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SCREENING AND CONFIRMATORY DETERMINATION OF CLENBUTEROL RESIDUES IN BOVINE MEAT MARKETED IN THE NORTHWEST OF MEXICO

MONITOREO Y CONFIRMACIÓN DE LA PRESENCIA DE RESIDUOS DE CLENBUTEROL EN CARNE DE BOVINO COMERCIALIZADA EN EL NOROESTE DE MÉXICO

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Abstract

It is essential from the public health point of view, that the Mexican regulatory agencies implement a monitoring surveillance program that not only uses preliminary screening methods for the determination of clenbuterol, but also uses confirmatory methods such as MS-MS techniques. Thus, the aim of this work was to monitor the presence of clenbuterol residues in bovine meat muscle collected from the local market. From 50 bovine meat samples analyzed by the ELISA test, 6 (12 %) presented clenbuterol residues from 3.06 to 6.12 µg/kg, 13 (26 %) from 0.5 to 1.83 µg/kg, 24 (48 %) from 0.1 to 0.5 µg/kg and only 7 (14 %) were below the limit of detection (LOD) of 0.1 µg/kg. The six samples with the highest clenbuterol residues were subjected to GC-MS-MS for confirmation. The results showed that clenbuterol was illegally used as a bovine growth promoter.

Resumen

El objetivo del presente trabajo fue determinar y confirmar la presencia de residuos de clenbuterol en carne de bovino comercializada en el noroeste de México. De un total de 50 muestras de carne de bovino obtenidas del mercado y analizadas por inmunoenayo específico ligado a enzima (ELISA), 6 muestras (12 %) presentaron residuos de clenbuterol en un rango de 3.06 a 6.12 µg/kg, 13 muestras (26 %) de 0.5 a 1.83 µg/kg, 24 muestras (48 %) de 0.1 a 0.5 µg/kg y sólo 7 muestras (14 %) se encontraron por debajo del límite de detección (LOD) de 0.1 µg/kg. Las seis muestras con los altos residuos de clenbuterol fueron sujetas a confirmación por medio de cromatografía de gases acoplada a un detector selectivo de masas-masas (GC-MS-MS). Los resultados encontrados confirmaron la presencia de clenbuterol, el cual fue ilegalmente utilizado como promotor de crecimiento de bovinos.

Keywords: Clenbuterol residues, bovine meat
Palabras clave: Residuos de clenbuterol, carne de bovino

INTRODUCTION

Clenbuterol is a synthetic drug belonging to the class of β₂-adrenergic agonists which is widely used in human and veterinary medicine as a bronchodilator, cardiotonic and tocolytic agent; however when administered in amounts above the level recommended as the therapeutic dose, this drug acts as a repartitioning agent and function as a growth promoter (Amendola et al., 2002; Ricks et al., 1984). However, clenbuterol may be used illegally as a growth promoter in meat-producing livestock. The use of clenbuterol as a growth promoter is prohibited in Mexico, as well as in other countries, since its residues may lead to a health risk for consumers.

The use of β-agonists for growth promoting purposes in farm animals is not permitted in the European Union (EU), the United States of America (USA) and most other countries. The maximum residue limits (MRL) set by the EU are 0.1 µg/kg for muscle and 0.5 µg/kg liver and the MRL recommended by the WHO and the Codex Alimentarius are 0.2 µg/kg for muscle and 0.6 µg/kg for liver (Codex Alimentarius, 1996). Nevertheless, several cases of food poisoning between 1989 and 1992 were reported in Spain and France when liver containing high levels of clenbuterol were consumed (Martínez-Navarro, 1990; Pulce et al., 1991). Additionally, in 2005, four cases of acute food poisoning, caused by the ingestion of lamb...
and bovine meat containing residues of clenbuterol were reported in Portugal (Barbosa et al., 2005).

In 2001, allegations about the illegal use of clenbuterol among Mexican meat producers created a state of alert of the regulatory agencies (Peña and Arias, 2001). Indeed, 132 cases of food poisoning with clenbuterol were associated to the consumption of bovine liver (García-López, 2002). Thus, the Mexican government issued a regulation in 2002 that included the technical specifications for a control program in the use of β-agonists in meat-producing livestock (SAGARPA, 2002). Additionally, this regulation stated that the official analytical methods for clenbuterol detection were ELISA, gas chromatography and HPLC (SAGARPA, 2002). However, these methods do not provide structural information of the analyte, thus are only useful for screening purposes and results should be confirmed by mass spectrometry (Cai and Henion, 1997; Kuiper et al., 1998). Moreover, this Mexican regulation is not in accordance with international regulations such as those of the EU or the USA, since it allows the use of some β₂-adrenergic agonists (SAGARPA, 2002). The compounds that were authorized as β₂-adrenergic agonists in the Mexican regulation were zilpaterol and ractopamine (Garcia-López, 2002).

Although immunoassay techniques are very sensitive, the potential lack of specificity is a drawback that may result in false positives since other compounds of similar chemical structures present cross-reactivities (Council Regulation 2377/90/EEC 1990; Kuiper et al., 1998). Therefore results of screening analysis should be confirmed by gas or liquid chromatography-mass spectrometric analysis (GC-MS) (Cai and Henion, 1997; Kuiper et al., 1998; Polettini et al., 1995; Whaites and Murby, 1999).

The EU suggested the use of tandem mass spectrometry, also known as MS-MS, for the acquisition of one parent ion and two product ions for the unambiguous GC-MS-MS identification of the forbidden β-agonists (Fiori et al., 2002). It is known that MS-MS can provide significantly more information than standard mass spectrometry (MS) in cases where the analytes exhibit a similar primary fragmentation.

This is because product ion mass spectra can be significantly different even for similar analytes, thus providing enhanced structural information with increasing selectivity of the analytes of interest (Zampronio et al., 2002). Additionally, MS-MS offers a number of advantages between analytes on the basis of chromatographic properties (retention time), parent ion (MS₁) and daughter ion (s) (MS₂) (de Wasch et al., 1998).

The aim of this work was to monitor the presence of clenbuterol residues in bovine meat muscle collected from the Mexican market by using screening and confirmatory methods.

**MATERIALS AND METHODS**

**Reagents**

Clenbuterol HCl, potassium dihydrogen phosphate, perchloric acid, bis(trimethylsilyl)trifluoro-acetamide (BSTFA), formic acid, ethyl acetate, methanol and n-heptane were from Sigma (St. Louis, MO, USA). Tris-buffer (hidroximetil- aminomethane) was from Bio-Rad (Richmond, CA, USA). The immunoenzymatic test was carried out by using an ELISA kit for clenbuterol and other β₂-adrenergic agonists (r-Biopharm, Darmstadt, Germany). Sodium hydroxide and hydrochloric acid were from Merck (Darmstadt, Germany). All solvents were HPLC grade.

**Standard solutions**

Clenbuterol hydrochloride stock solutions (1 mg/mL) were prepared in distilled water; two drops of 0.1M formic acid were added for stability before diluting to volume. These solutions were stored at 4 °C for no longer than two months. Standard working solutions (0.1 µg/mL) were prepared each day by dilution with methanol.

**Samples**

A total of 50 meat samples (beef top and ground beef) were collected in three occasions from 9 stores at the local market in the state of Sonora, Mexico. The first two samplings took place one month apart while the third sampling was 12 months later. Samples were kept frozen at -20 °C until analysis.

**Clenbuterol analysis by ELISA (screening test)**

The extraction and clean-up procedures were those described by the ELISA kit manufacturer (r-Biopharm, Darmstadt, Germany). Minced meat samples (5 g) were homogenized for 30 min with 25 mL of 50 mM tris-buffer (pH 8.5) in 50 mL centrifuge tubes by using an Ultra-Turrax homogenizer. To this slurry, 15 mL of n-heptane were added, homogenized for 5 min and centrifuged for 5 min at 5000 rpm at 4 °C. The supernatant was removed and the heptane extraction was repeated once again with 15 mL. Concentrated HCl (0.5 mL) was added to the meat extract and homogenized for 1 h. Then, 6 g of the homogenate were weighed in a 50 mL polypropylene tube and centrifuged at 5000 rpm for 15 min. The supernatant was collected into another tube and mixed with 300 µL of 1M NaOH for 15 min. Then, 4 mL of 500 mM KH₂PO₄ buffer (pH 3) were added, mixed briefly and stored at 4 °C for 16 h. After overnight storage, the sample was centrifuged for 15 min at 5000 rpm at 4 °C. Finally, the entire supernatant was purified by solid-phase extraction on a C₁₈ column.

The C₁₈ column was first rinsed with 3 mL of 100% methanol. After the methanol was eluted, the column was equilibrated with 2 mL of 50 mM potassium dihydrogen phosphate (KH₂PO₄) buffer (pH 3). Then, the sample was loaded and the column was rinsed with 2 mL of 50 mM...
Clenbuterol analysis by GC-MS-MS (confirmatory method)

The extraction and clean-up procedures were those described by the ELISA kit manufacturer with modifications. Briefly, minced meat samples (1 g) were homogenized with 1 mL of tris-buffer (50 mM, pH 8.5). Then, 2 mL of n-heptane were added, vortexed for 2 min and centrifuged at 10,000 rpm for 15 min at 4 ºC. The upper organic layer was discarded and the extraction was repeated. Concentrated perchloric acid (0.5 mL) was added to the meat extract, mixed for 20 min and centrifuged for 15 min at 10000 rpm at 4 ºC. The supernatant was collected in a tube containing 300 µL of 1M NaOH and mixed for 5 min. Then, 4 mL of 500 mM KH₂PO₄-buffer (pH 3) were added, the pH was adjusted to pH 6 and the extract was stored at 4 ºC for 1 h. Finally, the entire supernatant was purified by solid-phase extraction on a C₁₈ column. The C₁₈ column was first rinsed with 6 mL of 100 % methanol. After the methanol was eluted, the column was equilibrated with 2 mL of 1 M phosphate (KH₂PO₄) buffer (pH 3). Then, the sample was loaded, and the column was rinsed with 2 mL of 1 M phosphate (KH₂PO₄) buffer (pH 6). The sample was eluted with 1.5 mL of 100 % methanol and the eluate was evaporated to dryness under continuous flow of nitrogen at 45 to 50 ºC.

Derivatization

The sample residue was derivatized as reported by Fente et al. (1999). 100 µl of BSTFA and 100 µl of ethyl acetate were added to the residue and heated at 70 ºC for 30 min. Once the derivatization process was completed, the solution was evaporated to dryness under continuous flow of nitrogen and reconstituted in 25 µl of ethyl acetate. Finally, 3 µl of this solution were injected into the gas chromatograph.

GC-MS-MS conditions

The instrument was a GCQ plus ion trap mass spectrometer (Thermo Quest, Italy). Chromatographic separation was performed in a capillary column DB-5 of 15 m x 0.25 mm i.d. x 0.25 µm (J and W Scientific, Folsom, CA, USA). The injector temperature was set at 280 ºC and was operated in a splitless mode. The carrier gas (He) flow was 1 mL/min. The oven temperature program was raised from 120 ºC (0.1 min) to 245 °C at a rate of 15 ºC min⁻¹, then it was maintained at this temperature for 1 min and subsequently raised to a final temperature of 270 °C at 30 ºC min⁻¹. The interface and detector temperatures were 275 and 260 ºC, respectively. The GCQ acquisition was in electron-impact mode. The ion source was set at 200 ºC. The system was operated in the MS-MS mode using the fragment of the original molecular structure obtained of the MS₁, the precursor ion was m/z 262, the width was 4; the excitation volts were 1.15; and the product ions were 188-192, 225-229, and 262-264 as reported by Fiori et al. (2002).

RESULTS AND DISCUSSION

Determination of clenbuterol in bovine meat by ELISA (screening test)

In this study a commercial ELISA kit was used for presumptive clenbuterol detection and quantification, however performance of the kit was not clearly described by the manufacturer and validation of the actual performance of the immunoassay with the sample matrix was carried out. The ELISA technique showed very high correlation between clenbuterol concentration (ng/kg) and maximum % absorbance. The calibration curve constructed for the ELISA determinations (y = -18.396 Ln(x) + 184.68) followed a relationship with a highly significant (P < 0.01) coefficient of multiple determination (R² = 0.999), where y was maximum absorbance (%) and x was clenbuterol
Clenbuterol concentration (ng/kg). Thus this calibration curve was used for determining clenbuterol concentration in meat samples.

The efficiency of clenbuterol extraction by the ELISA technique was evaluated by analyzing clenbuterol fortified meat samples. Very good recoveries of 95 to 101% were observed at levels of 1 to 6 µg/kg of clenbuterol fortification. These clenbuterol recoveries were similar to those (93-94%) reported previously for bovine liver samples fortified with 0.5 to 2.0 µg/kg and analyzed by GC-MS-MS (Fiori et al., 2002) or those (95-113%) reported for liver samples fortified with 1.0-2.0 µg/kg and analyzed by ELISA (Degand et al., 1992). On the other hand, the only study that reported clenbuterol recovery from meat products was much lower (63%) at a fortification level of 0.4 µg/kg and determined by LC-MS-MS (Guy et al., 1999). Similarly, clenbuterol recoveries of 44-75% from urine samples spiked with 0.2 to 1.5 µg/kg were detected by using ELISA (Sawaya et al., 2000).

Therefore, for the quantitative determination of clenbuterol either in animal tissues or urine, it is important to perform recovery studies to ensure the accuracy of the method, since most important for the screening test is to obtain an acceptable low number of false negative results. A large number of false negative results were reported by the European residue control laboratories as a result of an interlaboratory study (Wolf et al., 1998). On the other hand,

Table 1. Clenbuterol concentrations in bovine meat samples determined by ELISA (n = 3). *Samples confirmed by GC-MS-MS. **LOD = Limit of detection (0.1 µg/kg). ^NC = Not collected.
other studies reported high rates of false positives in meat and urine samples, when the presumptive ELISA results were confirmed by GC-MS (Boyd et al., 1996). It has been reported that commercial ELISA kits for the analysis of clenbuterol present cross reactivity with other β-agonists of similar structures (Kuiper et al., 1998). Indeed, high cross reactivities were reported for the ELISA kit used in this study. Thus, confirmation of the ELISA test should always be carried out by chromatographic methods coupled to spectrometric methods.

The limit of detection (LOD) for the ELISA test was at the lowest concentration of clenbuterol standard used in the standard curve (0.1 µg/kg), although the manufacturer reported a LOD of 0.04 µg/kg. The reproducibility of the technique was very good since coefficients of variation determined for clenbuterol standards and meat samples were lower than 1.5 and 2.4 %, respectively.

From 50 bovine meat samples analyzed by the ELISA test, 6 (12 %) presented clenbuterol residues from 3.06 to 6.12 µg/kg, 13 (26 %) from 0.5 to 1.83 µg/kg, 24 (48 %) from 0.1 to 0.5 µg/kg and only 7 (14 %) were below the LOD of 0.1 µg/kg (Table 1). According to the maximum residue limited (MRL) recommended by the WHO and the Codex Alimentarius of 0.2 µg/kg, 38 (76 %) samples would be considered presumptive positive samples. However, the samples analyzed in this study presented clenbuterol concentrations much lower than those reported for cases of food poisoning documented in several countries (Martínez-Navarro, 1990; Pulce et al., 1991; Salleras et al., 1995; Brambilla et al., 1997; Sporano et al., 1998).

Samples implicated in documented cases of food poisoning in different countries reported clenbuterol concentrations of 161 to 291 µg/kg (Martínez-Navarro, 1990), 375 to 500 µg/kg (Pulce et al., 1991) and 19 to 5395 µg/kg (Salleras et al., 1995) in veal liver or 450 µg/kg (Brambilla et al., 1997) and 800 to 7400 µg/kg (Sporano et al., 1998) in meat samples. These documented cases presented clenbuterol concentrations much higher than the MRL set by the EC. On the other hand, no data were available on the concentrations of clenbuterol residues found in the liver samples implicated in food poisoning cases reported in Mexico. In fact, the only report available issued by Mexican officials, briefly described the food poisoning cases occurring in different parts of Mexico (García-López, 2002). Since the analytical techniques included in the Mexican regulations only consider presumptive tests for clenbuterol detection, presumably, cases of food poisoning were only verified by analyzing samples with the ELISA test. Additionally, Mexican regulations do not include MRL for clenbuterol residues in meat (SAGARPA, 2002), although unofficially, it is known that meat samples were considered positive when presenting clenbuterol residues above 2 µg/kg. For this reason, only the six samples (12 %) with the highest clenbuterol residues (>2.0 µg/kg) found in this study were subjected to confirmatory analysis by GC-MS-MS.
Identification of clenbuterol in bovine meat by GC-MS-MS (confirmatory method)

Clenbuterol was derivatized with N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), to form a mono-trimethylsilyl (TMS) derivative which gave a precursor ion of m/z = 262. This molecular ion was selected as the primary ion, that when subjected to MS/MS resulted in the products of fragmentation with m/z = 188, 190, and 225.

A clenbuterol-negative meat sample analyzed by GC-MS/MS showed the absence of the clenbuterol peak (Figure 1). On the other hand, a typical chromatogram of a clenbuterol fortified meat sample analyzed by GC-MS-MS showed the clenbuterol peak at retention time of 5.30 ± 0.02 min (Figure 2). Additionally, the typical product ions of m/z = 189, 191, and 225 were also observed (Figure 2). In this study, the six meat samples analyzed by GC-MS-MS were confirmed positive for clenbuterol. For the confirmation of clenbuterol residues in the meat samples, the relative intensities of the product ions corresponded to those of the standard analyte (± 10%). According to the European Commission (93/256/EEC), the relative abundances of all diagnostic ions monitored for the analyte should match those of the standard analyte or from spiked samples at comparable concentrations, preferably within a margin of ± 10%.

The illegal use of clenbuterol as a growth promoting agent has been well documented to have occurred in Europe, Asia, and North and Central America, spanning a time frame of nearly a decade. Much of the detected illegal use of clenbuterol in Europe have occurred in spite of the European ban of all anabolic compounds in animal production. Extensive illegal use of clenbuterol and other β-agonists have not been halted by the ban, and the problem of unsafe residues in food remains. In fact, the most recent food poisoning outbreak caused by clenbuterol residues in meat was reported in Portugal in 2005 (Barbosa et al., 2005). To the best of our knowledge, this is the first scientific report that presents data on clenbuterol residues in bovine meat from the market in the northwest of Mexico. Although samples were collected from the local market, these could have come from different parts of the country. Thus, this initial study should be useful as a first attempt to gain some insight in the illegal use of clenbuterol by some Mexican meat producers.

CONCLUSIONS

Since Mexico is a country that presents a very active international commercial meat market trade, it is essential from the public health point of view, to implement monitoring surveillance programs for clenbuterol residues according to international standards and regulations. Firstly, Mexican regulations should include MRL and confirmatory methods of analysis according to international standards. Secondly, interlaboratory studies should be carried out to evaluate proficiency testing and finally, a well documented national residue monitoring plan should be implemented by the Mexican regulatory agencies. The results presented in this study may trigger the implementation of effective surveillance monitoring programs to ensure the safety of the food supply.

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