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Detection of illegal clenbuterol use in calves using hair analysis Application in meat quality control

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Abstract

This study describes a real-life situation involving nine calves, 106 days old, which received oral doses of clenbuterol administered through their milk. Powdered skim milk containing 6.7 mg of clenbuterol was given daily for fifteen days under supervision (i.e. 100 mg per calf for the whole study) to seven calves, and two calves did not receive the drug. Hair samples and urine were taken and subjected to analysis by gas chromatography–mass spectrometry. Hairs were pulverized in a ball mill and 100 mg were incubated in a mildly acidic medium. The sample clean-up procedure involved solid-phase extraction on C_{18} cartridges. Metoprolol was used as the internal standard for quantitation, after formation of methylboronate derivatives. The calibration curve for clenbuterol in hair was linear in the range 20–5000 pg/mg. The limit of detection of clenbuterol was 16 pg/mg in hair and 0.14 ng/ml in urine. Hair testing was effective after 7–10 days of treatment, and concentrations were in the range of 20 to 4372 pg/mg. Urinalysis can detect clenbuterol for up to two weeks after discontinuation of the drug. Conveniently, this is around the time when the hair samples attain greatest sensitivity. Therefore, the combination of the two matrices appears to be the method of choice for testing for the illegal use of drugs in meat-producing animals. © 1997 Elsevier Science B.V.

Keywords: Clenbuterol

1. Introduction

Introduced into human therapy in the 1970s, β_2-agonists are in widespread use as bronchodilators. They are administered primarily as aerosols, nebulizers, pills and, in cases of emergency, by injection, with the principal drugs being salbutamol, salmeterol and terbutaline. However, there are many β_2-agonists that have a low therapeutic index and are not generally used in humans. These include albuterol, cimaterol, methyl-cimaterol, clenbuterol, methyl-clenbuterol, fenoterol, mabuterol, methyl-mabuterol, mapenterol, metaproterenol and tulobuterol [1]. Moreover, when used for purposes that were not envisaged, these drugs have resulted in a substantial increase in muscle mass. Delbeke et al. [2] reported that between 1988 and 1993, Belgian body-building enthusiasts frequently used clenbuterol in addition to anabolic steroids. Clenbuterol was documented in weightlifting more than a year before its appearance in other sports. Since then, numerous authors have investigated the performance-enhancing properties of such drugs [3–6] and have speculated on the possible uses of clenbuterol in winning Olympic medals [7,8].

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Similarly, β_2-agonists are also employed successfully in veterinary therapy, but for two different reasons. These drugs augment physical performance (horse racing, pigeon racing), or increase the mass of animals for human consumption. Being aware of possible abuses, the public representatives of the European Economic Community voted in July 1997 on a law to ban the use of β_2-agonists in all animals destined for human consumption [9]. Unfortunately, discontinuing the treatment several days before the animal is slaughtered (the time at which urine is taken) is sufficient to produce a negative result in urine tests. Naturally, the anabolic effects on the weight of the animal remain. A study by Salleras et al. [10] documented 113 cases of human clenbuterol intoxication in Spain in 1992 from ingestion of cow liver. Fortunately, no one died from the episode, but several people were seriously injured with myalgia, tachycardia, trembling and headaches. Certain organs, such as the liver, retain high concentrations of the drugs. Human consumption of β_2-agonists from treated animals is a significant public health issue that would merit more effective and powerful means to ensure that animals are not treated with the drugs. As toxicologists of many years standing, we have been studying toxins in human hair [11–14]. In this study, we examined the possibility of using animal hair to screen for and quantify clenbuterol. Other testing materials have been explored previously, e.g. urine, for horse racing [15], feces, for pigeons racing [16], and synovial liquid from cattle carcasses [17]. However, even if the methods themselves are sensitive, the quality of the test is mediocre at best, due to a short testing period. More recently, Polettini et al. [18] demonstrated that clenbuterol could be detected in guinea-pig hair. Each guinea pig received a dose of 0.12 mg/kg of clenbuterol intraperitoneally for fifteen days. Analysis was done by gas chromatography–mass spectrometry (GC–MS). The analytes were derivatized using trimethyl boroxime, forming methylboronate (MB) derivatives [19,20]. The derivatives produce a mass spectrum in which the principal ions are of a higher mass and are present in a much greater abundance than the ions from the BSTFA derivatives, thus increasing the detection sensitivity and the quality of the analysis. Because of the presence of two chlorine atoms on the clenbuterol molecule, detection by negative chemical ionization using ammonia as the reactant gas has also been proposed [21]. There are, of course, rapid immunoenzymatic (ELISA) detection kits available [16,17] and other authors propose identification by cryotrapping GC–Fourier transform infrared spectrometry [22]. Concerning extraction, many procedures exist, both in the liquid and the solid phase, although the latter seems to be preferred — either on silica (Extrelut) [17], by semi-preparative immunoaffinity chromatography [23] on a C_2 cartridge [24] or using a mixed phase [18].

The present study concerns a real-life situation involving nine calves, 106 days old, seven of which received oral doses of clenbuterol, administered through their milk. The milk, containing 6.7 mg of the drug, was given daily for fifteen days under official supervision (i.e. 100 mg per calf for the whole study). Two calves did not receive the drug. The results and conditions of the study are discussed below.

2. Experimental

2.1. Instrumentation

The apparatus used was a GC-17A gas chromatograph from Shimadzu, supplied by Touzart et Matignon (Courtaboeuf, France), an AOC 17 automatic injector with a QP-5000 mass selective detector. The analytical column was a CP SIL 8 CB, 25 m×0.25 mm I.D. (0.25 μm film thickness) from Chrompack (Les Ulis, France). Helium was used as the carrier gas at a flow-rate of 1.2 ml/min in the constant flow mode (i.e. 65 kPa at 80°C). The temperature of the detector was 300°C. Pulsed splitless injection (4 μl) was done at 280°C under a pressure of 100 kPa for 1 min. The initial oven temperature was 80°C for 2 min and was increased to 310°C at 15°C/min, where it was held for 2.67 min. The chromatography time was 20 min. Retention times of the compounds were 12.9 min for metoprolol-MB [internal standard (I.S.)] and 13.3 min for clenbuterol-MB. The relative retention time was 1.03. Scans were performed from 240 to 305 a.m.u. between 12.5 and 14 min. Quantitation was realized using the multiple ion chromatogram (MIC) by the sum of ions m/z 276 for metoprolol and 243 and 285 for clenbuterol.

For solid-phase extraction (SPE), we used C_{18}, 200 mg/3 ml (Isolute) cartridges from IST, supplied by Touzart and Matignon, and a Vac Elut sample processing station (Analytichem International) from Prolabo (Paris, France).

The ball mill (type MM2) was purchased from Retsch (Haan, Germany).

2.2. Reagents

Dichloromethane, ethyl acetate, methanol, 0.1 *M* hydrochloric acid, 1 *M* sodium hydroxide, anhydrous sodium sulfate (Na_2SO_4) and analytical grade sodium bicarbonate ($NaHCO_3$) were from Carlo Erba (Milan, Italy). Clenbuterol and metoprolol were purchased from Sigma–Aldrich (Paris, France), reference numbers C-5423 and M-5391, respectively. Trimethylboroxine, the cyclic anhydride of methylboronic acid, was also purchased from Sigma–Aldrich, reference number 32313-6.

2.3. Calibration and reagent solution

A stock solution containing 10 μg/ml of each drug (free-base) was prepared in methanol. Subsequent dilution was needed for clenbuterol and standard solutions at 1 and 0.1 μg/ml were also prepared and stored at 4°C for six months.

The reagent for conditioning the SPE columns was prepared by dissolving 1680 mg of sodium bicarbonate in 100 ml of 10% methanol in water (0.2 *M*), pH 8.6. This reagent was also used as a diluting buffer for the different samples.

The derivatizing solution was prepared by dissolving 15 μl of the anhydride in 5 ml of ethyl acetate. This solution was then filtered over 2–3 g of anhydrous sodium sulfate and stored at 4°C in an amber glass vial for not longer than one week.

Drug-free hair samples were collected to realize a standard at 5000 pg/mg of clenbuterol. A 500-μl stock solution of drugs was added to exactly 1 g of powdered hairs. After evaporation of the methanol at ambient temperature, the fortified hairs were homogenized using the ball mill at high speed for 10 min. Other points for the calibration curve were realized in the same way using different dilutions of the methanolic stock solution to obtain concentrations of 2000, 500, 200, 50 and 20 pg/mg. Drug-free materials were collected from drug-free calves. Biological matrices were pooled, extracted and analyzed using this method. No peak corresponding to compounds of interest was obtained.

2.4. Animals and drug administration

Nine black and white male calves, Holstein breed, 77 days-old and weighing 49 kg (±2) were put in light- and temperature-controlled stables at the Centre Expérimental de Sourche (Saint Symphorien, France). The individual cages were constructed of stainless steel and had a grated flooring that was 10 cm above the ground. This was to avoid any possible contamination of the animal's coat by urine and feces. Urine was collected every 24 h in order to track the disappearance of the toxin. A complete urinalysis was not performed because we were only trying to confirm or deny the presence of clenbuterol in the urine samples to determine if it was higher or lower than the limit of detection. The calves were fed 2.5–3.0 kg of powdered skimmed milk, provided by the VALS company (Champagné, France), per day. When they were 106 days old, the calves were administered clenbuterol per os. They weighed 105 kg (±10). Seven calves were given 3.33 mg of clenbuterol (in milk) both in the morning and at night, receiving a total dose of 100 mg in fifteen days. Two calves did not receive any clenbuterol and were raised under the same conditions as the others. The collection of hair was done using an electric clippers, Aesculap from Werke (Tuttlingen, Germany), which had a 7-cm blade. The hair was cut from the flanks of the animal in timed successive parallel passes without regard to the animal's black and white spots. No distinction was made regarding hair color because the aim of the study was to test the procedure in a real-life situation (considering that people who perform the sampling may not be trained in this technique and the individual variation in the distribution of an animal's color). Thus, we took hair without selecting for black hair, which should normally be the more concentrated. Approximately 3–5 g of hair were removed each time. Once a cut was done, hair was not cut again in this area. Then, the hairs were washed, finely cut with a scissors and pulverized.

2.5. Method of collection

Urine and hair were collected from the calves one day before the administration of clenbuterol in order to certify that all urine tests and measurements in hair for clenbuterol would be negative. Only a urine specimen was taken on the first day. From day three to day fifteen, hair samples were taken daily from all animals except one (sample collection for this calve would commence on day 54 and go on until day 95). The animals were then slaughtered to preserve all of their organs, the first animal being killed six days after the end of the treatment program, and then one animal every five days until day 47. The different tissues were frozen immediately to be used in another study. The carcasses that remained were subsequently destroyed.

2.6. Hair wash and pulverization

During analysis of human samples, decontamination procedures for drugs of abuse are very important. A similar contamination scenario seemed to be difficult for drugs that normally should be taken by mouth or by another therapeutic route [25,26]. However, in the absence of more information, we used the same procedures as those recommended for the analysis of drugs of abuse in human hair [11–14,27]. According to the Society of Hair Testing [28], the hairs should be washed in ethanol, then in two phosphate buffer baths (0.1 *M*, pH 5.0) for 3 min followed by two dichloromethane baths (also important to remove the external fat from the hair). The last wash is evaporated, derivatized and then analyzed according to our method to verify the absence of clenbuterol. After drying the hair at room temperature, it was finely cut with a scissors, then ground in a ball mill until a gray homogenous powder was obtained.

2.7. Extraction procedures

Powdered fortified hair (100 mg) or unknown samples were weighed in a conical vial and 10 μl of the I.S. methanolic solution were added (10 μg/ml), together with 2 ml of 0.1 *M* hydrochloric acid. After incubation at 56°C for 12 h, the vials were centrifuged at 1500 *g* for 5 min. The supernatant was transferred to a clean vial and 1 ml of 0.1 *M* hydrochloric acid was again added to the residue, shaken, centrifuged and the supernatant was removed. The two fractions were combined, neutralized with 1 *M* sodium hydroxide and buffered with 3 ml of 0.2 *M* sodium bicarbonate containing 10% aqueous methanol.

The columns were conditioned with 4 ml of methanol followed by 2 ml of bicarbonate buffer (pH 8.6). The prepared sample was then applied to the column and allowed to drain under vacuum. The columns were washed with 1 ml of water and then with 1 ml of 10% aqueous methanol, and dried by passing air through them for 15 min. The analytes were eluted with four 500 μl volumes of methanol. The eluate was evaporated under a stream of nitrogen at 40°C. Next, the residue was dissolved in 30 μl of derivatizing solution, incubated at 80°C for 15 min and 4 μl were injected.

The use of the bicarbonate buffer is intended to remove the activation solvent (methanol) and replace it with a liquid that is similar in composition to the sample matrix (salts, pH, solvent strength). A small concentration of methanol in the applied liquid sample (about 5%) improved reproducibility and reduced interferences [29,30].

Concerning the urine, a 4-ml sample was buffered with 4 ml of bicarbonate buffer (pH 8.6) and 10 μl of stock I.S. solution were added. The previously described SPE method used for hair was also used for urine. The extraction columns used were also the same, i.e., C_{18} 200 mg/3 ml, from IST.

3. Results

3.1. Precision and recovery

The relative standard deviations (R.S.D.s) of the within-day precision were always less than 6.2% ($n=8$ for each point on the calibration curve). R.S.D.s of the between-day precision study were 10.1, 8.9, 7.0, 5.3, 3.0 and 3.1% at 20, 50, 200, 500, 2000 and 5000 pg/mg respectively ($n=10$).

The overall recovery of clenbuterol was 91.5% at 200 ng/mg and 90.6% at 2000 ng/mg ($n=8$).

3.2. Linearity

Analysis of variance (ANOVA) was applied to determine if the system was linear, using an F-test. There are two contributions; one due to the regression and one that is not described by the linear model, i.e. residual. The residual variation can be divided into two contributions, i.e., lack of fit and pure analytical error. $F_{calc}=2493.4 \gg F_{theoretical}$; $F_{theoretical}$ is for (1, $n-2$) degrees of freedom=5.32, so the source of variation is well described by the regression, $F'_{cal\ (lack\ of\ fit)}=3.89<F'_{theoretical}$; $F'_{theoretical}$ is for [$(n-2)-n/2$, $n/2$] degrees of freedom=5.41, so the model (linear regression) can be considered to be correct [31–33].

3.3. Weighted linear regression

After affirmation of linearity, the linear regression model must be chosen. Over a wide dynamic calibration range, the y-errors tend to become larger as x increases, i.e. non-homogeneity of the variances. This can be easily confirmed by performing a Hartley's test, which will decide the type of linear regression, i.e., weighted or unweighted [32]. In the present case, r_{calc} was much higher than $r_{theoretical}(k,\nu)$, where k is the number of calibration points and $\nu=n-1$ degrees of freedom. Weighted linear regressions are characterized by the introduction of a weighting factor, ω, which is attributed to each point of the calibration range. The equation used was as follows:

$$Y_{\omega} = 0.00453114\, X_{\omega} - 0.00000284 \left[\left(\frac{\text{amount ratio (analyte/I.S.)}}{\text{response ratio (analyte/I.S.}}\right)\right]$$

3.4. Limits of detection (LODs)

The LOD is given by the mathematical formula: $\text{LOD}=m_{blank}+6s_{blank}$, where m_{blank} is the mean of the blanks and s_{blank} corresponds to the standard deviation of the blanks ($n=30$) [32–34].

Under these conditions, the LOD for clenbuterol in hair was 16 pg/mg, whereas the LOD in urine was 0.14 ng/ml.

3.5. Acidic solubilisation

Although clenbuterol is stable under strong alkaline conditions, unlike Polettini et al. [18], we prefer to use mild acidic solvation of the drugs. These mild conditions avoid creating a complex matrix when using the chromatographic system to analyze a dirty extract. This enables us to obtain a very pure extract, thus lowering the background chromatographic noise and enhancing sensitivity. The sample clean-up step is important, of course, but it is more important still to pretreat the hair, ensuring that is as effective and clean as possible.

3.6. Sample clean-up

The recovery of clenbuterol using C_{18} cartridges was 91.5% (200 pg/mg, $n=10$). It was 87.1% (10 ng/mg, $n=3$) using a mixed phase system, such as Bond Elut Certify [18]. Moreover, the protocol for manipulation of a classic reversed-phase C_{18} cartridge is simpler than that for a mixed phase. This improved the reproducibility: Intra-assay precision was 2.7% at 5 ng/mg compared to 11.5% for a mixed phase at the same concentration, $n=10$).

3.7. Chromatography

The inlet temperature of 280°C ensures the efficient flash vaporization of the sample. The large volume injected (4 μl), which can sometimes create a peak broadening effect, is compensated for by the nature of the injection solvent and the initial inlet conditions. A 4-μl volume of ethyl acetate give a vaporized volume of 617 μl, which is less than inlet liner volume, at 280°C under a pulsed splitless injection of 100 KPa. Thus, flashback troubleshooting should not occur. The initial flow-rate of 1.8 ml/min for 1 min transfers the vapor to the column in about 20 s. In order to obtain very narrow peaks, an initial column temperature of 80°C was judged to be sufficient to provide an adequate stationary phase focusing effect. Since retention of solutes is an exponential function of temperature, the lower the inlet temperature, the slower the speed at which solutes migrate into the column. Thus, when a

vaporized sample comes in contact with an adequate stationary phase, it is trapped in a narrow zone.

3.8. Detection

The very restricted mass sweep from 240 to 305 a.m.u. permits two things. First, the small variation in the mass analyzed allows for a significant ion retention time and leads to much greater sensitivity. Secondly, the two methylboronate derivatives formed have the essentials of their ionic repartition (electronic impact detection mode) on the same mass range (240 to 305 a.m.u.). The principle ions are m/z 276, 275, 291, 277, 290 and 246 for metoprolol-MB and m/z 243, 285, 245, 287, 300, 244, 286 and 302 for clenbuterol-MB. The identification specificity is, thus, excellent. Chromatograms of the total ion currents and specific ion extracts from 100 mg of standard calf hair spiked with 20 and 2000 ng/mg of clenbuterol are shown in Fig. 1 Fig. 2, respectively.

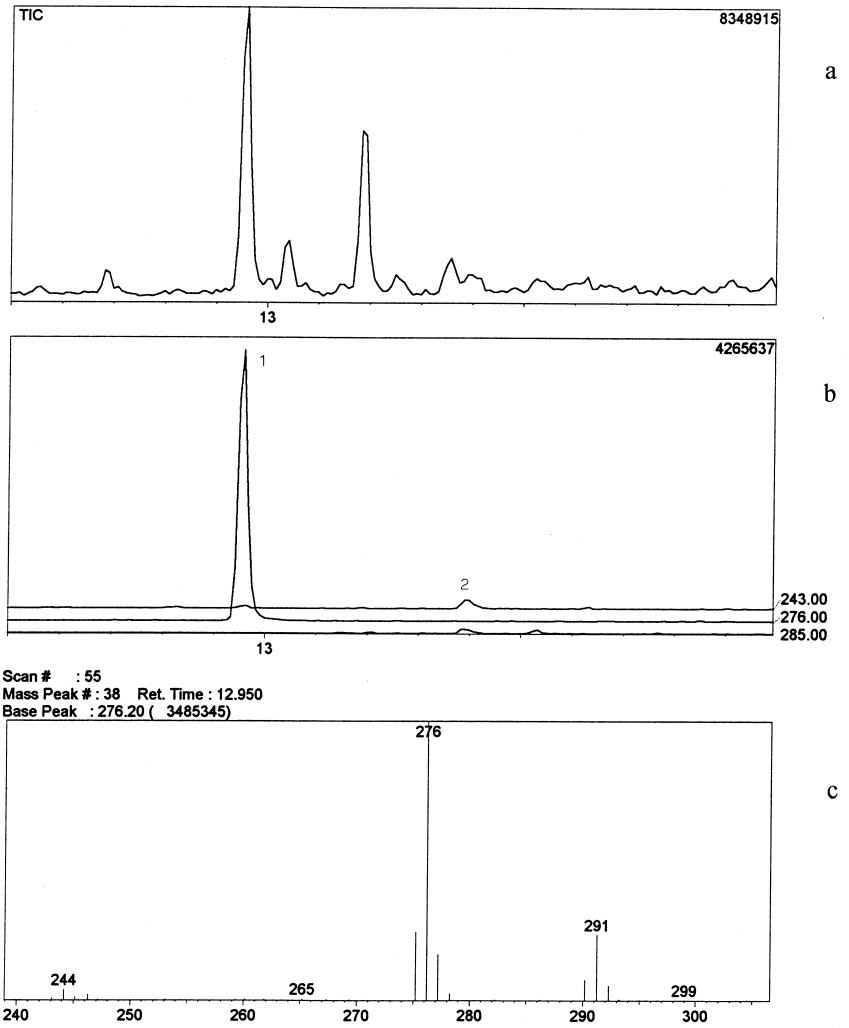

Fig. 1. Chromatogram of an extract of 100 mg of calf hair spiked with 20 pg/mg of clenbuterol. (a) Total ion current, (b) selected ions at m/z 276, 243 and 285 and (c) full mass spectrum of metoprolol-methylboronate. Peaks: 1=I.S., 2=clenbuterol-MB.

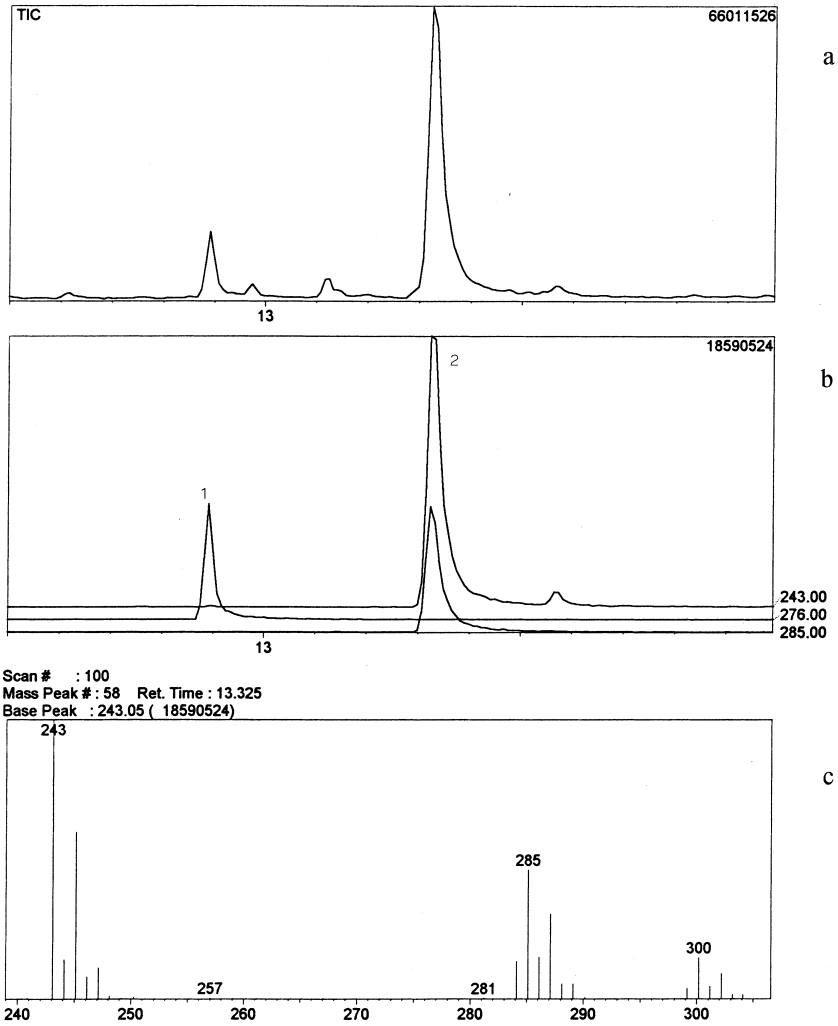

Fig. 2. Chromatogram of an extract of 100 mg of calf hair spiked with 2000 pg/mg of clenbuterol. (a) Total ion current, (b) selected ions at m/z 276, 243 and 285 and (c) full mass spectrum of clenbuterol-methylboronate. Peaks: 1=I.S., 2=clenbuterol-MB.

In Fig. 1C and Fig. 2C, the mass spectra of metoprolol-MB and clenbuterol-MB are shown. The chromatogram obtained for a 100-mg extract of a blank sample is shown in Fig. 3, while that for an extract of a real sample is shown in Fig. 4.

The concentrations of clenbuterol in hair and detection in urine for the nine different calves are presented in Table 1. The levels in the hair are expressed in pg/mg of hair ($n=3$). For the urine samples, we have indicated only whether they tested positive or negative for clenbuterol.

4. Discussion

We would like to point out that in the two untreated calves (Nos. 8 and 9), clenbuterol was not detected in either urine or hair. The results were markedly different, however, for calves 1–7, which received the complete treatment of 100 mg of clenbuterol over fifteen days. From the first day, we could detect the presence of clenbuterol in the urine, which is a normal consequence of the drug's pharmacokinetics. The urine from all remaining calves

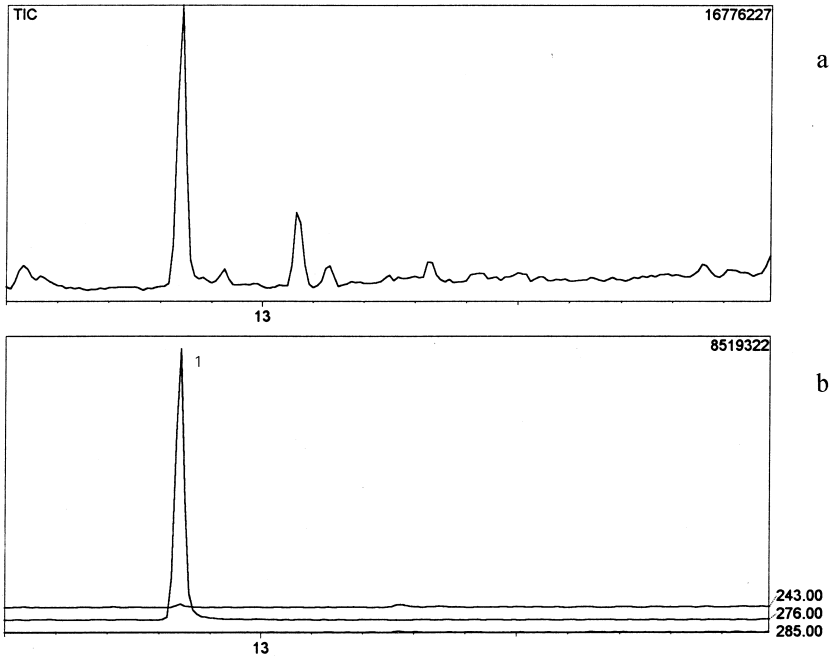

Fig. 3. Chromatogram of an extract of 100 mg of a blank calf hair sample. (a) Total ion current and (b) selected ions at m/z 276, 243 and 285. Peaks: 1=I.S..

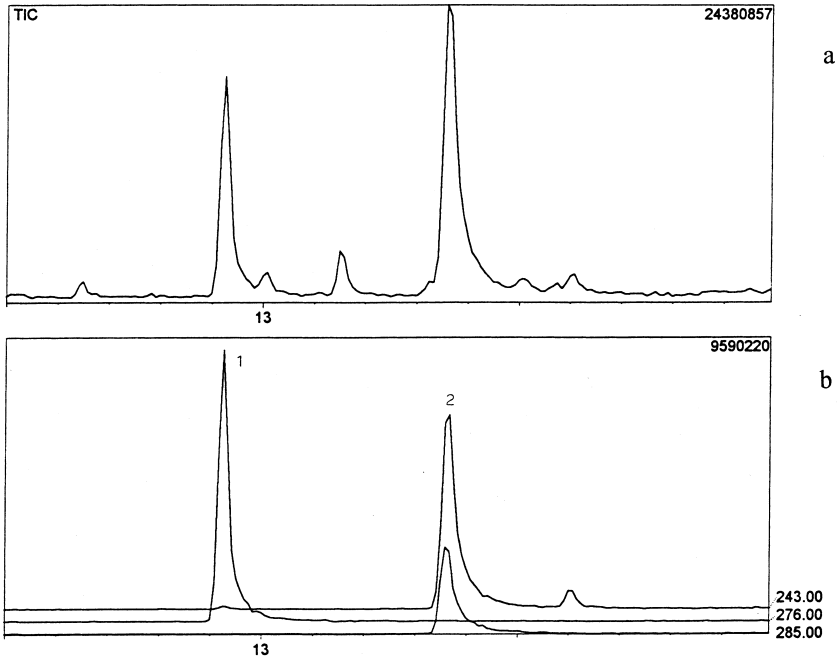

Fig. 4. Chromatogram of an extract of 100 mg of a real calf hair sample taken at day 41 from calf number 5. (a) Total ion current, (b) selected ions at m/z 276, 243 and 285. Peaks: 1=I.S., 2=clenbuterol-MB at a measured concentration of 1083 ng/mg.

Table 1
Concentration of clenbuterol in hairs and detection in urine of the nine different calves

Day	Calf 1		Calf 2		Calf 3		Calf 4		Calf 5		Calf 6		Calf 7		Calf 8		Calf 9	
	Hairs (pg/mg) (SD)	Urine	Hairs (pg/mg) (SD)	Urine	Hairs (pg/mg) (SD)	Urine	Hairs (pg/mg) (SD)	Urine	Hairs (pg/mg) (SD)	Urine	Hairs (pg/mg) (SD)	Urine	Hairs (pg/mg) (SD)	Urine	Hairs (pg/mg) (SD)	Urine	Hairs (pg/mg) (SD)	Urine
D−1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D 1	−	P	−	P	−	P	−	P	−	P	−	P	−	P	−	ND	−	ND
D 3	ND	P	ND	P	ND	P	ND	P	ND	P	ND	P	−	P	ND	ND	ND	ND
D 4	ND	P	ND	P	ND	P	ND	P	ND	P	98	P	−	P	ND	ND	ND	ND
D 5	ND	P	ND	P	ND	P	ND	P	ND	P	ND	P	−	P	ND	ND	ND	ND
D 6	ND	P	ND	P	ND	P	ND	P	ND	P	ND	P	−	P	ND	ND	ND	ND
D 7	ND	P	39 (3)	P	33 (6)	P	ND	P	ND	P	ND	P	−	P	ND	ND	ND	ND
D 8	ND	P	16 (4)	P	22 (3)	P	ND	P	ND	P	16 (2)	P	−	P	ND	ND	ND	ND
D 9	ND	P	28 (2)	P	25 (3)	P	ND	P	ND	P	16 (3)	P	−	P	ND	ND	ND	ND
D 10	31 (6)	P	16 (2)	P	50 (3)	P	25 (3)	P	21 (2)	P	24 (4)	P	−	P	ND	ND	ND	ND
D 11	20 (5)	P	27 (4)	P	334 (20)	P	18 (3)	P	49 (2)	P	24 (3)	P	−	P	ND	ND	ND	ND
D 12	16 (5)	P	17 (2)	P	200 (12)	P	17 (3)	P	71 (2)	P	23 (5)	P	−	P	ND	ND	ND	ND
D 13	40 (4)	P	38 (5)	P	339 (15)	P	17 (4)	P	79 (9)	P	60 (4)	P	−	P	ND	ND	ND	ND
D 14	128 (11)	P	36 (4)	P	334 (26)	P	98 (6)	P	78 (6)	P	64 (4)	P	−	P	ND	ND	ND	ND
D 15	42 (4)	P	100 (9)	P	430 (31)	P	100 (7)	P	88 (7)	P	190 (9)	P	−	P	ND	ND	ND	ND
D 21	45 (5)	P	150 (19)	P	105 (29)	P	87 (6)	P	170 (11)	P	225 (18)	P	−	P	ND	ND	ND	ND
D 26			198 (20)	P	1758 (52)	P	115 (9)	P	131 (9)	P	328 (17)	P	−	P	ND	ND	ND	ND
D 31					373 (26)	ND	340 (20)	P	150 (9)	ND	1978 (96)	P	−	P	ND	ND	ND	ND
D 36							2210 (140)	ND	1427 (87)	ND	2841 (124)	ND	−	ND	ND	ND	ND	ND
D 41									1083 (55)	ND	4372 (152)	ND	−	ND	ND	ND	ND	ND
D 47											3357 (179)	ND	−	ND	ND	ND	ND	ND
D 54													2650 (201)	ND	ND	ND	ND	ND
D 61													1500 (100)	ND	ND	ND	ND	ND
D 75													976 (54)	ND	ND	ND	ND	ND
D 82													1495 (102)	ND	ND	ND	ND	ND
D 89													1567 (95)	ND	ND	ND	ND	ND
D 95													746 (54)	ND	ND	ND	ND	ND

P: Positive.
ND: Not detected.
−: not realized.

was negative (the four that had not yet been slaughtered) on the 36th day of the study or 21 days after stopping treatment, however, it was still testing positive sixteen days after stopping the treatment. The direct implication of this is that if a cattle farmer uses clenbuterol, he would have to wait around two–three weeks to ensure that the animal's urine would not test positive.

Hair testing becomes effective after seven to ten days of treatment and the drug can be detected henceforth at low, but significant, levels. The growth rate of hair is 6–7 mm per month, while the thickness of the skin is between 2.5 and 3 mm for a three-month old calf. Hairs do not grow continuously but rather in cycles. Each cycle consists of a growing period (anagen), during which the follicle is actively producing hair, and a resting period (telogen), when the hair is retained in the follicle as dead hair that is subsequently lost. There is also a transitional period (catagen) between these two stages [35]. The hair cycle, and thus the hair coat, is controlled by the period of light, ambient temperature, nutrition, hormones, the animal's general state of health, genetics and other poorly understood intrinsic factors. During the anagen period, when clenbuterol is actively being incorporated into the structure of the hair, the follicle is located at 75–85% of the skin's depth, i.e., between 1.9 and 2.6 mm under the surface of the skin [36]. The rather low growth rate of the hair (compared to that of humans) would provide an

important delay between when the structure of the hair is established under the skin and when this hair is finally visible on the surface of the animal, or when this hair can be collected from the animal. Considering these factors, it is logical that positive identification of clenbuterol occurs about seven to ten days after the drug was first administered. Another direct consequence of the growth cycle of the hair on the detection of the drug is determined by the seasons. Hair follicle activity and, thus, hair growth rate, is maximal in summer and minimal in winter. For example, up to 50% of hair follicles may be in the telogen phase in the summer and may increase to 90% in winter. Maximal sensitivity for drug detection is thus obtained in summer when incorporation would be more effective. On the other hand, the more frequent replacement of hair in summer diminishes the time during which the drug can be detected. For animals housed indoors, these remarks are less true.

More remarkable, however, is that the concentration in the hair increased significantly after the treatment was stopped, with steep rises in peak concentrations between day 26 and day 36. However, we have no explanation of this phenomena. Since clenbuterol can be detected in urine for up than two weeks after discontinuation of the drug, it might be possible that redistribution of the drug occurred from different tissues and organs to the hair. A study with radiolabeled substances would be helpful to explore such a statement.

After the peak concentration, which was found on days 26–36, drug levels in hair decreased slightly with time, due to the perpetual replacement of the hair.

We will make one final comment on the results. Nakahara et al. [37] have shown that the incorporation of molecules in the hair is a function of three criteria: Affinity to melanin, liposolubility (affecting migration through the membranes) and the pH gradient between blood and hair. All the physicochemical constants favor clenbuterol (basic molecule that is very liposoluble, the hair being an acidic medium) [11,14,37,38]. Thus, it is normal that clenbuterol should accumulate more readily in black hair than white, this phenomena having been previously noted in the study by Polettini et al. [18] on black and white hair from guinea pigs. The calves used in our study were two-toned; the parallel rows of hair were clipped independent of hair color. Thus, samples may be non-homogeneous, explaining the variation in the results.

5. Conclusions

We have developed a simple and powerful method to quantify clenbuterol in calf hair. Seven calves received 100 mg of the product per os in 3.33 mg doses, twice daily for fifteen days. The concentrations measured in the hair were between 20 and 4372 pg/mg. Urine and hair analysis were shown to complement each other very well during the periods of time that cover the two matrices. It is true that urine remains the matrix of choice for many authors for revealing the on-going use of clenbuterol. Urinalysis can detect clenbuterol for up to two weeks after discontinuation of the drug. Further investigations should focus on (a) analysis of homogeneous black hair samples and white hair samples, (b) elucidation of the mechanism involved in the steep rises observed between day 26 and day 36 using labeled substances.

Hair, not being very biodegradable, stores a powerful record of what has been administered to animals. Thus, the joint analyses of urine and hair would cover all possible periods and would be an effective means of combating clenbuterol use in animal products that are destined for human consumption. Lastly, it would seem that the International Olympic Committee might find the sensitivity of the two combined analyses, and the non-invasiveness of the procedures, useful when testing for banned substances.

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