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Journal of Chromatography B, 726 (1999) 133–139

JOURNAL OF CHROMATOGRAPHY B

Determination of clenbuterol residues in bovine hair by using diphasic dialysis and gas chromatography–mass spectrometry

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Received 12 October 1998; received in revised form 21 December 1998; accepted 29 December 1998

Abstract

A method for the determination of clenbuterol (4-amino-3,5-dichloro-α[(*tert.*-butylamino)methyl]-benzyl alcohol hydrochloride) in hair of living cows has been developed. Hair samples were digested in an alkaline medium. The diphasic dialysis technique is a semi-permeable membrane technology developed for the direct extraction of relatively low-molecular-mass analytes such as clenbuterol. In this case, we used sodium citrate buffer to homogenize the digested hair, dichloromethane was used as the extraction solvent at 37°C, and stirring was applied at 150 rpm for 4 h. The analysis was carried out using gas chromatography–mass spectrometry. The calibration curve for clenbuterol in hair was linear in the range from 12.5 to 400 ng g^{-1}. The detection limit of clenbuterol was 5 ng g^{-1} and the quantification limit was 12.5 ng g^{-1}, in hair. A good inter-day reproducibility was obtained (R.S.D.=7.08%). The repeatability and intra-day reproducibility (50 ng g^{-1} of hair, n=10) show R.S.D.s of 7.1 and 9.5%, respectively.

Keywords: Hair; Cattle and diphasic dialysis; Clenbuterol

1. Introduction

During the last decade, it has been demonstrated that many drugs including clenbuterol (4-amino-3,5-dichloro-α[(*tert.*-butylamino)methyl]-benzyl alcohol hydrochloride) can be found in the hair of both humans and animals. Recent studies show that clenbuterol concentrates in hair melanin granules. The ability to analyze clenbuterol in hair from animals is very advantageous because it provides an ''in vivo'' piece of evidence prior to sacrifice and facilitates the action of veterinary inspectors. For this reason, new analytical methods have been developed [1–3]. Such methods include liquid–liquid procedures for the extraction of the analyte, which involve large amounts of organic solvents.

The diphasic dialysis technique is a semi-permeable membrane technology developed for the direct extraction of relatively low-molecular-mass analytes from different substrates [4]. The diphasic dialysis membrane procedure has previously been applied to the extraction of clenbuterol from matrices of animal origin [5,6].

In this paper, a method is described for the determination of clenbuterol in hair of living calves treated with promoting growth doses. An optimized

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00014-6

two-phase dialysis technique was used to simplify both steps: extraction and purification. Confirmatory analyses were performed by gas chromatography–mass spectrometry of the clenbuterol trimethylsilyl derivatives.

2. Experimental

2.1. Chemicals

Citric acid monohydrate, hydrochloric acid 37%, monopotassium phosphate, disodium phosphate, orthophosphoric acid, Tween 80 (10%), ethyl acetate, dichloromethane, *tert*.-butylmethyl ether, hexane, chloroform, diethyl ether, sodium hydroxide and the derivatization agent bis(trimethylsilyl) trifluoroacetamide (BSTFA) were purchased from Merck (Darmstadt, Germany). All reagents were of analytical grade. Visking dialysis tubing was of regenerated cellulose, size 20/32, with a molecular exclusion size of 10 000 Da, (Serva, Feinbiochemical, Heidelberg, Germany). Microreaction vessels of 1.0 ml were supplied by Supelco (Belfonte, PA, USA). Spasmobronchal® was from Boehringer Ingelheim (0.03 mg clenbuterol/ml).

2.2. Standard solutions

Internal standard metoprolol and clenbuterol were from Sigma (St. Louis, MO, USA). Metoprolol stock solutions were prepared in methanol at a concentration of 100 μg ml^{-1}. Clenbuterol stock solutions were prepared in 0.01 *M* hydrochloric acid at a concentration of 100 μg ml^{-1}. These solutions were stored at 4°C for no longer than two months. Standard working solutions were prepared each day by dilution with methanol.

2.3. Apparatus

A thermostated incubator shaker, model G25&R25 (New Brunswick Scientific, Edison, NJ, USA) and a nitrogen evaporation system, with a thermostated heating plate (Liebisch Bielefeld, Germany) were used.

Gas chromatograph, Hewlett-Packard (Avondale, PA, USA) model 5890 Series II; gas carrier, helium. Chromatographic separation was performed in a capillary column (HP-5MS; 5% phenyl methylsiloxane, 0.25 μm film thickness) from Hewlett-Packard (30 m×0.25 mm I.D.). The injector and interface were kept at temperatures of 280 and 300°C, respectively. The GC oven was programmed from 110 to 180°C at a rate of 4°C min^{-1} and subsequently to 300°C at 30°C min^{-1}, maintaining the final temperature for 5 min. The gas chromatograph was coupled to a Hewlett-Packard mass detector model 5972, operating in selected ion monitoring (SIM) mode, with selected m/z 86, 243, 262, 277 and 333 ions. The injection mode was splitless for 1.85 min.

2.4. In vivo animal assays

2.4.1. Assay A: Therapeutic treatment

Two Holstein calves, aged two months (A and B) were used.

Two i.m. injections containing 4 ml of Spasmo Bronchal (Boehringer Ingelheim; 0.03 mg ml^{-1} clenbuterol) were administered per day for ten days.

2.4.2. Assay B: Promoting growth treatment

Calf B, which had previously been treated therapeutically, received additional promoting growth treatment.

After day 10, and for 29 extra days, the animal was fed with 0.5 mg kg^{-1} of clenbuterol-doped feed. The mean dose of clenbuterol administered was 1.5 mg day^{-1}.

2.5. Sampling

In both treatments, the collection of pigmented hair (1 g) was performed from the back of the animal with a razor blade, every ten days, starting on day 10 and ending after two months. Once a cut was made in a specific area, no further hair samples were taken from the same area. No distinction was made regarding the hair length because the aim of this study was the development of a method that can be used in real life.

Clenbuterol-free hair samples were also taken from cows that were not treated with clenbuterol.

Samples were kept refrigerated.

2.6. Sample preparation

Decontamination procedures prior to hair analysis are necessary to avoid false positives. The hair was placed in a single-use plastic syringe after removing the piston. The hair was washed by filling the syringe with an aqueous solution of Tween 80 (10%) and pressing the piston into the syringe. Hair was rinsed three times with the above-mentioned solution, to remove potential exogenic clenbuterol contamination. The last wash was evaporated, derivatized and then analyzed to verify the absence of clenbuterol. The hair was subsequently dried at 40°C and cut into very small pieces (ca. 1 mm) with standard scissors.

2.7. Extraction of clenbuterol from hair

The hair (500 mg) was introduced into a 20-ml test tube and digested with 2 ml of 1 *M* NaOH at 80°C for 1 h. It was vortex-stirred twice during the process. With this digestion procedure, the complete destruction of hair structure was assured.

The digested hair was placed in a 500-ml beaker and the pH was adjusted to 12 using sodium citrate buffer at pH 4.8, prepared according to Sorensen [7] with 88.2 ml of 0.1 *M* disodium citrate and 11.8 ml of 0.1 *M* HCl. The volume of the digest after pH adjustment was close to 20 ml. Then, a previously wetted 25 cm long piece of dialysis tubing, with an exchange surface of ca. 196 cm^2, and containing 25 ml of extraction solvent (dichloromethane), was introduced in the beaker. The extraction was performed by stirring at 150 rpm and 37°C for 4 h.

The dialysis contents were then poured into a separation funnel, the aqueous phase was removed and the organic phase was dried on filter paper with anhydrous sodium sulphate.

The extract was placed in a round-bottomed beaker and concentrated to dryness under a continuous flow of nitrogen. The residue was treated with BSTFA to obtain the trimethylsilyl derivative; 100 μl of a 2-μg ml^{-1} metoprolol solution was added as an internal standard. The derivatization process was performed according to van Rhijn et al. [8], adding 100 μl of BSFTA and ethyl acetate (1:1, v/v) to the evaporated extracts. The mixture was heated at 60°C for 40 min and, once the derivatization was completed, it was evaporated under nitrogen and redissolved in 25 μl of ethyl acetate. Finally, 1 μl of this solution was injected into the gas chromatograph.

2.8. Validation of the extractions

The assays of diphasic dialysis optimization were carried out with 500 mg of hair from untreated calves spiked with 50 ng g^{-1} of clenbuterol ($n=3$).

With the diphasic procedure optimized, checking the linearity of the assay was performed on 500 mg of hair from untreated calves, spiked with 12.5, 50, 150, 300 and 400 ng g^{-1} of clenbuterol.

In both cases, the hair was gently shaken at room temperature for 1 h to allow binding of clenbuterol to the hair material [3].

3. Results and discussion

3.1. Diphasic dialysis extraction

The extraction technique of diphasic dialysis was optimized for the analysis of clenbuterol in calf hair by gas chromatography–mass spectrometry (GC–MS). Extraction parameters were optimized to improve the percentage recovery and the cleanliness of the extract, i.e. the absence of chromatographic peaks that might interfere in the GC–MS analysis. The optimization parameters were: pH, extraction solvent, stirring rate (rpm), temperature and extraction time. All of the assays were performed three times with 500 mg of hair spiked with 50 ng g^{-1} of clenbuterol and the results shown are average values.

Initially, *tert.*-butylmethyl ether was used as the extraction solvent, with an extraction time of 4 h, temperature of 37°C and a stirring rate of 150 rpm (optimal conditions for clenbuterol extraction from liver [6]).

The first parameter adjusted was the pH. From our previous work [5,6], we know that a pH of around 9 at the end of the extraction process is needed to achieve a satisfactory extraction with diphasic dialysis. The best results were obtained using sodium citrate buffer to homogenize the hair digest at pH 12. We assume that, during the extraction process, the sulphydryl radicals present in the hair are able to

lower the pH from 12 to a final value of 9. Using different HCl solutions or phosphate buffers, we were unable to adjust the final pH close to 9.

Several solvents (*tert.*-butylmethyl ether, chloroform, dichloromethane, hexane and diethyl ether) were tested. Solvents were chosen according to their lack of miscibility with water so as to avoid the transfer of aqueous homogenate into the dialysis tubing. The best extraction solvent was found to be dichloromethane, for which relatively clean extracts (without interfering peaks) were obtained. The percentage recoveries were 80% (average from three extractions, coefficient of variation, R.S.D.=6%).

The extraction was not effective at 100 rpm (recovery, 49.3%; average from three extractions, R.S.D.=8%), but using a higher stirring rate, 200 rpm, part of the extraction solvent was evaporated before the end of the extraction process and, thus, the recovery decreased. The optimum stirring rate was found to be 150 rpm.

Different extraction times, ranging from 1 to 6 h, were assayed. The recovery improved with increasing extraction times, reaching a maximum at an extraction time of 4 h. When longer extraction times were used, the extraction did not improve and the extracts were noticeably dirtier.

Furthermore, several extraction temperatures were tested, keeping the other parameters constant (sodium citrate buffer, dichloromethane, 150 rpm, 4 h). At 35°C, the recovery was minimum, and at 38°C, the solvent evaporated before the end of the extraction process and clenbuterol recovery decreased. The best percentage recovery obtained was 80%, when the temperature was set to 37°C.

The percentage recovery obtained with diphasic dialysis is higher [3] or similar to that found by other authors [1]. Additionally, this method does not require the operator to perform much work and requires a low volume of organic solvents, mainly because extraction and purification of the extracts are performed in a single step.

3.2. Gas chromatography–mass detection

The analysis of the obtained extracts was carried out by GC–MS. It was performed according to the method proposed by van Rhijn et al. [8] but using a temperature of 300°C at the interface rather than 280°C.

The structures of clenbuterol and metoprolol and their corresponding BSTFA derivatives [9] are shown in Fig. 1.

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

The method proved to be linear from 12.5 to 400 ng clenbuterol g^{-1} of hair ($y=1023x-1054$, $r=0.997$, linearity test was significant with $p<0.001$). An inter-day reproducibility of the calibration curves was performed over three days, with three replicates for each point. A good inter-day reproducibility of the slope of the calibration was obtained (R.S.D., 7.08%) (Table 1). The intra-day repeatability and the reproducibility ($c=50$ ng g^{-1} of hair, $n=10$) were satisfactory with R.S.D.s of 7.1 and 9.5%, respectively. The LOD (signal to noise/ratio=3) of this technique is 5 ng g^{-1} and the LOQ is 12.5 ng g^{-1}. The LOD and LOQ of this technique are smaller than those obtained by other authors, such as Gaillard et al. [10], because larger quantities of hair can be used with diphasic dialysis than can be used with SPE extraction, as, according to our personal experience, it is not possible to introduce a large amount of sample because this leads to poor recoveries and reproducibilities.

Fig. 3 shows the chromatogram (clenbuterol peak at 23.54 min, metoprolol peak at 24.17) and mass spectrum with peaks for the characteristic ions of clenbuterol (m/z 86, 243, 262, 277, 333). These plots correspond to a sample of real hair from a calf treated with clenbuterol (clenbuterol concentration was 58.7 ng g^{-1} of hair).

3.3. In vivo animal assays

Results are shown in Table 2. Only black hair was collected because clenbuterol should accumulate more readily in black than in white hair [11,12]. Therapeutic doses of clenbuterol were detected from ten days up to two months after the treatment, however, the detected quantities were not sufficient to be quantified. For calf B, quantification of the residues was possible only 30 days after the start of the treatment program, since the low growth rate of the hair promotes an important delay between the time when the structure of the hair is achieved under

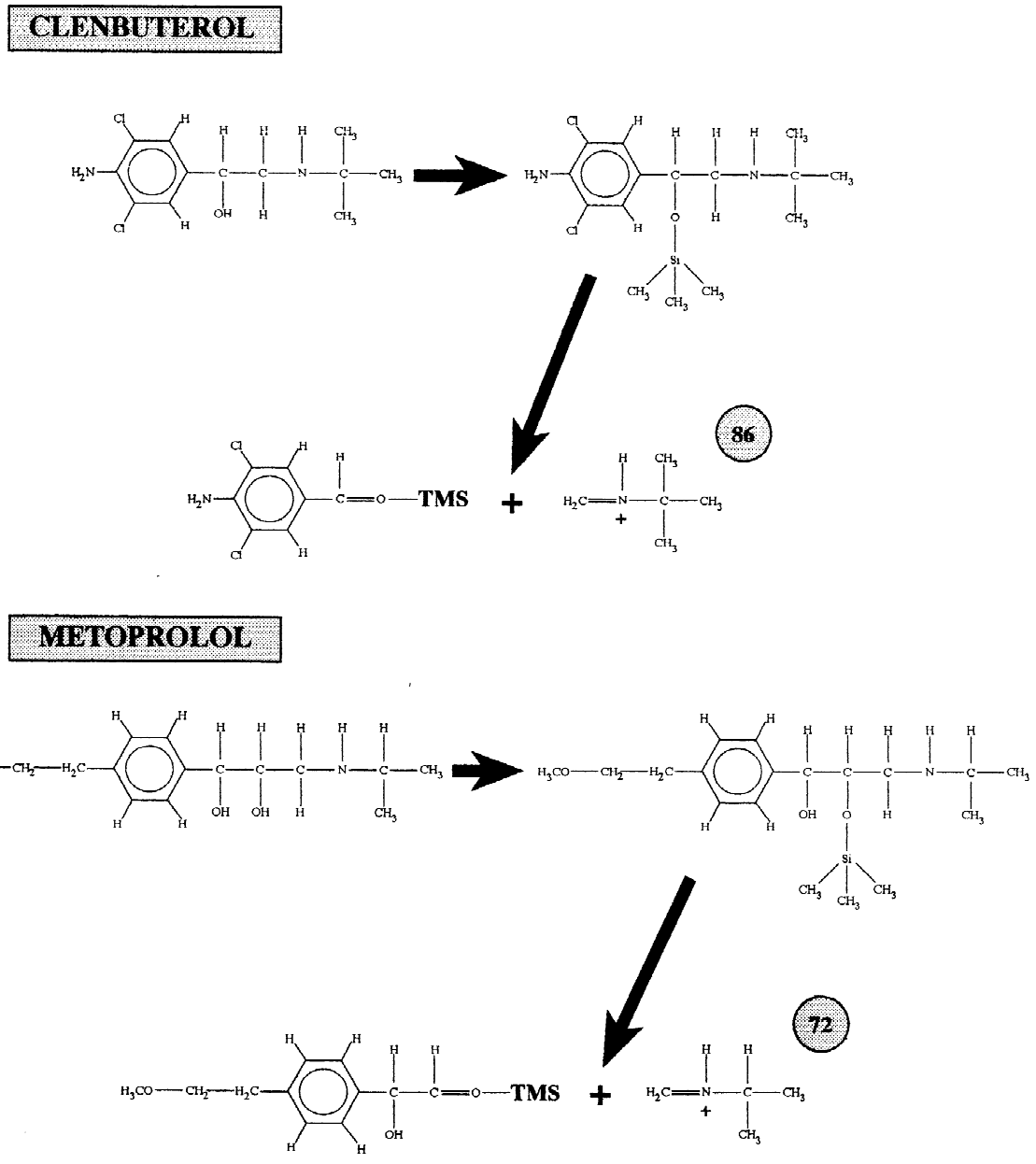

Fig. 1. Structures of clenbuterol, metoprolol and their corresponding BSTFA derivatives and characteristic ions.

the skin and the time when this hair is finally visible on the surface of the animal and when this hair can be collected. Residue accumulation reached a maximum on the 60th day (20 days after the last ingestion of β-agonist). Appelgren et al. [2] and Gaillard et al. [10] have also observed an increase in the accumulation of this residue in hair until a maximum is reached, followed by a decrease in the amount detected. A possible explanation of this phenomenon is that redistribution of the drug occurred from different tissues and organs to the hair. Dürsch et al. [11] observed an accumulation of clenbuterol in

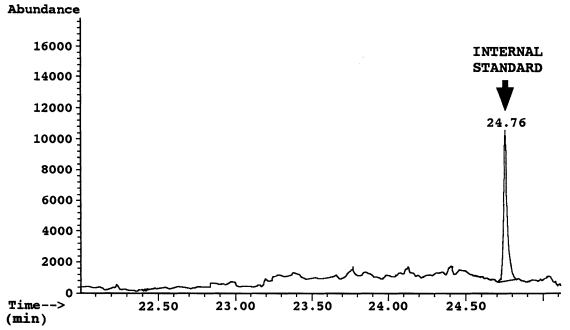

Fig. 2. Chromatogram of a hair blank.

black hair of calves during the first two weeks after treatment. Residues could be quantified in smaller and smaller quantities until 110 days after the first dose, which might be due to the perpetual replacement of hair. Clenbuterol was detected in hair until the 140th day after the start of the treatment.

4. Conclusions

We have developed a method to quantify clenbuterol in calf hair. To perform the clenbuterol extraction from hair by diphasic dialysis and further analysis by gas chromatography–mass spectrometry, dichloromethane was used as the extraction solvent, following an initial adjustment of the pH to 12 by the addition of sodium citrate buffer, and at a temperature of 37°C and a stirring rate at 150 rpm for 4 h. This method has been used for clenbuterol concentration measurements in hair from two calves, one treated with therapeutic doses of clenbuterol and the other with growth promotor doses. Quantification of clenbuterol residues in hair was only possible for the latter calf, and a delay of 30 days after the start

Table 1
Interday reproducibility of the calibration graph for clenbuterol extracts of hair

Day	Calibration graph	Slope	Calibration coefficient
1	$y=1023x-1054.2$	1023	0.997
2	$y=1126x1565.1$	1126	0.993
3	$y=983x+8495.6$	983	0.995
	Mean	1044	
	R.S.D. (%)	7.06	

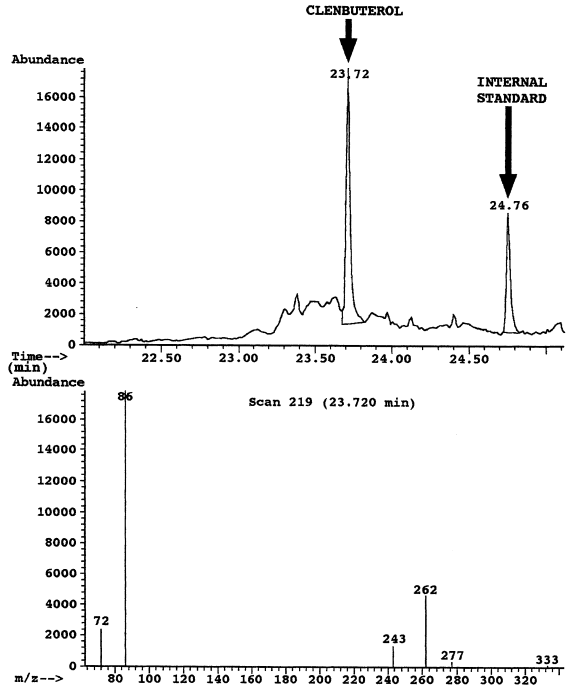

Fig. 3. Chromatogram and mass spectrum showing ions characteristic for clenbuterol (m/z 86, 243, 262, 277, 333) acquired in SIM mode by GC–MS. This sample was a hair sample extracted by diphasic dialysis from an animal treated with clenbuterol, at a concentration of 58.7 ng g^{-1} of hair.

Table 2
Accumulation of clenbuterol in hair of calves following (A) therapeutic treatment and (B) therapeutic and growth-promoting treatment

Sampling days	ng clenbuterol g^{-1} hair	
	Calf A	Calf B
Day 10	P (no quantification)	P (no quantification)
Day 20	P (no quantification)	P (no quantification)
Day 30	P (no quantification)	13
Day 40	P (no quantification)	175.37
Day 50	P (no quantification)	136.75
Day 60	P (no quantification)	301.25
Day 70	ND	75.00
Day 80	ND	47.50
Day 90	ND	73.75
Day 100	ND	36.25
Day 110	ND	27.50
Day 120	ND	P (no quantification)
Day 130	ND	P (no quantification)
Day 140	ND	P (no quantification)

[a] P, Positive; ND, Not detected.

of the treatment was observed. Clenbuterol was detected in hair until the 140th day after the start of the treatment. On the basis of the data presented here, we can propose this method for the detection of fraudulent treatments with clenbuterol of food for animals, because hair is a non-biodegradable matrix and stores a record of what has been administered to the animals.

Acknowledgements

This work was supported by the project FEDER-CICYT 1FD97/0198. The authors wish to thank Consuelo Busto Peteiro for technical collaboration. Special appreciation is extended to Dr. Luis Liz, for clarifying, correcting and proof-reading the text.

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