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Determination of residues of the β-agonist clenbuterol in liver of medicated farm animals by gas chromatography–mass spectrometry using diphasic dialysis as an extraction procedure

P. González[a], C.A. Fente[b,*], C. Franco[c], B. Vázquez[c], E. Quinto[c], A. Cepeda[c]

[a]*Laboratorio de Salud Pública de Lugo, Consellería de Sanidade e Servicios Sociais, Xunta de Galicia, Lugo, Spain*
[b]*Area de Tecnología de los Alimentos, Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Campus de Lugo, Universidad de Santiago de Compostela, Lugo, Spain*
[c]*Area de Nutrición y Bromatología, Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Veterinaria, Campus de Lugo, Universidad de Santiago de Compostela, Lugo, Spain*

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Abstract

A method has been developed for the rapid confirmation of clenbuterol in cow liver using gas chromatography coupled with detection by mass spectrometry of the trimethylsilyl derivatives of clenbuterol. The technique used for the extraction was diphasic dialysis. It was observed that the best suitable solution to homogenize the liver the barium hydroxide–barium chloride buffer, the optimal extraction solvent was *tert.*-butylmethyl ether at an extraction temperature of 37°C, and stirring should be applied at 150 rpm for 4 h. This extraction method improves clenbuterol recovery up to values of 99.3%. With the use of the barium buffer, derivatization is performed more efficiently and the detection and quantification limits can be decreased to values close to 250 ppt and 500 ppt, respectively.

Keywords: Clenbuterol

1. Introduction

Selective β-agonists such as clenbuterol can be called partitioning agents because they accelerate animal growth [1], and also alter the animal carcass composition [2,3].

Illegal use of clenbuterol in meat production has prompted the development of test programmes. Reports from such programmes state that outbreaks of food poisonous to humans resulted from the illegal administration of this substance to consumption animals [4,5]. Concentrations of clenbuterol in liver in the range reported to be associated with toxicity to consumers [4] (>100 ng/g) were found in animals which had received growth-promoting doses of drug [6]. They also reported that the liver is the target organ for clenbuterol residue analysis. Even 15 days after drug withdrawal, the livers of all animals dosed with therapeutic concentrations of the compound contained detectable amounts of clenbuterol residues.

Detection of nanogram concentrations of this drug in animal tissues (such as liver) has been published by several analytical techniques. Such techniques include enzyme immunoassays (EIA) [6,7], gas chromatography–mass spectrometry (GC–MS) [8],

*Corresponding author.

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and high-performance liquid chromatography (HPLC) [7,9,10].

The technique of diphasic dialysis permits the direct extraction of the analytes in the organic extract [11,12]. It has been used in bovine urine for the screening of clenbuterol by HPTLC. We have developed a two-phase dialysis technique for the confirmatory analysis of clenbuterol in bovine urine by gas chromatography coupled with mass detection [13].

In this paper, a method for the rapid determination of clenbuterol in liver is described. An optimized diphasic dialysis technique was utilized as a simplification of the extraction and purification steps. For confirmatory analysis, gas chromatography coupled with mass detection for the trimethylsilyl derivatives of clenbuterol was used.

2. Experimental

2.1. Chemicals

tert.-Butylmethyl ether, toluene, chloroform, ethyl acetate, *n*-hexane, disodium phosphate, monopotassium phosphate, sodium bicarbonate, sodium carbonate, triethylamine, hydrochloric acid, and the derivatizing agent bis(trimethylsilyl)trifluoracetamide (BSTFA) were purchased from Merck (Darmstadt, Germany). Barium hydroxide 8-hydrate was from Panreac (Barcelona, Spain). Clenbuterol and the internal standard metoprolol were from Sigma (St. Louis, MO, USA). All reagents were analytical grade. Dialysis tubing was of the type 20/32, of regenerated cellulose with a molecular exclusion size of 10 000 Da (Visking, Serva, Feinbiochemical, Heidelberg, Germany). Sep-Pak C_{18} columns were from Millipore Waters Chromatography (Bedford, MA, USA). Micro-reaction vessels of 1.0 ml were supplied by Supelco (Bellefonte, PA, USA).

2.2. Standard solutions

Clenbuterol stock solutions with a concentration of 100 μg/ml were prepared in 0.01 *M* hydrochloric acid. These solutions could be stored under cooling for no longer than two months.

Standard work solutions were freshly prepared every day with 0.01 *M* hydrochloric acid.

2.3. Apparatus

Thermostated incubator shaker Model G25 & R25 New Brunswick Scientific (Edison, NJ, USA).

Nitrogen evaporation system, with thermostated heating plate, Liebisch (Bielefeld, Germany).

Gas chromatograph: Hewlett-Packard Model 5890 Series II. Gas carrier, helium. Chromatographic separation was performed in a capillary column SP 5 Hewlett-Packard (25 m×0.25 mm). The injector and interface were kept at temperature of 280°C and 300°C, respectively. The gas chromatograph oven was programmed from 110°C to 180°C at a rate of 4°C/min and subsequently to 300°C at 30°C/min, keeping that final temperature for 5 min. The gas chromatograph was coupled to a Hewlett-Packard mass detector Model 5972, operating in single ion mode (SIM), with the selection of ions 86, 243, 262, 277 and 333, and with split-less at 1.85 min.

2.4. Sample preparation

Clenbuterol was extracted from liver samples using diphasic dialysis. Extraction solvent (*tert.*-butylmethyl ether, 25 ml) was placed in previously-wetted dialysis tubing 25 cm long and with an exchange surface of ca. 196 cm^2.

A buffer solution [Cl_2Ba–$Ba(OH)_2$, pH 13.8] was prepared by addition of excess $Ba(OH)_2$ to a 0.2 *M* HCl solution. This solution remained stable for 30 days. A 50 ml volume of the buffer solution was added to 10 g of liver doped with 2 ppb clenbuterol and homogenized in a 500 ml beaker. The dialysis tubing containing the extraction solvent was introduced in the mixture, and extraction was performed under stirring at 150 rpm at a temperature of 37°C, for 4 h.

After the extraction process, the contents of the dialysis tubing were poured into a separation funnel, the aqueous phase removed and the organic phase dried of aqueous residues on filter paper with anhydrous sodium sulphate.

The extract was then placed in a round-bottom beaker and concentrated to dryness under nitrogen flow. The residue was treated with BSTFA to obtain

the trimethylsilyl derivative. An amount of 2 ppb of metoprolol was added and used as an internal standard. The derivatization process was performed according to the method of van Rhijn [14] by addition of 100 μl of a mixture 1:1 of BSFTA and ethyl acetate to the evaporated extracts. The mixture was then heated to 60°C for 40 min and, after completion of the derivatization process, the product was evaporated under nitrogen, redissolved in ethyl acetate and injected into the chromatographic system.

3. Results and discussion

3.1. Extraction procedure

Two-phase dialysis is a new extraction technique based on a cellulose membrane. It was developed in Spain by Domínguez et al. [11] for the extraction of low molecular mass organic substances from natural and synthetic products. This technique has been utilized for the screening, via HPTLC (high-performance thin-layer chromatography) [12] and confirmatory analysis with GC–MS [13], of clenbuterol in bovine urine.

In the present work this extraction technique was optimized for the analysis of clenbuterol in cow liver by gas chromatography coupled to mass spectrometry.

The liver is a quite more complex matrix than the urine, for which some extraction parameters, such as pH or extraction solvent, were already difficult to adjust.

For each assay, 10 g of clenbuterol-free cow liver doped with 2 ppb clenbuterol was used. The extraction parameters [such as liver homogenizate pH, extraction solvent, stirring rate (rpm), temperature, and extraction time] were optimized to improve the recovery and cleanliness of the extract (such as the absence of chromatographic peaks that might interfere on a GC–MS analysis). During the experiments only one parameter at a time was allowed to vary, keeping the others constant. All the assays were performed three-fold, and the results shown here are mean average values.

Initially *tert.*-butylmethyl ether was used as an extraction solvent, with an extraction time of 4 h, temperature of 35°C and a stirring rate of 150 rpm (optimal conditions for clenbuterol extraction from urine [13]).

The first parameter to be adjusted was the pH. Our personal experience in clenbuterol extraction from urine, is that a pH around 9 is optimal for its extraction. During the homogenization of liver with water the pH dropped to 6, and triethylamine had to be added to the mixture to keep the pH around 9. However, during the 4 h of stirring used for this extraction, the pH decreased again to the initial values, and extraction did not take place. In view of this, we decided to use a buffer solution to keep a constant pH throughout the process.

A 50 ml volume of phosphate buffer (pH 9) was used to homogenize the liver sample. During the homogenization process the pH dropped rapidly to values close to 7, and therefore triethylamine was added to adjust it. Several pH values were tested, taking into account the following observations: at pH 9 a characteristic peak of clenbuterol is visible, but some other peaks in the same range are observed which could interfere with the detection; at pH 10 and 11 extremely noisy chromatograms are obtained, with no peak for clenbuterol.

In view of the results obtained, other buffers were tested, such as 0.2 *M* sodium carbonate–0.2 *M* sodium bicarbonate, with pH 9–10. This buffer showed an additional problem of forming foam due to stirring during the extraction process and no extraction of clenbuterol was achieved.

The best results were obtained when the barium hydroxide–barium chloride buffer at (pH 13.8) was used. When performing the homogenization, the pH was always close to 10, decreasing to 9 during the extraction. A recovery close to 80% was achieved in every case (81.2% was the average from three extractions, coefficient of variation, C.V.=2.1%).

Several solvents (*tert.*-butylmethyl ether, chloroform, *n*-hexane, toluene and ethyl acetate) were tested. These were chosen because of their nonmiscibility with water to avoid the transfer of liver homogenate to the interior of the gut. For these experiments, the following constant extraction conditions were chosen: barium hydroxide–barium chloride buffer, T=35°C, stirring rate=150 rpm, extraction time=4 h.

On extraction with chloroform and ethyl acetate

the external surface of the dialysis tubing was highly impregnated with fat and the recovery was very low (10%). With ethyl acetate, evaporation of the extraction solvent was slow and the derivatization process became more difficult because of simultaneous crystallization. With *n*-hexane and toluene the results obtained were very clean, but recovery was very low (15%). The best extraction solvent was found to be *tert.*-butylmethyl ether, with which relatively clean extracts were obtained (without interfering peaks) and the extraction percentages were excellent.

With respect to stirring rate, keeping the other parameters constant, the most suitable stirring rate was found to be 150 rpm. When a lower rate of 100 rpm was used, the extraction was less effective (50%), and at a higher rate of 200 rpm, evaporation of part of the extraction solvent took place, and the recovery decreased to 50%.

In addition, several extraction times were studied (barium hydroxide–barium chloride buffer, *tert.*-butylmethyl ether, 35°C, 150 rpm). The recovery improved with increasing extraction time (1 h, 25%; 2 h, 60%; 3 h, 78%) and reached a maximum of 81.2% after 4 h. For longer times, extraction did not improve and the amount of contaminants in the extracts was noticeably increased.

The last parameter studied was the extraction temperature. Several extraction temperatures were tested, keeping the other parameters constant (barium hydroxide–barium chloride buffer, *tert.*-butylmethyl ether, 150 rpm, 4 h). At 20°C and 30°C no extraction occurred, at 35°C extraction was very good (81.2%) but at 37°C the recovery was even higher, close to 100% (99.3% average from three extractions, coefficient of variation, C.V.=1.05%). At 40°C and above, the colour of the liver homogenate changes and an unpleasant odour appears during the extraction process. The recovery decreased slightly to values close to those obtained at 35°C.

In summary, to perform clenbuterol extraction by diphasic dialysis and further analysis by gas chromatography and mass detection, the best suited solution to homogenize the liver is barium hydroxide–barium chloride buffer with pH 13.8, the optimal extraction solvent is *tert.*-butylmethyl ether at an extraction temperature of 37°C, and stirring should be applied at 150 rpm for 4 h.

In previous studies performed in our laboratory using Sep-Pak C_{18} cartridges in the extraction method [15], 65% recoveries (average from 10 extractions) have been obtained. The extraction with two-phase dialysis improves clenbuterol recovery to values closed to 100% (99.3%, average from three extractions under optimized conditions). It must be taken into account that the concentration range necessary to get results measurable by GC–MS, when using C_{18}, has to be at least 2 ppb. However, using the membrane technique it is possible to work with a concentration lower than 1 ppb (even with 0.25 ppb good results were obtained). This is really interesting because the clenbuterol residues are in very low concentration in the liver of consumption animals.

Another important advantage of the dialysis technique is the reduction in the price and in the sample processing time, mainly because extraction and purification of the extracts are performed in a single step.

3.2. Gas chromatography–mass spectrometry

The analysis of the extracts obtained was performed by gas chromatography coupled to a mass spectrometry detector. The method proposed by van Rhijn [14] was used, but setting the temperature of the interface at 300°C instead of 280°C.

The structures of clenbuterol and metoprolol and their corresponding BSTFA derivatives are shown in Fig. 1.

The use of barium buffer has the advantage that the derivatization is more efficient and crystallization is not observed in any case, and also the detection and quantification limits can be decreased to values close to 250 ppt and 500 ppt, respectively.

A standard curve for clenbuterol analysis using the optimized diphasic dialysis technique was obtained for liver samples overloaded with 0.5, 1, 2, 3 and 4 ppb of clenbuterol. The equation of the first-order regression analysis for these liver samples was ($y=1.61e^{+000}x+1.76e^{-001}$, $r=0.999$).

Repeatibility of the method was determined analyzing a liver sample overloaded with 2 ppb of clenbuterol. The sample was analyzed on five different occasions, each time in duplicate. The repeatibili-

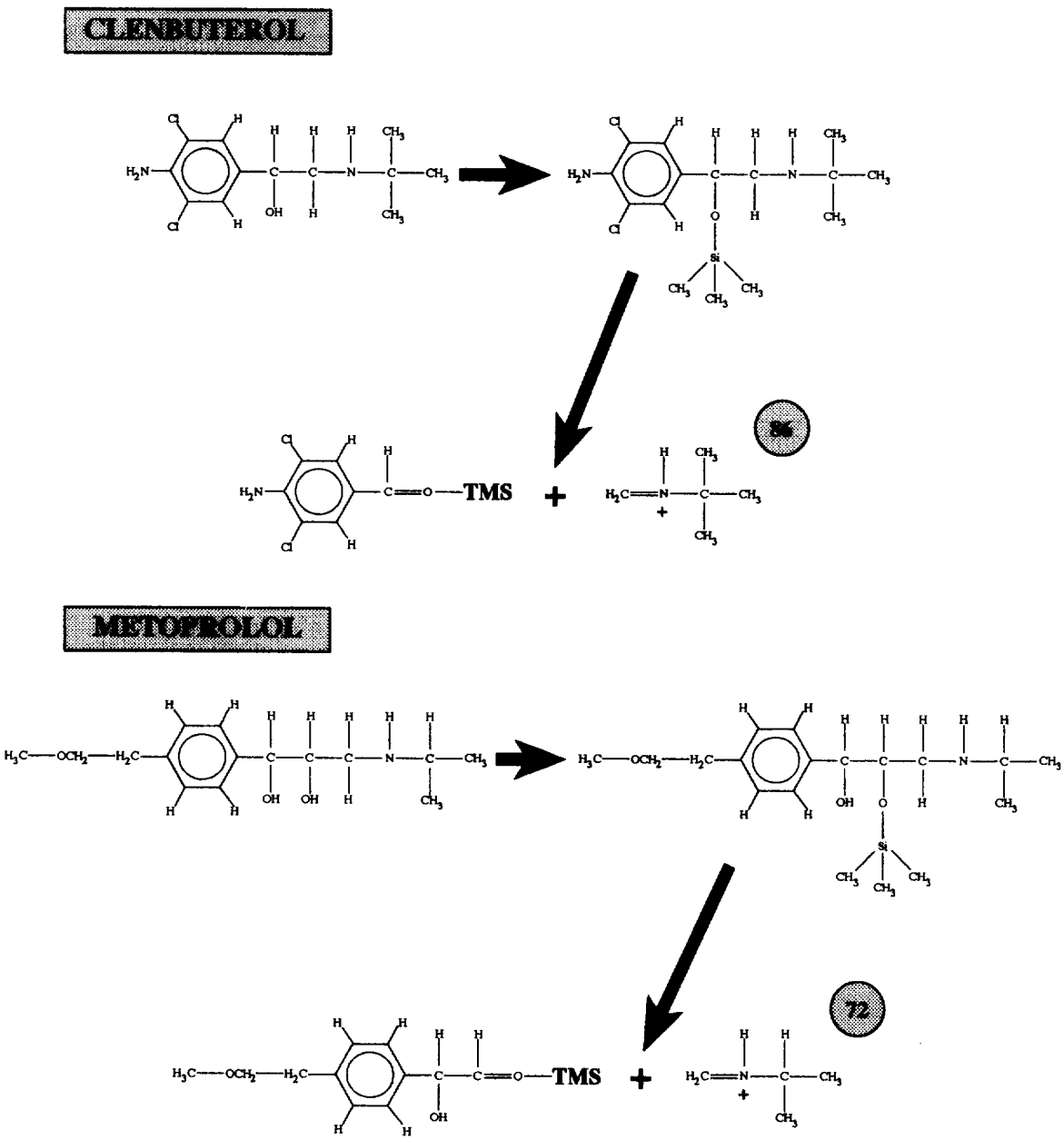

Fig. 1. Structures of clenbuterol, metoprolol and their corresponding BSTFA derivatives and characteristic ions.

ty seemed to be acceptable with a coefficient of variation, C.V.=2.67%.

Fig. 2 shows the liver blank chromatogram to verify potential interferences in the analysis.

Fig. 3 shows the chromatogram (clenbuterol peak at 23.54 min, metoprolol peak at 24.17) and mass spectrum with characteristic ions of clenbuterol (86, 243, 262, 277, 233). This chromatogram corresponds to a real liver sample from an animal treated with clenbuterol, which was positive on the screening analysis by HPTLC. For the extraction, diphasic dialysis was used, finding an amount of 2.1 ppb.

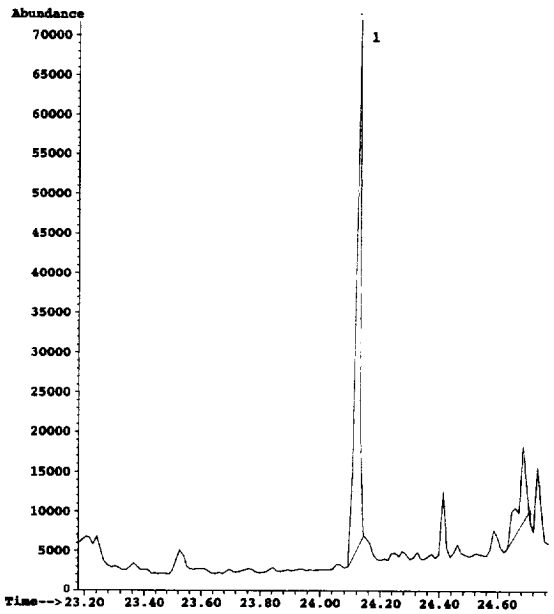

Fig. 2. Liver blank chromatogram.

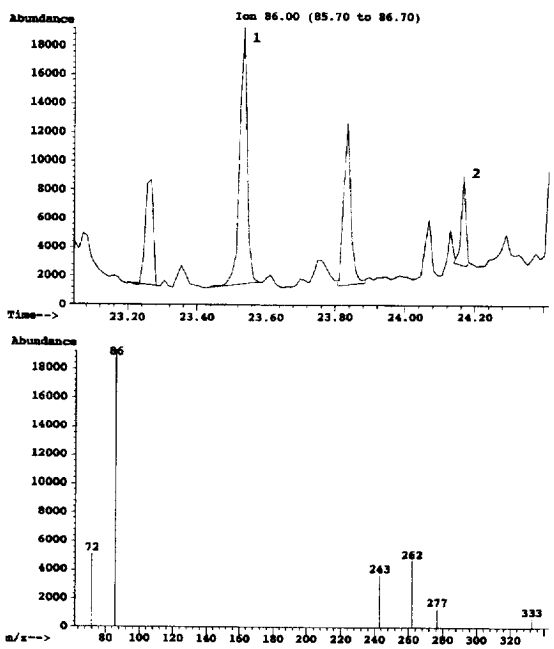

Fig. 3. Chromatogram (1=clenbuterol peak at 23.54 min, 2= metoprolol peak at 24.17) and mass spectrum showing ions characteristic of clenbuterol (m/z 86, 243, 262, 277, 333) acquired in SIM mode by GC–MS. This sample was a liver sample from an animal treated with clenbuterol. Extracted was by diphasic dialysis, and the analysis showed a concentration of 2.1 ppb of clenbuturol.

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