A fast immunoassay for the screening of β-agonists in hair†

The Analyst

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Hair has been shown to be an excellent site for the accumulation of clenbuterol residues. Compared with other matrices, hair sampling is very easy and this might result in large numbers of samples. In this study, a simple digestion–extraction procedure was combined with a sensitive clenbuterol ELISA, which resulted in an easy screening procedure suitable for the detection of at least four β-agonists. Hair from untreated cows (n = 40) resulted in low blank levels (0.9 ± 0.7 and 0.5 ± 0.2 ng g^{-1} for black and white hair, respectively). The detection limits for clenbuterol, bromobuterol, mapenterol and mabuterol were determined as 1–1.5 ng g^{-1} for white and 3–4 ng g^{-1} for black hair. The accumulation of mabuterol and mapenterol in black and white hair from treated calves was demonstrated by GC-MS. The screening assay is not suitable for the detection of cimbuterol (owing to the low extraction efficiency) and for salbutamol and terbutaline (owing to the low cross-reactivity of the antibodies used for the ELISA and the low extraction efficiency). Black hair samples from cows treated with clenbuterol were still found to be positive (> 5 ng g^{-1}) at 23 weeks after treatment. The fast screening procedure is a powerful means to detect and track the illegal use of clenbuterol, bromobuterol, mabuterol and mapenterol in animal production.

Introduction

β-Agonists are illegally used in meat production to improve carcass composition by decreasing fat for the benefit of muscle mass. Within the European Union the use of any drug to improve animal growth is banned.[1] In The Netherlands, urine, which can be taken in both farm- and slaughter-houses, is still the most frequently analysed material for the detection of growth promoters. However, after a withdrawal time of 3–4 days, the concentration of β-agonists is below the limit of detection of most assays, which makes this sample material suitable only for detection during treatment and less suitable for the detection of illegal use.[2]

The eye has been described as the most accumulating tissue for β-agonists[3] and analysis of retinal extracts has been shown to extend the detection period to about 20 weeks.[4] However, eyes can only be sampled at the slaughterhouse. Pigmented hair has been described[5–8] as a suitable alternative for the detection of clenbuterol. After a therapeutic treatment [0.8 μg of clenbuterol per kg body mass (b.m.), twice daily for 10 d], clenbuterol could be detected from day 4 of clenbuterol application to 60 d after withdrawal of the treatment.[6] The highest concentrations were found in black hair (100 ng g^{-1}) and the lowest in white hair (5 ng g^{-1}). Using a growth promoting dose (1 mg d^{-1}), which is about 10 times the therapeutical dose, the maximum concentration found in hair of cattle was 1720 ± 528 ng g^{-1}, which is much higher than the maximum concentration found after a therapeutic dose (60 ± 39 ng g^{-1}).[8] Applying a high dose (5 μg kg^{-1} b.m., twice daily for 3 weeks), clenbuterol could be detected in pigmented hair up to 18 weeks and in white hair up to 8 weeks after treatment.[7] According to these experiments, hair analysis is a powerful means to detect and track the illegal use of clenbuterol in animal production. However, the external nature of this matrix allows for the possibility of contamination with the drug either from exposure to animal excreta or by malicious application.[9] By applying a series of washes, hair samples taken 24 h after contamination were easily distinguished from those taken from medicated cattle.[9] In hair samples taken from medicated cattle, over 98% of the detected clenbuterol was present in the washed hair fraction. After external exposure to clenbuterol containing urine [1 l of spiked urine (200 ng ml^{-1}) sprayed on to each side of the rear flanks of an animal], hair samples taken 24 h after the treatment contained clenbuterol in both the washed hair samples (40%) and the washes (60%) and could be distinguished from those taken from medicated cattle. However, differentiation was not possible 1 month later, when almost all clenbuterol was found in the washed hair fraction. This was most likely due to exposure of the animals to rain. These data suggest that hair samples can be contaminated by external contamination and differentiation between such contamination and medication, by applying washing procedures, is not always possible. Therefore, a positive hair result can be used only to identify an animal or a farmer for special attention (first screening). To confirm clenbuterol treatment, eye samples could be collected at slaughter.

Hence, hair samples could be an excellent material for the first screening of cattle for the possible misuse of clenbuterol. Because hair samples are easy to obtain, such a first screening might result in a large number of samples. So far, prior to the application of most previously described assays, hair sample treatment is laborious, which is partly due to the washing procedures applied to remove potential exogenic clenbuterol contamination.[8,10] Omitting these washing procedures, the sample preparation can be performed much more easily. For the digestion of hair, enzymes (using papain[8]) or basic solutions (pH 12)[9,10] have been described. The enzymatic digestion procedure takes about 16 h whereas basic digestion can be performed within 15 min (at 95 °C). Depending on the method applied for detection, different sample clean-up procedures are described. Godfrey *et al.*[8] used immunoaffinity chromatography prior to the application of a chemiluminescent ELISA and Gleixner *et al.*[10] applied an overnight extraction in *tert*-butyl methyl ether (T-BME) prior to ELISA.

In this study, the combination of the fast basic digestion with a shortened T-BME extraction procedure was evaluated for application to unwashed hair samples in our previously

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developed clenbuterol ELISA.[11] Hair samples from untreated cows (n = 40), clenbuterol treated cows (n = 12) and calves treated with a combination of clenbuterol, mabuterol and mapenterol were used. The selected digestion–extraction procedure was tested for its performance on seven β-agonists.

Experimental

Materials

Clenbuterol hydrochloride and goat anti-rabbit immunoglobulin (IgG; whole molecule) were obtained from Sigma (St. Louis, MO, USA), clenbuterol-d_6 was synthesized at the Laboratory of Organic Chemistry, Agricultural Universtity, Wageningen, The Netherlands and salbutamol sulfate and terbutaline sulfate were supplied by Bufa-Chemie (Castricum, The Netherlands). Mabuterol and mapenterol were prepared at Rephartox (Maarssen, The Netherlands) and cimbuterol was a gift from Professor F. André, (Ecole Veterinaire Nantes, Nantes, France). Bromobuterol was a gift from the National Institute of Public Health and the Environment (RIVM), Laboratory for Residue Analysis, Bilthoven, The Netherlands. T-BME was obtained from Merck (Darmstadt, Germany). Horseradish peroxidase grade I (EC 1.11.1.7) was supplied by Boehringer (Mannheim, Germany). Flat bottomed microtitre ELISA plates (96-well) were purchased from Greiner (Nurtingen, Germany). Tetramethylbenzidine (TMB) peroxidase substrate was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA). Antibodies raised against clenbuterol conjugated to bovine serum albumin and salbutamol–horseradish peroxidase conjugate were prepared as described previously.[11] BondElut Certify columns were obtained from Varian (Harbor City, CA, USA).

Equipment

For the ELISA, a Wellwash Model 4 micro plate washer (Denley Instruments, Billinghurst, UK) and an Argus 400 micro plate reader (Canberra Packard, Downers Grove, IL, USA) were used. The GC-MS system (Hewlett-Packard, Avondate, PA, USA) consisted of a Model 5890, series II gas chromatograph, an Engine Model 5989 B mass spectrometer (electron impact/chemical ionization mode), a Model 7673-A autoinjector and a Chemstation. The column used was a CP-Sil 5CB-MS capillary column (15 m× 0.25 mm id; Chrompack, Middelburg, The Netherlands) with a film thickness of 0.25 μm and helium was used as the carrier gas. The inlet pressure was 69 kPa at 90 °C. A volume of 2 μl was injected (splitless mode) and the oven temperature was programmed from 90 °C (2 min) to 210 °C at 12.5 °C min^{-1}, maintained at 210 °C for 5 min and then increased at 15 °C min^{-1} to 300 °C. The transfer line was kept at 280 °C. For electron impact ionization, the source was maintained at 250 °C and the quadrupole at 120 °C; in the chemical ionization mode, these temperatures were 150 and 100 °C, respectively.

ELISA

The preparation of polyclonal antibodies and enzyme conjugate and the performance of the ELISA have been described previously.**[11]** A short protocol is as follows. Microtitre plates were coated overnight with 100 μl aliquots of goat anti-rabbit IgG [10 μg ml^{-1} in 50 mM sodium carbonate buffer (pH 9.6)] at 4 °C. The plates were washed four times with PBST [5.4 mM sodium phosphate–1.3 mM potassium phosphate–150 mM NaCl (pH 7.4)–0.05% Tween-20]. Aliquots of 50 μl of diluted clenbuterol standard solutions (0.05–5 ng ml^{-1}) or 50 μl of sample (extract) were added to the wells. Next, 25 μl of diluted salbutamol–HRP (final dilution in PBST 1 : 75 000) and finally 25 μl of diluted antiserum (final dilution in PBST 1 : 10 000) were added. The plate was incubated for 1 h at 4 °C and after washing (four times with PBST), the bound peroxidase was assessed by adding 100 μl of a tetramethylbenzidine (TMB)–peroxidase substrate system. After incubation in the dark for 20–30 min at room temperature, the reaction was stopped by adding 100 μl aliquots of 1 M phosphoric acid and the coloured product of the peroxidase reaction was measured at 450 nm.

Samples

In February 1997, different coloured (black, brown and white) blank hair samples were obtained from untreated cows and calves (n = 40) located at the DLO Institute for Animal Science and Health (ID-DLO, Lelystad, The Netherlands).

Three hair samples (one blank and two positives) from calves were obtained from an animal experiment in which calves were orally treated with a mixture of clenbuterol, mabuterol and mapenterol (each 0.4 μg kg^{-1} b.m.) for a period of 6 weeks. Directly after the treatment (no withdrawal period), hair samples were taken. This animal experiment was performed at IVVO-DLO in June 1993 and samples were stored at −20 °C.

Twelve hair samples were obtained from two adult cows (animals 102 and 104) treated with clenbuterol at twice the recommended therapeutic dose for 28 d (by addition of the drug to the animals' daily ration). Before and during treatment and after different withdrawal periods (0.3, 4, 6, 10, 13, 23 and 33 weeks after the treatment), an amount of hair (about 5 g) was removed by means of electric clippers from the flank region of each animal. This animal experiment was performed at the Veterinary Sciences Division of the Department of Agriculture for Northern Ireland (Belfast, UK) in the period October 1994–July 1995.

Sample preparation for screening

Of the hair samples, 0.1 g was weighed into a test-tube (9× 1.4 cm id) and 2.5 ml of 5 M NaOH were added. The mixture was heated for 10 min in a water-bath at 95 °C and after cooling to room temperature, 5 ml of T-BME were added. The tube was closed with a stopper and mixed for 30 min (head over head). The layers were separated by centrifugation (5 min at 1000 rpm) and 2.5 ml of the upper layer (equivalent to 0.05 g of hair and containing the extracted β-agonists) were evaporated at 40 °C under a stream of nitrogen. The residue was dissolved in 0.5 ml of PBST (0.1 g of hair per millilitre).

Sample preparation for GC-MS

Hair (0.5 g) was weighed into a screw-capped tube (50 ml) and 20 ng of deuterated internal standard (clenbuterol-d_6) and 4 ml of 5 M NaOH were added. The tube was heated for 30 min in a water-bath at 95 °C and the tube was shaken every 10 min. After cooling to room temperature, the pH was adjusted to 6 by the addition of concentrated phosphoric acid. The sample was transferred into an activated BondElut Certify column, which was then washed successively with 1 ml of 1 M acetic acid, 6 ml of methanol and 2 ml of acetone–chloroform (1 + 1 v/v). The analytes were eluted with 7.5 ml of a mixture of ethyl acetate and ammonia and, after evaporation under a stream of nitrogen at 40 °C, the residue was dissolved in 100 μl of a derivatization solution (MSTFA with 1% TMCS). After mixing, heating for 30 min at 60 °C, cooling to room temperature and evaporation, the residue was dissolved in 15 μl of dried toluene and 2 μl of the solution were injected into the GC-MS system.

Results and discussion

The clenbuterol ELISA has been described previously,[11] where it was used for different kinds of sample materials (cattle feed, urine, faeces, liver, kidney, blood, muscle, bile and retina). Hair was not included in that study. In this ELISA, the working range of the calibration curve is from 0.05 ng ml^{-1} (80% B/B_0) to 2 ng ml^{-1} (at 20% B/B_0) with 0.3 ng ml^{-1} at 50% B/B_0 (see Fig. 1). In this study, the ELISA showed high cross-reactivity towards mapenterol, mabuterol and cimbuterol and low cross-reactivity towards salbutamol and terbutaline (see Fig. 1 and Table 1). In the previous study, the cross-reactivity towards other β-agonists was also determined [e.*g.*, cimaterol (6%), carbuterol (5%), pirbuterol (4%) and tulobuterol (2%) and no cross-reactivity towards fenoterol and ractopamine (< 0.1%)].

For the screening of hair samples for the presence of clenbuterol and other cross-reacting β-agonists, we aimed at a level of determination in the low ppb range. Therefore, with 0.3 ng ml^{-1} of clenbuterol at 50% B/B_0 on the calibration curve, a 10-fold sample dilution would be acceptable (0.1 g ml^{-1} of hair). For small series of samples (n = 20), the sample preparation procedure can be performed within 2 h. The most labour intensive step is weighing the hair samples into the test-tubes.

As a first check of the sample preparation procedure, standard solutions of clenbuterol were added to the sodium hydroxide solution (at a level of 1 ng ml^{-1}), extracted with T-BME, of which half was evaporated and dissolved in PBST. A recovery of 101 ± 7% (n = 5) was found. This experiment was repeated and, as for the hair samples, the sodium hydroxide solution (with clenbuterol added) was heated for 10 min at 95 °C. Here the recovery was 99 ± 4%. Therefore, clenbuterol could be extracted completely from the basic aqueous solution by T-BME and the heat treatment had no negative effect on the clenbuterol. The same experiments were performed for salbutamol added to the sodium hydroxide solution at a level of 25 ng ml^{-1}. Here the recoveries were less than 2%, which means that the extraction procedure is unsuitable for salbutamol.

Next, a blank black hair sample was analysed without and with the addition of seven β-agonists (see Table 1). Standard solutions of the β-agonists were added to the hair sample and, to allow the drugs to interact with the sample, the sodium hydroxide solution was added after about 1 h. The blank value of this sample was determined as 0.2 ng g^{-1} (clenbuterol equivalents). As shown in Table 1, clenbuterol, bromobuterol, mapenterol and mabuterol could be extracted with good efficiency from the hair sample. Although cimbuterol showed a high cross-reactivity in the clenbuterol ELISA (50%), the extraction efficiency was low (7%) and therefore the overall procedure was not suitable for this compound. Salbutamol and terbutaline are β-agonists towards the clenbuterol ELISA and showed low cross-reactivity (7%) and also the extraction efficiency was very low (< 2%). The procedure described here is not suitable for these β-agonists also. Therefore, it was demonstrated that the procedure worked well for the screening of hair samples for the presence of clenbuterol, bromobuterol, mapenterol and mabuterol.

To determine the limit of detection (LOD) of the screening procedure, blank hair samples of different colours (white, brown and black) were analysed for background (blank levels). As shown in Table 2, the LOD in black hair was 2.9 ng g^{-1} and the limit of determination (LDM) was 5.0 ng g^{-1}. In white hair the LOD was 1.1 ng g^{-1} and the LDM was 1.7 ng g^{-1}.

Finally, positive hair samples obtained from two different animal experiments were analysed and the results were compared with GC-MS results. In 1993, calves (n = 20) were treated orally with a mixture of clenbuterol, mabuterol and mapenterol (each 0.4 μg kg^{-1} b.m.) for a period of 6 weeks and directly after treatment (no withdrawal period) various kinds of samples (urine, faeces, liver, bile and retina) were taken. Unfortunately, because the possibilities of hair analysis were unknown at that time, hair samples were taken from only two treated calves (calves 2 and 3) and one blank calf. The light and dark hair of these samples were separately analysed by the screening procedure (eight times) and by GC-MS (in duplicate) and the results are given in Table 3. Using GC-MS, all three β-agonists were found, which is the first proof that mabuterol and mapenterol accumulate in the hair. However, at the time of analysis deuterated internal standards for mabuterol and mapenterol were not available and only clenbuterol was quantified. As demonstrated previously, the ELISA detects all three compounds and, compared with GC-MS, higher concentrations would be expected. As shown in Table 3, the concentrations found by the ELISA are higher than those quantified by GC-MS (only clenbuterol quantified). As expected, the dark hair samples from the treated calves contained higher concentrations than the light hair samples.

The results obtained with the other sample materials from the 20 treated calves have been published previously.[11] Briefly, using the same ELISA, the mean concentrations found in urine (direct method), faeces, liver, bile and retina/choroid were 24,

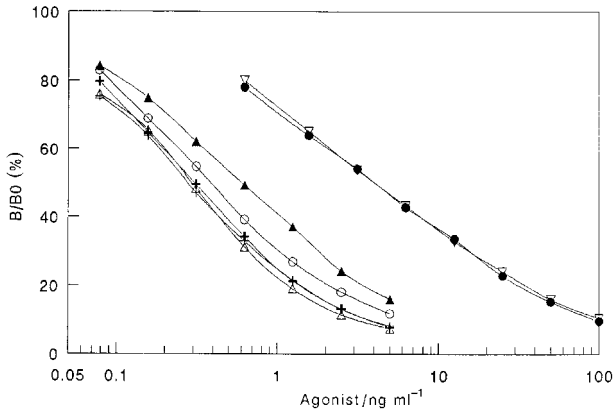

Fig. 1 Calibration curves for seven β-agonists in the clenbuterol ELISA: +, clenbuterol; △, bromobuterol; ○, mapenterol; +, mabuterol; ▲, cimbuterol; ●, salbutamol; and ▽, terbutaline.

Table 1 Extraction efficiency, corrected for cross-reactivity, of β-agonists from spiked hair extracts

β-Agonist	Level spiked/ ng g^{-1}	n	Clenbuterol equivalents/ ng g^{-1}	Cross-reactivity (%)	Extraction efficiency (%)
Clenbuterol	5	8	5.0 ± 0.4	100	101 ± 7
Bromobuterol	5	8	5.3 ± 0.3	108	98 ± 5
Mapenterol	5	8	2.9 ± 0.2	75	77 ± 5
Mabuterol	5	8	4.4 ± 0.6	100	88 ± 12
Cimbuterol	5	8	0.2 ± 0.1	50	7 ± 3
Salbutamol	100	8	0.1	7	< 2
Terbutaline	100	8	0.1	7	< 2

Table 2 Blank values obtained with hair samples from untreated cows (n = 40)

		Clenbuterol equivalents (ng g^{-1})			
Colour of hair	No. of samples	Mean	s	Limit of detection (LOD)[a]	Limit of determination (LDM)[b]
Black	19	0.88	0.68	2.9	5.0
White	18	0.52	0.20	1.1	1.7
Brown	2	0.36, 0.29	—	—	—
White + Blue	1	0.81	—	—	—

[a] Mean + 3 s. [b] Mean + 6 s.

Table 3 Concentrations found in hair samples of calves treated with a combination of clenbuterol, mabuterol and mapenterol (calves 2 and 3) and a blank calf

Hair sample	Colour	ELISA: clenbuterol equivalents/ng ml^{-1}	GC-MS: clenbuterol/ng ml^{-1}
Blank calf	Light	0.6	nd[a]
	Dark	0.6	nd[a]
Calf 2	Light	14 ± 2	12
	Dark	48 ± 21	39
Calf 3	Light	15 ± 2	13
	Dark	84 ± 19	32

[a] Not detected.

Table 4 Concentration of clenbuterol found with the screening procedure and GC-MS in black hair samples from two treated adult cows

Cow No.	Sampling date	Week after treatment	Clenbuterol concentration/ng g^{-1}	
			ELISA	GC-MS
104	4.10.1994[a]	—	0.9	nd[c]
104	25.10.1994[b]	—	> 100	590
104	16.12.1994	6	> 100	290
104	10.1.1995	10	> 100	430
104	30.1.1995	13	> 100	nq[d]
104	14.4.1995	23	9 ± 3	46
104	20.6.1995	33	3	1
102	4.11.1994	0.3	> 100	150
102	1.12.1994	4	> 100	550
102	16.12.1994	6	> 100	270
102	14.4.1995	23	36 ± 12	51
102	26.6.1995	33	0.8	4

[a] Negative control taken before treatment. [b] Sample taken during the third week of the 4 week treatment. [d] = Not quantified. [c] = not detected.

11, 9, 8 and 61 ng g^{-1}, respectively. Although only hair samples from two calves were taken, the levels found in dark hair indicated that they are in the higher range as also found in the retina/choroid.

The hair samples obtained from the two with clenbuterol treated adult cows (at twice the recommended therapeutic dose) were also analysed using the screening procedure and also GC-MS (Table 4). The samples were analysed in duplicate with both methods. During and after the treatment period, high concentrations (150–590 ng g^{-1}) were found by GC-MS. In the ELISA, these samples were out of range (> 100 ng g^{-1}). The hair samples obtained at 23 weeks after the treatment were analysed eight times by ELISA and the mean concentrations were 9 ± 3 ng g^{-1} (cow 104) and 36 ± 12 ng g^{-1} (cow 102). The higher concentrations found by GC-MS in these samples could not be explained. However, it was clearly shown that even at 23 weeks after treatment with clenbuterol, black hair samples were still found positive.

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