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Analysis of clenbuterol residues in pig liver using liquid chromatography electrospray ionization tandem mass spectrometry

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Abstract

The liquid chromatography-electrospray ionization tandem mass spectrometry method was developed for determination of clenbuterol residues in liver tissue as a regulatory matrix for the control of clenbuterol abuse as an anabolic. To investigate the level of clenbuterol residues during the withdrawal period, male food-producing pigs were exposed to subchronic repeat oral administration of a clenbuterol growth-promoting dose for 28 days. The analytical procedure showed acceptable validation results for all liver spiked samples analyzed and proved to be useful as a quantification and confirmation method in supporting regulatory enforcement programs of clenbuterol misuse monitoring. The highest level of clenbuterol in the liver of treated animals was recorded on day 0 of treatment cessation (21.58±14.29 ng/g), followed by 6.59±3.11 ng/g on day 3, 0.83±0.27 ng/g on day 7, and 0.44±0.08 ng/g on day 14 of withdrawal. At the end of the study period (day 35), the concentration of clenbuterol was below the method limit of detection (<0.1 ng/g).

Keywords: clenbuterol residues; liquid chromatography-electrospray ionization tandem mass spectrometry; liver; pigs.

Introduction

The use of β_2-adrenergic agonist clenbuterol (4-amino-α-[t-butylaminomethyl]-3,5-dichlorobenzyl alcohol) as a growth promoter for fattening purposes has been banned in the European Union since 1988 (European Council 2003). Clenbuterol as a pharmacologically active substance influences body composition of many animal species by reducing fat deposition and increasing carcass protein. Because of these effects, clenbuterol has been illegally used as an anabolic in food-producing animals (Warriss et al. 1990, Kuiper et al. 1998, Meyer 2001). By contrast, illicit use of clenbuterol leads to the accumulation of its residues in edible tissues, associated with several instances of intoxication in humans (Martinez-Navarro 1990, Pulce et al. 1991, Brambilla et al. 1997, Garay et al. 1997, Ramos et al. 2003, Woodward 2005). Persistence of clenbuterol residues in biological material during and after exposure to therapeutic and anabolic doses of clenbuterol, and the time needed for clenbuterol residues to decrease below the maximum residue limit (MRL) of 0.5 ng/g for edible tissues (e.g., liver) have been tested in different matrices of various animal species (Smith 2000). The high potency of growth promoting effects and rapid metabolism of clenbuterol require highly sensitive and validated analytical methods for quantification and confirmation of its residues in biological material to detect its abuse in food-producing animals. Whereas screening of clenbuterol residues can be done using rapid enzyme-linked immunosorbent assay (ELISA) (Elliott et al. 1993, Reig and Toldrá 2008), the quantification and confirmation require unambiguous structural determination involving the use of mass spectrometry (Doerge et al. 1996, Williams et al. 2004, Fesser et al. 2005). Control of group A substances (clenbuterol) is a high priority because of public health concern; relatively large numbers of samples have to be analyzed and more stringent criteria (according to Decision 657/2002/EC) (2002/657/EC 2002) used in view of the serious public health implications of positive results (Stolker and Brinkman 2005). Consequently, liquid chromatography-mass spectrometry (LC-MS) is often the method of choice in the analysis of trace levels of polar contaminants. In the case of clenbuterol, quantification and confirmation by LC-MS in different matrices with detection limits of 0.01–0.5 μg/kg have been reported (Guy et al. 1999). In our study, we used the liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) method for confirmation and quantification of residual clenbuterol in pig liver tissue as a regulatory matrix for control purposes and interpreted results according to the established MRL of 0.5 ng/g for clenbuterol.

Materials and methods

Reagents

Analytically pure chemicals were used in the study. Organic solvents were of HPLC grade (Merck, Darmstadt, Germany). Demineralized water was obtained by use of the MilliQ system (Millipore, Milford, MA, USA). For construction of calibration curve and blank liver sample fortifications, clenbuterol

standard C5423 (Sigma-Aldrich GmbH, Steinheim, Germany) was purchased.

Apparatus

The LC-ESI/MS/MS equipment had the following performance characteristics: Agilent Technologies 1100 series HPLC instrument (Waldbroonn, Germany) and Applied Biosystems (Darmstadt, Germany) Qtrap mass spectrometer with a TurboIon spray (electrospray ionization) interface. The whole system was controlled by the Applied Biosystems Analyst (v.1.3.) software.

Animal treatment and sampling procedure

The experimental protocol was designed according to the Act on Animal Welfare, as stated in the Official Gazette of the Republic of Croatia (2006). The study included male pigs of known bred, aged 90 days, body mass 50 kg, farm-bred and kept under the same hygienic conditions. Animals (n=15) were randomly allocated into five groups of three animals each and treated with 20 µg clenbuterol/kg body mass orally daily for 28 days. One group of pigs (n=3) was left completely untreated (control). On days 0, 3, 7, 14, and 35 upon withdrawal of clenbuterol administration, the pigs were sacrificed and the liver was collected and stored at -20°C until analysis.

Analytical procedures

Method validation A 10-point matrix-based calibration graph with clenbuterol concentrations equivalent to 0.0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, and 50 ng clenbuterol/g liver was established. The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the value of the mean of multiple injections (n=10) of blank liver samples plus three- and 10-fold standard deviation, respectively. The specificity of the method was studied by analyzing blank pig liver samples to probe for interfering peaks in the selected ion chromatograms at the expected retention time of clenbuterol. The recovery of the assays was determined by the analysis of blank liver tissue fortified with clenbuterol at levels of 0.5, 1.0, 5.0, and 10.0 ng/g (n=6). The procedure was repeated twice within the same test with the same conditions for determination of repeatability. Statistical data analysis was performed by use of STATISTICA ver.6.1 software (StatSoft Inc., Tulsa, OK, USA).

Sample preparation A 2.5±0.1 g portion of liver was homogenized in 10 ml of 25 mM sodium citrate buffer (sulfatase >10,000 units/g solid and β-glucuronidase >300 unit/mg) and incubated at 37°C for 1 h. The resulting suspension was centrifuged (4000 *g*) for 5 min and the supernatant was carefully decanted and purified using solid phase extraction (SPE). The columns were activated with 3 ml 5% NH_4OH in methanol and 3 ml methanol, and balanced with 3 ml 10 mM NH_4OAc buffer (pH=5). The supernatant was added and the columns were washed with 0.6 ml 10 mM NH_4OAc buffer (pH=5), 0.6 ml 1 M formic acid, dried for 30 s and finally washed with 0.6 ml methanol. The analytes were eluted with the addition of 2 ml 5% NH_4OH in methanol. The eluate was evaporated to dryness by use of rotation vacuum evaporator and the residues were dissolved in 0.5 ml of 5% methanol solution in 0.1% formic acid.

Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) analysis HPLC parameters: Analytical column Xterra MS C18, 2.1 mm× 50 mm, 3.5-µm particles preceded by a 10-mm guard column of the same type and diameter; chromatographic run: flow 0.35 ml/min; eluent A: 2 mM ammonium acetate in water; eluent B: 2 mM ammonium acetate in acetonitrile; a gradient elution program was employed: 0 min 10% B, 7 min 90% B, 9 min 90% B and 15 min 10% B; injection volume: 80 µl; column temperature: 25°C.

Mass spectrometer parameters: TurboIon spray ion source, positive mode, ion spray voltage: 5500 V with a capillary temperature of 300°C; scan type: MRM.

For confirmatory analysis, one precursor (*m/z* 277) and two product diagnostic ions (*m/z* 259 and 203) were measured under the above conditions, at a collision energy setting of 15 eV in the collision cell. Quantification of clenbuterol concentration in liver tissue was performed with the most intensive transition (*m/z* 277>203) vs. the external standard monitored and extrapolation using 10-point (0.1–50 ng/g) calibration curve established by spiking blank liver samples with clenbuterol standard. All pig liver samples of the treated and control groups were analyzed in triplicate and clenbuterol concentrations were calculated by taking average recoveries into account.

Results and discussion

Validation results

The calculated LOD and LOQ were 0.1 ng/g and 0.15 ng/g, respectively. Specificity of the method was supported by the absence of any interfering signal at the expected retention times in the extracted ion chromatograms of the product ions of clenbuterol. The values obtained for the method recovery and repeatability with relative standard deviation (% RSD) are presented in Table 1. Method validation resulted in mean recoveries ranging from 86.6% to 92.1%, and repeatability ranging from 82.1% to 86.1% with RSD of 6.1%–8.7% and 7.7%–11.4%, respectively. Chromatograms for the clenbuterol confirmatory ions (*m/z* ions 203 and 259) for real liver sample (treated pig, on day 7 of withdrawal) and clenbuterol standard (2.5 ng/g) are shown in Figure 1. The ratio (mean±SD; n=6) of the peak areas of 277>259 transition to the major transition (277>203) for real liver samples and clenbuterol standard was 0.33±0.01 and 0.30±0.01, with RSD of 8.0% and 6.5%, respectively. The values of ratios of the peak areas for MRM transition between real liver samples and clenbuterol standard (Figure 1) were within 10% of the absolute value produced by an authentic standard, being appropriate for regulatory confirmation (EU criteria) and consistent with a previous

Table 1 Validation results of method recovery and repeatability for pig liver spiked with clenbuterol standard at concentration level of 0.5, 1, 5, and 10 ng/g.

Validation parameter	Spiked clenbuterol concentration (ng/g)	Determined clenbuterol concentration (ng/g)	Mean recovery R (%)	Relative standard deviation RSD (%)
Recovery (n=6[a])	0.5	0.433	86.6	6.1
	1	0.921	92.1	7.3
	5	4.385	87.7	8.7
	10	8.95	89.5	7.9
Repeatability (n=18[a])	0.5	0.411	82.1	7.7
	1	0.861	86.1	10.5
	5	4.245	84.9	11.4
	10	8.42	84.2	10.2

[a]Number of fortified replicates per each concentration level.

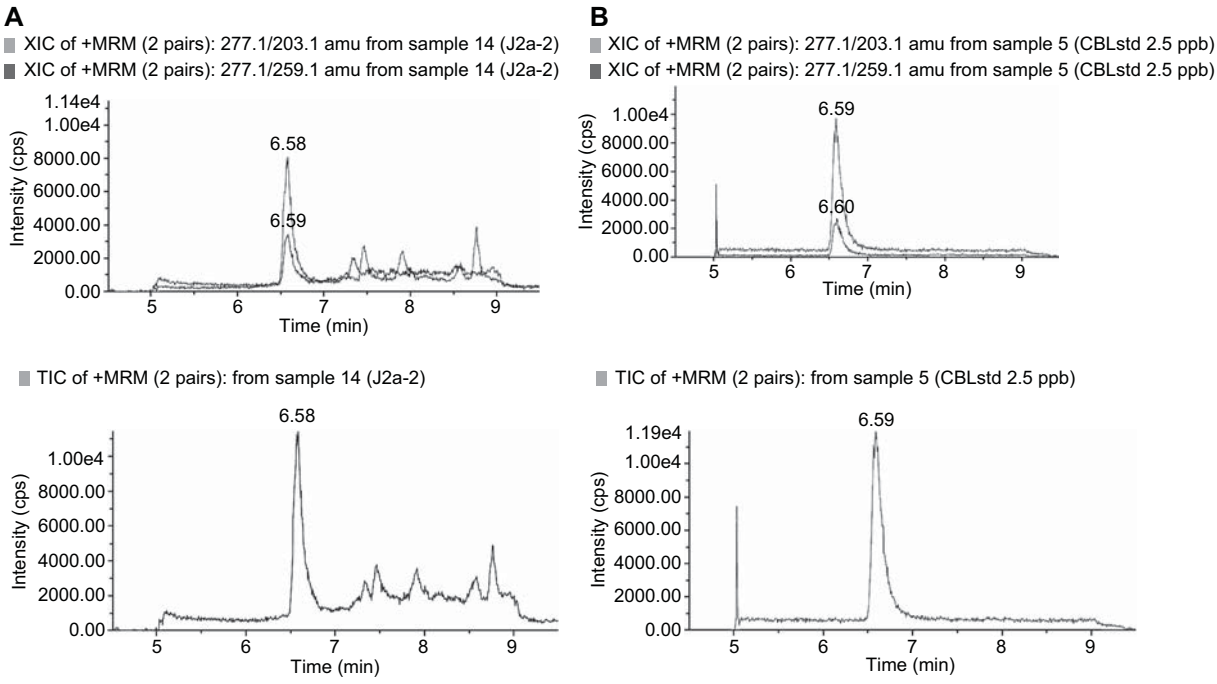

Figure 1 The confirmatory chromatographic signals of clenbuterol obtained from LC-ESI/MS/MS analysis of the liver from treated pig (A) and clenbuterol standard (B), shown as separate overlain MRM functions (first row) and sum signal (second row).

report (Doerge et al. 1996). The analytical procedures using LC-ESI/MS/MS as a quantification and confirmation method showed acceptable validation results for all liver spiked samples analyzed.

Concentration of clenbuterol residues in liver according to days of withdrawal period

The determined concentration of accumulated clenbuterol residues in liver tissue of treated male pigs on days 0, 3, 7, 14, and 35 of withdrawal is presented in Figure 2. The concentration gradually decreased from 21.58±14.29 ng/g on day 0 to 0.44±0.08 ng/g on day 14 of withdrawal period, which is in agreement with data reported for ruminants (Appelgren et al. 1996) and mature female pigs (Gojmerac et al. 2002a). The mean clenbuterol concentration measured on day 0 of treatment was approximately 43-fold MRL for liver of 0.5 ng/g; on day 14 of treatment cessation it was around the MRL for liver, whereas on day 35 of withdrawal it was below 0.10 ng/g, i.e., below the method detection limit.

These results indicated more than 90% of clenbuterol residues in the liver to undergo degradation in the first 7 days of treatment withdrawal, which is consistent with literature reports from studies in swine (Smith 2000), cattle (Elliott et al. 1993), and broiler chicken (Malucelli et al. 1994). In our previous study, pigs weighing 80–100 kg were administered 10 µg clenbuterol/kg body weight twice daily for 25 days. On day 0 of treatment withdrawal, clenbuterol concentration measured by the screening method (ELISA) ranged from 32.42 to 58.30 ng/g (Gojmerac et al. 2002b). In the

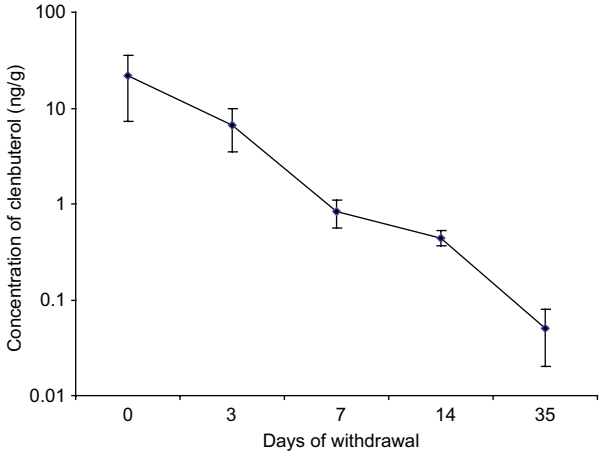

Figure 2 Mean (±SD) clenbuterol concentration (ng/g) in pig[a] liver determined by validated LC-ESI/MS/MS method on days during the withdrawal period.
[a]Male pigs (n=15) known bred aged 90 days, body mass 50 kg.

present study, the clenbuterol concentrations recorded on 0 day of treatment withdrawal were lower; this concentration variation could probably be explained by difference in the experimental animal body weight and different mode of treatment. The results obtained in the present study are consistent with clenbuterol concentrations recorded in calf liver, which was found to decrease from 46 ng/g on day 0 to 0.5 ng/g on day 14 of treatment cessation (Meyer and Rinke 1991). This study found the liver to be more suitable as a matrix for clenbuterol detection than urine and plasma as control matrices (Pleadin et al. 2009), thus liver tissue being considered appropriate for clenbuterol abuse monitoring (Sauer et al. 1995). The sensitivity and specificity of the chosen sample preparation and LC-ESI/MS/MS analysis conditions allow for the unambiguous identification and quantification of the target analyte in all liver samples collected from experimental animals.

Conclusion

A quantitative and confirmatory LC-ESI/MS/MS method was developed to investigate the level of clenbuterol residues in the liver of treated pigs and was found suitable for use in the regulatory enforcement programs of clenbuterol abuse monitoring. The validation results demonstrated the efficiency of the sample preparation method and subsequent mass spectrometric determination of clenbuterol in the liver. Using this method, clenbuterol residues in the pig liver could be detected and quantified after more than 2 weeks of anabolic clenbuterol dosage withdrawal.

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References

2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official J.* **2002**, *L221*, 8.

Act on Animal Welfare No. 135. Official Gazette of the Republic of Croatia, **2006**.

Appelgren, L. E.; Bondesson, U.; Fredriksson, E.; Larsson, C. I.; Jansson, D. S. Analysis of hair samples for clenbuterol in calves. *Fleischwirtschaft* **1996**, *76*, 314–316.

Brambilla, G.; Loizzo, A.; Fontana, L.; Strozzi, M.; Guarino, A.; Soprano, V. Food poisoning following consumption of clenbuterol-treated veal in Italy. *J. Am. Med. Ass*. **1997**, *278*, 635–640.

Doerge, D. R.; Churchwell, M. I.; Holder, C. L.; Rowe, L.; Bajic, S. Detection and confirmation of β-agonists in bovine retina using LC/APCI-MS. *Anal. Chem*. **1996**, *68*, 1918–1923.

Elliott, C. T.; McEvoy, J. G. D.; McCaughey, W. J.; Shortt, H. D.; Crooks, S. R. H. Effective laboratory monitoring for the abuse of the beta-agonist clenbuterol in cattle. *Analyst* **1993**, *118*, 447–448.

European Council. Directive 2003/74/EC of the European Parliament and of the Council of 22 September 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. *Off. J. Eur. Communities* **2003**, *L262*, 17.

Fesser, A. C. E.; Dickson, L. C.; Macneil, J. D.; Petterson, J. R.; Lee, S.; Gedir, R. Determination of β-agonists in liver and retina by liquid chromatography-tandem mass spectrometry. *J. AOAC Int.* **2005**, *88*, 61–69.

Garay, J. B.; Jimenez, J. E. H.; Jimenez, M. L.; Sebastian, M. V.; Matesanz, J. P.; Moreno, P. M.; Galiana, J. R. Clenbuterol poisoning – clinical manifestations and analytical findings in an epidemic outbreak in Mostoles, Madrid. *Rev. Clin. Esp.* **1997**, *197*, 92–95.

Gojmerac, T.; Mandić, B.; Pleadin, J.; Mitak, M. Determination of clenbuterol in pig liver following prolonged administration of a growth-promoting dose. *Food Technol. Biotechnol.* **2002a**, *40*, 343–346.

Gojmerac, T.; Pleadin, J.; Žurić, M.; Lojkić, M.; Ćurić, S. Effects of repeated growth-promoting doses of clenbuterol on the hepatic function of female pigs. *Vet. Hum. Toxicol.* **2002b**, *44*, 269–271.

Guy, P. A.; Savoy, M. -C.; Stadler, R. H. Quantitative analysis of clenbuterol in meat products using liquid chromatography-electrospray ionisation tandem mass spectrometry. *J. Chromatogr. B* **1999**, *736*, 209–219.

Kuiper, H. A.; Noordam, M. Y.; Dooren-Flipsen, M. M. H.; Schilt, R.; Roos, A. H. Illegal use of β-adrenergic agonists: European Community. *J. Anim. Sci.* **1998**, *76*, 195–207.

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

Martinez-Navarro, J. F. Food poisoning related to consumption of illicit beta-agonist in liver. *Lancet* **1990**, *336*, 1311.

Meyer, H. H. D. Biochemistry and physiology of anabolic hormones used for improvement of meat production. *Apmis* **2001**, *109*, 1–8.

Meyer, H. H. D.; Rinke, L. M. The pharmacokinetics and residues of clenbuterol in veal calves. *J. Anim. Sci.* **1991**, *69*, 4538–4544.

Pleadin, J.; Gojmerac, T.; Bratoš, I.; Lipej, Z.; Novosel, D.; Vulić, A. Clenbuterol residues in plasma and urine samples of food-producing pigs during and after subchronic exposure to a

growth-promoting dose. *Food Technol. Biotechnol.* **2009**, *47*, 67–74.

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

Ramos, F.; Cristino, A.; Carrola, P.; Eloy, T.; Manuel Silva, J.; Castilho, A. C.; Silveira, M. I. N. Clenbuterol food poisoning diagnosis by gas chromatography-mass spectrometric serum analysis. *Anal. Chim. Acta* **2003**, *483*, 207–213.

Reig, M.; Toldrá, F. Veterinary drug residues in meat: concerns and rapid methods for detection. *Meat Sci.* **2008**, *78*, 60–67.

Sauer, M. J.; Pickett, R. J. H.; Limer, S.; Dixon, S. N. Distribution and elimination of clenbuterol in tissues and fluids of calves following prolonged oral administration at a growth-promoting dose. *J. Vet. Pharmacol. Therap.* **1995**, *18*, 81–86.

Smith, D. J. Total radioactive residues and clenbuterol residues in swine after dietary administration of [14C]clenbuterol for seven days and preslaughter withdrawal periods of zero, three, or seven days. *J. Anim. Sci.* **2000**, *78*, 2903–2912.

Stolker, A. A. M.; Brinkman, U. A. Th. Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals – a review. *J. Chromatogr. A* **2005**, *1067*, 15–53.

Warriss, P. D.; Kestin, S. C.; Rolph, T. P.; Brown, S. N. The effects of the beta-adrenergic agonist salbutamol on meat quality in pigs. *J. Anim. Sci.* **1990**, *68*, 128–136.

Williams, L. D.; Churchwell, I.; Doerge, D. R. Multiresidue confirmation of β-agonists in bovine retina and liver using LC-ES/MS/MS. *J. Chromatogr. B* **2004**, *813*, 35–45.

Woodward, K. N. Veterinary pharmacovigilance. Part 2. Veterinary pharmacovigilance in practice – the operation of a spontaneous reporting scheme in a European Union country – the UK, and schemes in other countries. *J. Vet. Pharmacol. Therap.* **2005**, *28*, 149–170.