected in 2007-2009 from turkeys at public slaughterhouses in Central-Northern Italy. The 280 collected samples were analysed with a new and effective method of extraction and purification based on HPLC analysis with UV-DAD detection. The average extraction recoveries were 80.60 ± 1.57%. All the samples were below the quantification limit (0.010 µg/mL). At 95% confidence level the percentage of positive turkeys breeding-farms was below 14%. This allows extending positive evaluations on the safety of edible tissues of Italian turkey.

Keywords: Clenbuterol, Food Safety, HPLC, Ocular Bulb, Turkey

INTRODUCTION

β_2-receptor agonist adrenergic drugs as clenbuterol are currently used as bronchodilator for the treatment of asthma in humans, and as bronchodilator as well as tocolytic agent in veterinary medicine. The β_2-adrenergic agonist activity influences the mechanisms that regulate the division of the nourishing substrata, the consequence of this action is the increased protein synthesis and lipolysis and the following improving in feed conversion efficiency (Moloney and Beerman, 1996; Sanz Pèrez, 1996; Strydom et al., 2009). This ability of β_2-agonist has been named "repartitioning action". In livestock, this anabolic effect requires doses 5-10 times higher than therapeutics ones in bronchial diseases (Miller et al., 1988; Witkamp, 1996). In spite of these "positive" effects, the use of these substances in animal husbandry is potentially dangerous because their presence in the edible tissues of treated animals can adversely affect human consumers. For example, animal-derived foods containing clenbuterol have caused several outbreaks of food poisoning in Spain (Martinez-Navarro, 1990), France (Pulce et al., 1991) and Italy (Maistro et al., 1995; Sporano et al., 1998; Brambilla et al., 2000). Because of its possible harmful effects to humans, none of the European Member States has authorised the use of β_2-agonists for repartitioning purposes (Kuiper et al., 1998).

Moreover, in Italy, epidemiological data about the use of clenbuterol in turkey breedings is not available.

The aim of this work was to check and to monitor the presence of the drug in samples of ocular bulb collected during slaughtering at public structures from turkeys coming from different breeding placed in Northern and Central Regions of Italy, in the period 2007-2009. Another purpose is to test a new practical method to extract and purify the clenbuterol from the ocular bulb based on HPLC analysis with UV-DAD detection.

MATERIALS AND METHODS

Sampling

In the present study we have chosen the different breedings localised in the Middle-Northern Italy, joined from the same birdseed industry and from the same center of butchery: in this way we have a complete control of the whole cycle of production. Thereafter, we have selected in a random way 20 breedings. In the slaughterhouse we have randomly sampled 14 animals from each of the 20 breedings previously selected.

The samples, withdrawn from 280 turkeys, were collected between June 2007 and June 2009. In the designated slaughterhouses the head was withdrawn from each subject, refrigerated and transported to laboratory where the ocular bulbs were excised, frozen promptly and stored at -20 ± 1°C until analysis. At the moment of analysis the ocular bulbs were thawed and after incision, the fluid tissues of the eyes was withdraw by a syringe, and the retina and choroid layers were removed by scraping. The different parts of two eyes of single turkey were combined and homogenised.

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Analysis of clenbuterol

Clenbuterol standard was purchased from Sigma-Aldrich (Milan, Italy). Water, acetonitrile and tertiary butylmethil ether were HPLC-grade (Baker).

The extraction method is based on the method described by Meyer and Rinke (1991) appropriately modified. Aliquots (1 mL) were acidified by addition HCl 2.5 N (150 µL) and the lipids were removed with 4 mL of tertiary butylmethil ether (TBME). After gentle and continuous shaking (10 min) and the centrifugation at 1000 x *g* (5 min, at room temperature) the ether phase was eliminated. The whole aqueous phase was combined with 150 µl of NaOH 5 N, and the clenbuterol was extracted with 4 mL of TBME. This extraction was repeated other two times and the three organic phases were evaporated with UNIVAPO. The residue was dissolved in 500 µL of H_3PO_4 0.01 M and aliquots (50 µL) of this solution were analysed by HPLC.

High-Performance Liquid Chromatography

The HPLC apparatus (Beckman Coulter, Cassina de' Pecchi, Milano, Italy) consisted of a piston pump, an automatic autosampler, a variable wavelength ultraviolet detector and computer for data collection and analysis. The software Gold release 4.0 was used for instrument control and data acquisition (Beckman Coulter, Cassina de' Pecchi, Milano, Italy). Sample separation was conducted on a 4.6x250 mm column packed with 5 µm reversed-phase silica (Luna C18, Phenomenex, Castelmaggiore, Bologna, Italy). Clenbuterol was eluted with a mobile phase that consisted of acetonitrile and 10 mM phosphoric acid (20:80 vol/vol). The flow rate was 0.7 mL/min. UV detection was performed at a wavelength of 245 nm.

Fortifications of eyes were obtained adding 50 µL of dilute standards to aliquots of 1 mL of eye to obtain concentrations ranging between 0.010 and 10 µg/mL from blank animals.

All the laboratory activities were performed within the Quality Management System of the Department of Veterinary Public Health and Animal Pathology which has been certified ISO 9001-2000.

Statistical analysis

The validation of the analytical methods was performed by confirming the response linearity, assessed on the basis of R^2, coupled with a study of the reproducibility and repeatability. The recoveries were evaluated by comparison between peak areas for fortified eyes and standards. The standard deviation and the coefficient of variation (CV%) were calculated for each set of standards and fortified samples.

The statistical analysis, performed in order to calculate both the probability of detect at least one positive and the confidence intervals, was based on the binomial probability distribution (Armitage, 1971; Cannon and Roe, 1982). The binomial is the discrete distribution that describe the probability to have r positives in a sample of n specimens (with $0 \leq r \leq n$), given the percentage of positives in the population is equal to p (with $0 \leq p \leq 1$). The population size was considered to be infinite, according to the very large number of both turkey breeding farms and animals in Central-Northern Italy. The binomial probability distribution has the following formula:

$$Pr(r) = \binom{n}{r} p^r (1-p)^{n-r}$$

where $\binom{n}{r} = \frac{n!}{r!\,(n-r)!}$ is the binomial coefficient and Pr(r) is the probability to find r positives in the sample of size n from an infinite population.

According to this distribution, the probability of failure to detect one positive in a sample of n specimens, if the proportion of positives in the population is equal to p, has been calculated as follows:

$$Pr(0) = \binom{n}{0} p^0 (1-p)^n = (1-p)^n$$

The 95% confidence limits for the proportion of positives (p) are two values of p (the inferior and the upper limit of the confidence interval) chosen in order to have 5% probability only that the proportion of positives in the population is out of the confidence interval itself. It was exactly calculated according to the binomial probability distribution mentioned above; the normal approximation has not been used, although it represents a considerable simplification, because it is suitable only for large sample size (Armitage, 1971). Calculations were performed using Microsoft® Office Excell 2003.

RESULTS

Under our experimental conditions, clenbuterol showed a retention time of 10.5 min. Chromatograms obtained after extraction did not contain substance that would interfere at retention time of clenbuterol (Fig. 1).

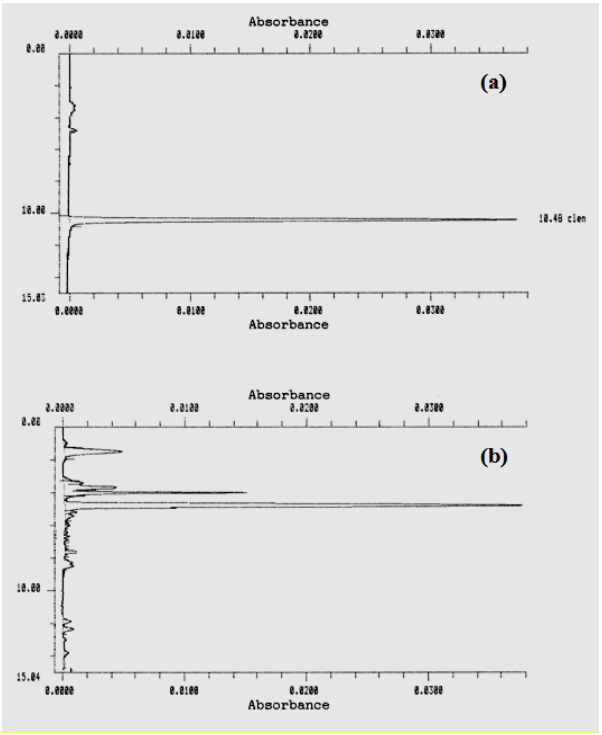

Figure 1 - Representative chromatograms of Clenbuterol standard (**a**) and drug-free eye (**b**).

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The correlation coefficient in linearity evaluations generally exceeded 0.99. The analysis of precision and accuracy evaluated on different days allowed determination of mean coefficients of variation equivalent to 3.12%, and the average extraction recoveries were 80.60 ± 1.57%. The limits of detection (LOD) and quantification (LOQ) were 0.005 and 0.010 µg/mL, respectively.

This method of extraction and purification is characterised by facility and rapidity of execution, and it is relatively not much expensive.

All the 280 samples analysed were below LOQ. The calculated probability of failure to detect any positives examining a sample size (n) of 14 animals if the real population prevalence is 20% (p=0.2), was 0.0488 (4.88%) while, if the real prevalence was at least 50% (p=0.5), the probability to failure to detect any positives approximated zero. Regarding the percentage of positive breeding farms, being all the examined farms negatives and the sample size equal to 20 (number of examined farms), the calculated 95% confidence interval for the cited percentage was 0 - 14%.

DISCUSSION

The clenbuterol is licensed and marked in Europe solely for tocolysis in parturient cows and for tocolysis and treatment of respiratory ailments in equidae (EMEA, 2000).

Despite its limitations in therapeutic use, there is continued concern in European Community over the illegal use of the clenbuterol. For residues surveillance of β_2-agonist, it is essential to identify the better matrices for detection of residue, with respect to potential accumulation of the drug, to the facility of sampling, and of extractability of residues (Kuiper et al., 1998).

Clenbuterol was well adsorbed after oral administration to laboratory animal, humans and the target species. In most species peak blood concentration were achieved 2 to 3 hours after oral dosing (EMEA, 2000). The substance was widely distributed to the tissue. In all species, excretion was predominantly via the urine as unmetabolised clenbuterol (EMEA, 2000).

Many studies have demonstrated and confirmed that clenbuterol accumulates in melanine-containing tissues, such as hair and retina (Elliott et al., 1993c; Meyer and Rinke, 1991; Sauer and Anderson, 1994; Panoyan et al., 1995; Polettini et al., 1995; Sauer et al., 1995), providing a highly effective matrix for detecting administration of growth-promoting doses of clenbuterol, even after prolonged drug withdrawal (Elliott et al., 1993a). For inspection in the slaughterhouse, the hair and the ocular bulb represent the appropriate target organs, since the clenbuterol residues persist longer in these tissues after anabolic treatment (Meyer and Rinke, 1991; Elliott et al., 1993b; Malucelli et al., 1994; Sauer et al., 1995; Smith and Paulson, 1997; Gowik et al., 2000). Concerning clenbuterol, experiments have shown that the concentrations in the retina exceed those in the liver by at least two orders of magnitude and that the elimination half-life is approximately five times higher than in liver (Gowik et al., 2000).

The European ban on these drugs in stockfarming has determined a considerable illegal use of β_2-agonist drugs, clenbuterol in particular, in livestock (EU, 1996a; EU, 2003).

Considering the important repercussions on the public health, each European Member State is obliged to monitor animals and meat for residue of legally and illegally used veterinary drugs and to present a National Residue Monitoring Plan (EU, 1996b). In relation to Italian Animal National Program (PNR, 2011), analyses for clenbuterol determination are requested in liver of turkey and poultry.

Despite the ocular bulb is not an official matrix for turkey, we have adopted the eye for monitoring because it accumulates relatively more clenbuterol than other tissues; consequently, the eye seems to be a superior indicator of the absence of residues in all tissues. In fact, also in turkeys, the eyes may be a useful tissue to detect illegal drug use even after long period of withdrawal (Gowik et al., 2000). Furthermore, the eye is not an edible matrix which allowed easy sampling, handling and extractability of compound.

Our method of extraction and purification resulted to be characterised by facility and rapidity of execution, and it is relatively not much expensive.

It is for these features that this matrix may be useful for the detection of residues of clenbuterol in slaughterhouse.

All samples analysed were below LOQ. Taking into account the drugs widespread administration techniques in avian breeding farms which makes it unlikely the use of clenbuterol for some individuals only, the prevalence of treated animals in a specified farm is probably about 100% and should not be less than 20%. The probability of failure to detect any positives is less than 0.05 if the real prevalence is at least 20%. If the real prevalence is at least 50%, as it is likely the case, the probability to failure to detect any positives approximates zero. Subsequently, the examined breeding farms have to be considered fully negative. The farm positivity confidence interval, allows us also to state that the percentage of breeding farms possibly using clenbuterol in Central-Northern Italy was at most 14%.

The negativity found in all samples analyzed in the places of greatest accumulation of the molecule allows to extend positive evaluations on the safety of turkey edible tissues in Italy.

REFERENCES

Armitage, P, 1971. Statistical Methods in Medical Research. Blackwell Scientific Publications, Oxford and Edinburg.

Brambilla, G, Cenci T,, Franconi F,, Galarini R,, Macrì A,, Rondoni F,, Strozzi M, and Loizzo, A, 2000. Clinical and pharmacological profile in a clenbuterol epidemic poisoning of contaminated beef meat in Italy. *Toxicol. Lett.*, 114: 47-53.

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Journal homepage: http://jwpr.science-line.com/

Cannon, RM and, Roe, RT, 1982. Livestock Disease Surveys: a field manual for Veterinarians. Australian Government Publishing Service, Canberra.

Elliott, CT, Crooks, SRH,, McEvoy, JGD, McCaughey, WJ,, Hewitt, SA, Patterson D and Kilpatrick D, 1993a. Observations on the effect of long term withdrawal on carcass composition and residue concentrations in clenbuterol-medicated cattle. *Vet. Res. Commun*., 17: 459-468.

Elliott, CT, McCaughey, WJ and Short, HD, 1993b. Residue of β-agonist clenbuterol in tissues of medicated farm animals. *Food Addit. Contam.*, 10: 231-244.

Elliott, CT, McEvoy JD, McCaughey, WJ, Crooks, SR, and Hewitt, SA, 1993c. Improved detection of the β-agonist clenbuterol by analysis of retina extracts. *Vet. Rec.*, 132: 301-302.

EMEA, 2000. Clenbuterol Summary Report (2) EMEA/MRL/723/99-FINAL. European Agency for the Evaluation of Medicinal Products, Committee for Veterinary Medical Products. http://www.ema.europa.eu.

EU 2003. Amending Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists. Directive 2003/74/EC of the European Parliament and of the Council of 22 September 2003. Off. J. Eur. Union, L262, Pages 0017-0021.

EU 1996a. Concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of ß-agonists, and repealing Directives 81/602/EEC, 88/146/EEC and 88/299/EEC. Council Directive 96/22/EC of 29 April 1996. Off. J. Eur. Union L 125, 23/05/1996 Pages 0003-0009.

EU 1996b. On measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. Council Directive 96/23/EC of 29 April 1996. Off. J. Eur. Union, L 125, 23/5/1996, pages 0010-0032.

Gowik, P, Julicher, B, Ladwig, M, and Behrendt, D, 2000. Measurement of β-agonist residues in retinal tissue of food producing animals. *Analyst*, 125: 1103-1107.

Kuiper, HA, Noordam, MY, Van Dooren-Flipsen, MMH, Schilt, R, and Roos, AH, 1998. Illegal use of beta-adrenergic agonists: European Community. *J. Anim. Sci.*, 76: 195-207.

Maistro, S, Chiesa E,, Angeletti R, and Brambilla G, 1995. Beta blockers to prevent clenbuterol poisoning. *Lancet*, 346: 180.

Malucelli, A, Ellendorff F and Meyer, HHD, 1994. Tissue distribution and residues of clenbuterol, salbutamol, and terbutaline in tissues of treated broiler chickens. *J. Anim. Sci.*, 72: 1555-1560.

Martinez-Navarro, JF, 1990. Food poisoning related to consumption of illicit β agonist in liver. *Lancet*, 336: 1311.

Meyer, H HD and Rinke, LM, 1991. The pharmacokinetics and residues of clenbuterol in veal calves. *J. Anim. Sci.*, 69: 4538-4544.

Miller, M F, Garcia, DK, Coleman, ME, Ekeren, PA, Lunt, DK, Wagner, KA, Procknor, M, Welsh, TH Jr and Smith, SB, 1988. Adipose tissue, Longissimus muscle and anterior pituitary growth and function in clenbuterol-fed heifers. *J. Anim. Sci.*, 66: 12-20.

Moloney AP and Beermann, DH, 1996. Mechanisms by which β adrenergic agonist alter growth and body composition in ruminant. In: Enne G, Kuiper HA and Valentini A. Residues of Veterinary Drugs and Mycotoxins in Animal Product. New methods for Risk Assessment and Residue Control. Proceedings of the first electronic mail conference. ISBN 90-74134-27-0 pp. 124-136. Wageningen Pers, The Netherlands.

Panoyan, A, Delahaut, P, Ayotte, C, Lamothe, P, Huy, O and Adam, A, 1995. Immunodetection of clenbuterol in the hair of calves. *J. Agric. Food Chem.*, 43: 2716-2718.

PNR 2011. Piano Nazionale per la ricerca dei Residui negli animali e nei prodotti di origine animale Anno 2011. Ministero del Lavoro, della Salute e delle Politiche Sociali. pp. 100-101.

Polettini, A, Segura, J, Gonzalez, G, De La Torre, X and Montagna, M, 1995. Clenbuterol and beta-adrenergic drugs detected in hair of treated animals by ELISA. *Clin. Chem.*, 41: 945-946.

Pulce, C, Lamaison, D, Keck, G, Bostvironnois, C, Nicola, J and Descotes, J, 1991. Collective and human food poisonings by clenbuterol residues in veal liver. *Vet. Hum. Toxicol.*, 33: 480-481.

Sanz Pèrez, B, 1996. Problemi di salute pubblica provocati dall'uso del clenbuterolo. *Ingegneria Alimentare.*, 3: 9-16.

Sauer MJ, Pickett, RJ H, Limer, S and Dixon, SN, 1995. Distribution and elimination of clenbuterol in tissues and fluids following prolonged oral administration at a growth promoting dose. *J. Vet. Pharmacol. Ther.*, 18: 81-86.

Sauer, MJ and Anderson, SP (1994). In vitro and in vivo studies of drug residue accumulation in pigmented tissues. *Analyst.*, 119: 2553-2556.

Smith, DJ,and Paulson, GD, 1997. Distribution, elimination, and residues of [^{14}C] clenbuterol HCl in Holstein calves. *J. Anim. Sci.*, 75: 454-461.

Sporano, V, Grasso, L, Esposito, M, Oliviero, G, Brambilla, G and Loizzo, A, 1998. Clenbuterol residues in non-liver containing meat as a cause of collective food poisoning. *Vet. Hum. Toxicol.*, 40: 141-143.

Strydom, PE, Frylinck, L, Montgomery, JL and Smith, MF, 2009. The comparison of three β-agonist for growth performance, carcass characteristics and meat quality of feedlot cattle. *Meat Sci.*, 81: 557-564.

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Journal homepage: http://jwpr.science-line.com/

Witkamp, RF, 1996. The use of β_2 agonists as growth promoters in food-species. Toxicological aspects and possible health risks to the consumer. In: Scientific conference on growth promotion in meat production. Brussels 29/11 to 1/12 1996. Page 297-317.

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