Immunodetection of Clenbuterol in the Hair of Calves

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To validate the use of hair as a target for the detection of clenbuterol, hair was collected from two groups of calves receiving clenbuterol at two doses of 10 ($n = 3$) and 100 μg/kg ($n = 3$) for 10 days. Clenbuterol was extracted from hair by an immunochromatography procedure and quantified by an enzyme immunoassay. Following the 10 μg/kg dose, clenbuterol could be detected at day 20 after the last dose. However, at the 100 μg/kg dose, clenbuterol could be found in hair 40 days after the last treatment. These data confirm that hair is a target of choice for the detection of clenbuterol as a drug residue when this drug is found undetectable in urine and tissue.

Keywords: *Clenbuterol; meat residue; hair analysis; affinity chromatography; enzyme immunoassay*

INTRODUCTION

Clenbuterol, a therapeutic agent in veterinary medicine, has been reported to be used illegally in the meat-producing industry. At supratherapeutic doses, this β_2-agonist is a repartitioning substance, increasing muscle growth and decreasing fat deposition. Under such conditions, clenbuterol residues in meat constitute a potential health risk for consumers (Martinez-Navarro, 1990). However, detecting clenbuterol residues at the time of slaughter is limited by two main factors: rapidly decreasing tissue clenbuterol concentrations to subnanogram levels during the withdrawal period and the unavailability of sensitive analytical methods to detect tissue residues at this time (Girault et al., 1990).

In a recent study (Adam et al., 1994), we showed that clenbuterol, like other illicit drugs (Baumgartner et al., 1989), could be identified in the hair of rats until 40 days after the last dose. This observation pleads for the detection of its chronic illicit use.

The purpose of the present work was to document the hair disposition of clenbuterol in calves.

MATERIALS AND METHODS

1. Materials. Clenbuterol, sodium dodecyl sulfate (SDS), Tween 20, Tris base, Tris-HCl, and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). Protein G Sepharose (MabTrap G) and Sepharose 4B were from Pharmacia (Uppsala, Sweden). Enzyme immunoassay grade alkaline phosphatase was acquired from Boehringer Mannheim Biochemica (Mannheim, Germany), and A/C Nunc immunoplates were supplied by Nunc (Roskilde, Denmark). Econo columns were from Bio-Rad Laboratories (Richmond, CA).

The Speed Vac was from Savant Instruments Inc. (Farmingdale, NY), the ELISA processor from the Behring institute (Marburg, Germany), and the Rotavapor from Buchi Labs (Flawil, Switzerland). A Hewlett-Packard gas chromatograph Model 5890, mass selective detector Model MSD-5970, and HP-5 column (Avondale, PA) were used for the gas chromatography/mass spectrometry (GC/MS) step. Sep-Pak C_{18} cartridges were obtained from Waters Chromatography (Milford, MA), and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was supplied by Aldrich Chemical Co. (Milwaukee, WI). The other reagents were all of analytical grade purchased from Fisher Scientific (Muskegon, MI).

2. Methods. 2.1. Animal Treatment. Two groups of calves (mean weight 139 ± 27 kg) respectively received 10 ($n = 3$) and 100 μg/kg ($n = 3$) of clenbuterol subcutaneously for 10 days. Hair was collected on days 2, 4, 6, 8, and 10 of the treatment period as well as on days 10, 20, 30, and 40 of the washout period.

Concomitantly, hair on the left side of the animals was shaved before the first clenbuterol treatment. Growing hair samples were collected from this area on days 20 and 40 of the washout period.

2.2. Analytical Procedures. The analytical procedures used have been described previously for the quantification of clenbuterol in rat hair (Adam et al., 1994).

a. Sample Preparation. The hair (approximately 100 mg) was washed with an aqueous solution of 1% SDS and rinsed with distilled water. Clenbuterol was extracted from the cleaned hair by 3 mL of methanol.

Growing hair was analyzed sectionally by aligning it on a dissection board and cutting it into 1-cm sections, starting from the root up to the tip, before the washing step. Then, each section of hair was washed and extracted separately.

b. Immunoextraction and Quantification of Clenbuterol by Enzyme Immunoassay (EIA). Following methanol evaporation, the residue containing clenbuterol was resuspended in 500 μL of a 50 mM phosphate buffer (pH 7.4) and immunoextracted by affinity chromatography, using monoclonal anti-salbutamol immunoglobulin (IgG), which displays 75% cross-reactivity with clenbuterol, 7% with terbutaline, and less than 1% with other β_2-agonists (Adam et al., 1990).

The immunoextracted material was quantified in triplicate by a competitive EIA (Adam et al., 1991). The identity of the immunoreactive material was finally confirmed by GC/MS (Adam et al., 1994).

c. GC/MS. The residue of the immunoextraction procedure was dissolved in 2 mL of 0.2 M phosphate buffer (pH 6.9) and applied on a Sep-Pak plus C_{18} cartridge previously primed with 5 mL of methanol and 10 mL of distilled water. After two washings with 5 mL of water and hexane, elution was undertaken with 5 mL of methanol. After the addition of 20 μL of a 5% v/v aqueous solution of hydrochloric acid, the methanolic solution was evaporated to dryness under a stream of nitrogen. Clenbuterol was extracted from the residue by 5

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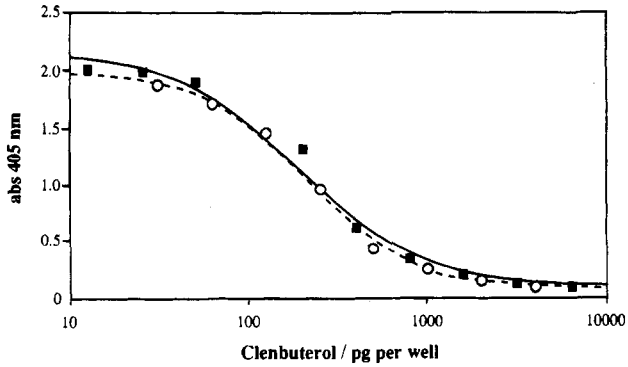

Figure 1. Calibration curve for the enzyme immunoassay of clenbuterol (■) and successive dilutions of hair extracts (○).

mL of 1-chlorobutane after 1 mL of distilled water and 250 mg of potassium carbonate (pH 12) were added. The organic layer was evaporated to dryness. The residue was dissolved in 200 μL of methanol, transferred into 250-μL vials, and evaporated to dryness. The monotrimethylsilyl (TMS) derivative was obtained by the addition of 10 μL of MSTFA to the stoppered vial under nitrogen and heating at 70 °C for 10 min. The reaction mixture was analyzed by GC/MS as described elsewere (Adam et al., 1994). Ions at m/z 86.05, 264.05, and 262.20 were monitored (dwell time 50 ms) in the selected ion monitoring (SIM) mode.

RESULTS

1. Accuracy and Precision of the Extraction Procedure. The efficiency of the immunoaffinity extraction step has been assessed by documenting the recovery of tritiated clenbuterol spiked in the hair extract. The recovery was 94.8 ± 3.8% (n = 3).

The accuracy and the precision of the extraction procedure were documented using spiked samples with 1 ng/50 mg of hair. The residues of evaporation were analyzed by EIA. The overall recovery of clenbuterol revealed a yield of 83.6 ± 2.95% (n = 3).

2. Immunoidentity of Clenbuterol Extracted from Hair. Immunoidentity of the material extracted from hair was assessed by testing the parallelism of the calibration and dilution curves for hair from calves receiving clenbuterol. As shown in Figure 1, the curves (ED_{50} = 241 pg/well; minimum detection level = 3.0 pg/well) displayed slope factors of −1.20 and −1.18, respectively. These values confirmed the immunoidentity of the extracted material as clenbuterol.

3. GC/MS Identification of Clenbuterol in Hair. One of the immunoextracted residues was analyzed by GC/MS to confirm the identity of extracted clenbuterol. The results of GC/MS analysis in the SIM mode (ion traces of m/z 264.05, 262.20, and 86.05) are presented in Figure 2. Clenbuterol, as its TMS derivative, was identified in the immunoextracted material containing 10 ng of clenbuterol (Figure 2b) by comparison with a blank matrix (Figure 2a) and authentic clenbuterol standard spiked in the blank matrix (Figure 2c). The ratio of selected ions at m/z 262.20 and 264.05 over m/z 86.05 fell between 0.7 and 12.9% variation.

4. Clenbuterol Levels in Hair. In calves receiving 100 μg/kg clenbuterol (Table 1) hair samples revealed a mean value of 7.90 ± 1.41 ng/g as early as day 6 of treatment. The clenbuterol accumulation increased to 21.30 ± 10.40 ng/g by day 10. During the washout period, a peak concentration of 56.60 ± 21.50 ng/g was reached by day 20 after the last clenbuterol dose. The residues decreased to 19.20 ± 2.48 ng/g on day 40. In calves that received 10 μg/kg of this β_2-agonist, a peak clenbuterol concentration of 12.70 ± 9.98 ng/g was detected on day 20 of the washout period (Table 1), although subsequent samples did not show any measurable amounts.

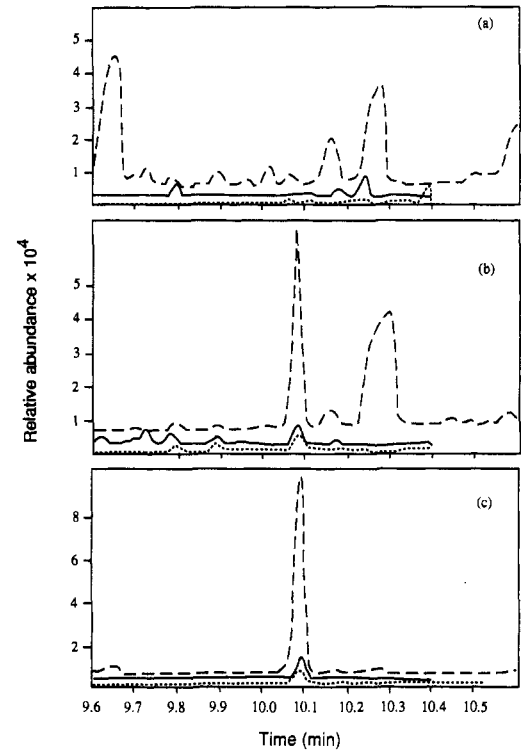

Figure 2. Selected ion current chromatograms of a blank extract (a), a hair extract from calves receiving clenbuterol (b), and a blank extract spiked with 10 ng of clenbuterol (c). The ions monitored in each tracer are as follows: (– – –) (m/z 86.05), (—) (m/z 262.20) and (- - -) (m/z 264.05) corresponding to the same retention time of TMS derivatives of clenbuterol.

Table 1. Mean Concentrations in Hair after Different Periods in Calves Treated with 100 (A) and 10 μg (B) of Clenbuterol

period (days)		clenbuterol concn (ng/g ± SD) A (n = 3)	B (n = 3)
treatment	0	ND	ND
	2	ND	ND
	4	ND	ND
	6	7.90 ± 1.41	ND
	8	12.90 ± 6.87	ND
	10	21.30 ± 10.40	ND
washout	10	33.90 ± 11.40	ND
	20	56.60 ± 21.50	12.70 ± 9.98
	30	36.20 ± 11.10	ND
	40	19.20 ± 2.48	ND

[a] ND, nondetectable.

5. Clenbuterol Levels in Growing Hair. Analysis of growing hair sampled on days 20 and 40 of the washout period revealed differences in clenbuterol concentration (40.30 ± 1.98 and 27.50 ± 1.39 ng/g, respectively; $p < 0.05$). When clenbuterol was quantified in different segments of growing hair (0–1, 1–2, 2–3 cm), the highest concentration of the immunoreactive residue was detected in 1–2-cm sections (Figure 3).

6. Clenbuterol Hair Disposition. To assess the uniformity of clenbuterol disposition, seven different hair samples taken from calf 4 (10 μg kg^{-1} day^{-1} of clenbuterol) on day 20 of the washout period were extracted and quantified separately. The mean detected clenbuterol concentration was 20.80 ± 1.28 ng/g of hair. The interassay variability was 6.15%.

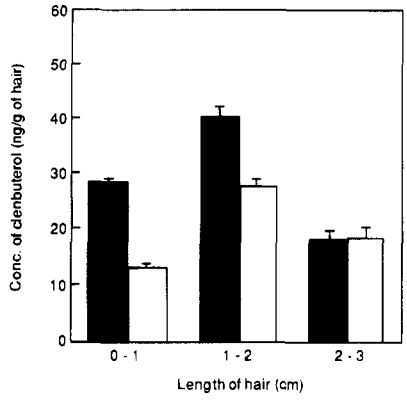

Figure 3. Comparative measurement of clenbuterol concentration in growing hair (0–1-, 1–2-, 2–3-cm segments) at 20 (■) and 40 days (□) of the washout period.

DISCUSSION

To meet standard quality assurance requirements for clenbuterol-free meat, we report here the detection of this β_2-agonist in carcasses by monitoring its residue levels in hair. Effectively, following chronic clenbuterol treatment in calves, its accumulation in hair appears to be time-and dose-dependent. Clenbuterol in hair is found at the nanograms per gram level from day 6 after administration at a 100 μg/kg dose and is increased for some time thereafter. To assess the capability of detecting its illegal use, monitoring of this drug was carried out at different times in the washout period. Clenbuterol could be found in hair for up to 40 days after its withdrawal. The clenbuterol profile displayed a peak concentration of 56.60 ± 21.50 ng/g on day 20 of the washout period. The drug concentration also peaked on day 20 after a 10 μg/kg dose. The time course of drug accumulation in hair shows that clenbuterol concentration is highest in 1–2-cm hair sections. The specific mechanism of drug accumulation is unknown, but the same concentration pattern has been reported previously for cocaine and opiates (Baumgartner et al., 1982).

Selecting hair for the detection of clenbuterol contamination is an interesting alternative to testing urine from the calf bladder or tissue samples as reported earlier (Pou et al., 1994). In conclusion, we report here that hair is an interesting target to check the illicit abuse of clenbuterol in calves. This approach requires minimal sample preparation. The immunoextraction step coupled with enzyme immunoassay represents a reliable screening methodology to detect clenbuterol residues.

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