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The β-agonist clenbuterol in mane and tail hair of horses

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Summary

Reasons for performing study: **The β_2-agonist clenbuterol is commonly administered for therapeutic purposes in the horse, but its use an an anabolic agent is illegal. Clenbuterol can be detected in blood and urine for a relatively short period after administration and detection in hair could enhance the analytical range and be used to determine the history of clenbuterol application.**

***Hypothesis*: That detection in mane or tail hair is possible over an extended period.**

Methods: **Four horses received 0.8 µg clenbuterol hydrochloride/kg bwt b.i.d. for 10 days. Four other horses were used as untreated controls. Blood, urine, mane and tail hair samples were taken on Day 0 (before) and 5, 10, 30, 35, 40, 60, 90, 120, 150 and 360 days after start of treatment. Gas chromotography/high resolution mass spectrometry (GC/HRMS) was developed for clenbuterol analysis: limit of detection was 0.2 pg/mg; intra-assay repeatability limit r = 0.06 (confidence level 95%); interassay repeatability limit r = 0.03 (confidence level 95%). Prior to treatment, clenbuterol was absent from all samples analysed.**

***Results*: Clenbuterol was detectable as early as Day 5 in tail and mane hair of Segment 1 (0–20 mm from the roots) and was maximal on Day 90. However, as time progressed, shift into lower 20 mm segments was observed. On Day 360, the maximum concentration (up to 21 pg/mg) was located in Segment 13, i.e. 26–28 cm from roots of hair. Clenbuterol was not detectable in blood or urine after Day 30. Mane and tail hair results were very similar.**

Conclusions: **The study showed that the β-agonist clenbuterol can be found in mane and tail hair of horses after extended periods.**

Potential relevance: **It will be possible to detect clenbuterol in breeding and show horses where anabolic drugs have been used illegally to improve conformation. This method may also be helpful to monitor therapeutic clenbuterol treatment.**

Introduction

'Doping' in horses is defined as the application of drugs for better performance. This is illegal in most countries (Anon 2002). Drug usage is monitored routinely during competition by analysis of urine or blood samples. In recent years, it is purported that the growth and development of some young horses has been accelerated by the use of anabolic drugs to gain advantages for sale and promotion of breeding stallions. Random tests for illegal drug application have been carried out but have rarely yielded positive results, possibly because treatment was discontinued early enough to avoid urine or blood detection. The ß-agonist clenbuterol cannot be detected in horse urine later than 11 days after withdrawal, and in blood it is undetectable 4 days after the last application (Hagedorn *et al.* 1995; Harkins *et al.* 2001). It was our hypothesis that detection in mane or tail hair would be possible over an extended period, as shown in rat hair (Adam *et al.* 1994), poultry feathers (Malucelli *et al.* 1994), cattle (Gaillard *et al.* 1997; Fente *et al.* 1999) and human hair (Gleixner *et al.* 1996). Recently, Popot *et al.* (2000) published results of a study of 2 horses in which clenbuterol was detectable in mane and tail for 10 months after treatment. Furthermore, Dunnett (2002) has emphasised the diagnostic potential of hair analysis to assess residues in stock production, prepurchase examination and sports antidoping control and misuse in horses. Due to the slow growth and usually long hair in horses' tails, this should provide a powerful reservoir of material for detection of past usage of the β-agonist clenbuterol.

Materials and methods

A total of 8 geldings, age 5–7 years, were included in the study. They were unexposed to β-agonists for at least 3 years prior to the study. Four were treated with the β-agonist clenbuterol and 4 served as controls. In the treated groups, 2 horses had a black coat with black tail and mane, one a brown coat with lighter tail and mane and one a brown coat with black tail and mane. In the control group, one black horse and one brown had a black tail and mane and the other 2 horses were bay (brown coat) with a lighter tail and mane.

Ventipulmin Gel[1], containing 0.025 mg clenbuterol hydrochloride, 1.8 mg methyl-4-hdroxybenzoate and 0.2 mg propyl-4-hydroxybenzoate as conserving agent/ml, was used for treatment. This product is a bronchospasm dilator licenced in Germany for restricted equine medical application.

The study was initiated in October 2001. In the morning and afternoon of the first day of the experiment, the 4 horses selected for treatment received clenbuterol hydrochloride 0.8 µg/kg bwt *per os*. This amounted to a gel volume of 3.2 ml/100 kg bwt per application and is a typical therapeutic dosage. Treatment was continued for a period of 10 days b.i.d. (morning and evening). Application of clenbuterol directly into

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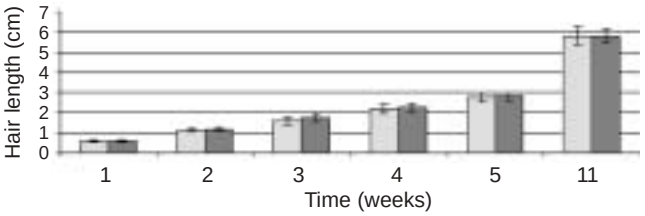

Fig 1: Mean ± s.d. hair growth/week (cm/week) of the tail (■) and mane (■) of 4 horses and 3 ponies over an 11 week period.

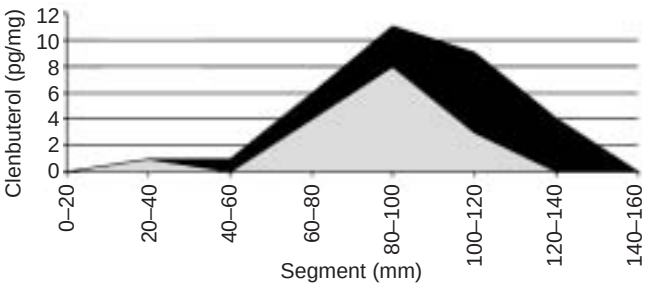

Fig 2: Clenbuterol concentration (pg/mg) after a 10-day oral treatment with 1.6 µg/kg bwt/day clenbuterol hydrochloride in the different tail hair segments on Day 150 in one horse with black (■) and one horse with pale brown (■) tail hair.

the mouth results in uncontrolled expectoration and loss of medication, and the gel was therefore mixed with feed. For feeding, the horses were isolated into individual boxes and the gel administered in a small portion of standard horse feed. After controlled complete intake of the food/gel mixture, the remaining feed ration was given in 2 successive portions, to assure that any remnants of the gel which might still remain in the trough were taken up. Complete uptake was confirmed after each feeding by visual inspection of the troughs.

Blood, urine, mane hair and tail hair samples were taken on Day 0 (the day before the first application) and on Days 1, 5, 10, 30, 35, 40, 60, 90, 120, 150 and 360. Samples were always collected in the afternoon before the evening feeding and clenbuterol application was on Days 1, 5 and 10. For each sample, 20 ml blood was drawn from the external jugular vein into heparinised tubes and centrifuged at 1560 ***g*** for 20 mins at 4°C. The plasma was frozen and stored at -18°C for analysis. Urine was collected during spontaneous micturition in one-way plastic cups. An aliquot of 50 ml was frozen in plastic bottles at -18°C prior to analysis. A bundle of mane and tail hair approximately 0.7 cm thick was collected from the crest of the mane and base of tail at each time point. Care was taken to ensure that the hair included the root. The bundles of hair were sealed in plastic bags and kept at room temperature for further processing.

The study was approved by the state authorities responsible for animal welfare (License No. 509c-42502-00/335).

Hair growth

In a pilot study preceding the main experiment, the rate of growth of mane and tail hair length was measured for a period of 11 weeks (August to October) in 7 horses. The group consisted of 3 ponies and 4 horses (4 geldings and 3 mares) age 1–20 years. For this test, a 3 x 3 cm area was shaved at the dorsal radix of the tail and the middle of the mane on each animal. The mean length of 15 individual hairs was measured at weekly intervals.

Analysis of hair samples

Analysis of hair samples followed the procedure described below at the Institute of Doping Analysis and Sports Biochemistry, Kreischa, an IOC-accredited laboratory. All segments from treated horses were analysed; in control samples, only those segments that corresponded to segments from treated horses with the highest clenbuterol concentrations were analysed (Tables 1 and 2). For each sample, pulled hairs were aligned exactly at their roots and cut into 20 mm segments. Subsequently, each segment was cut with scissors into fragments approximately 1 mm in length and stored at room temperature for further processing. The number of hairs in each

TABLE 1: Mean ± s.d. clenbuterol concentrations (pg/mg) in successive 20 mm segments of tail hair of horses before and after a 10-day oral treatment with 1.6 µg/kg bwt/day clenbuterol hydrochloride. In each hair sample, the segment with maximum concentration of clenbuterol is in bold text

Segment† (mm)	Day after start of treatment							
	0‡	5	10	30	60	90	120	150	360
0–20	0.00 ± 0.00*	**11.75 ± 4.35***	**23.25 ± 5.68***	**19.75 ± 7.89***	0.50 ± 1.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
>20–40		0.00 ± 0.00	0.03 ± 0.05	2.50 ± 1.29	**20.25 ± 8.38***	1.50 ± 1.00	0.00 ± 0.00	0.50 ± 0.58	0.00 ± 0.00
>40–60				0.00 ± 0.00	6.00 ± 3.92*	**19.75 ± 10.50***	4.00 ± 2.94	1.00 ± 0.82	0.00 ± 0.00
>60–80					0.00 ± 0.00	10.25 ± 4.50*	**20.75 ± 8.73***	5.50 ± 4.20	0.00 ± 0.00
>80–100						0.05 ± 0.10	9.75 ± 7.85*	**15.50 ± 9.88***	0.00 ± 0.00
>100–120						0.00 ± 0.00	0.50 ± 1.00	9.50 ± 6.24*	0.00 ± 0.00
>120–140							0.00 ± 0.00	1.50 ± 1.91	0.00 ± 0.00
>140–160								0.00 ± 0.00	0.50 ± 1.00
>160–180									2.50 ± 3.00
>180–200									4.50 ± 3.11
>200–220									7.00 ± 2.45
>220–240									12.50 ± 3.00
>240–260									**16.75 ± 4.79**
>260–280									11.25 ± 6.18
>280–300									6.50 ± 3.87
>300–320									2.75 ± 3.59
>320–340									1.08 ± 1.08
>340–360									0.25 ± 0.50
>360–380									0.00 ± 0.00

†From the root of the hair. ‡Day 0 = day before start of treatment. *Samples where corresponding control samples were analysed. All controls remained below the limit of detection (LOD).

TABLE 2: Mean ± s.d. clenbuterol concentrations (pg/mg) in successive 20 mm segments of mane hair of horses before and after a 10-day oral treatment with 1.6 µg/kg bwt/day clenbuterol hydrochloride. In each hair sample, the segment with maximum concentration of clenbuterol is in bold text

Segment[†] (mm)	Day after start of treatment								
	0	5	10	30	60	90	120	150	360
0–20	0.00 ± 0.00*	**11.50 ± 6.61***	**22.75 ± 12.84***	**17.00 ± 10.55***	2.00 ± 2.71	1.50 ± 1.00	0.00 ± 0.00	0.50 ± 1.00	0.00 ± 0.00
>20–40		0.05 ± 0.06	0.10 ± 0.14	7.25 ± 5.80	**18.00 ± 9.56***	1.50 ± 1.29	1.25 ± 0.96	0.50 ± 0.58	0.00 ± 0.00
>40–60			0.00 ± 0.00	0.03 ± 0.05	5.75 ± 3.95*	**15.00 ± 10.42***	2.75 ± 0.96	0.75 ± 0.50	0.00 ± 0.00
>60–80				0.00 ± 0.07	0.00 ± 0.00	11.50 ± 9.95*	**14.50 ± 7.55***	4.75 ± 3.30	0.00 ± 0.00
>80–100						0.10 ± 0.20	13.25 ± 8.10*	**17.25 ± 11.95***	0.00 ± 0.00
>100–120						0.00 ± 0.00	1.00 ± 1.41	9.50 ± 4.93*	0.00 ± 0.00
>120–140							0.00 ± 0.00	1.75 ± 2.06	0.05 ± 0.10
>140–160								0.25 ± 0.50	0.30 ± 0.48
>160–180								0.00 ± 0.00	1.50 ± 1.29
>180–200									3.50 ± 2.65
>200–220									4.75 ± 3.77
>220–240									5.25 ± 3.77
>240–260									**9.25 ± 6.85**
>260–280									5.75 ± 4.43
>280–300									6.50 ± 7.85
>300–320									4.13 ± 4.77

[1]From the root of the hair. Day 0 = day before start of treatment. *Samples where corresponding control samples were analysed. All controls remained below the limit of detection (LOD).

segment was not counted, but an equal weight was analysed from each segment. An aliquot of 50 mg of each segment was placed into an ultrasonic bath containing 0.5 mol/l KOH for 3 h at 60°C to give complete hydrolysis. Bambuterol was added as an internal standard. This solution was then extracted on a solid-phase column (Oasis HLB)[2] controlled by an automated processor (Gilson Aspec XL)[3]. After 2 subsequent conditioning steps (1 ml methanol then 1 ml water), the alkaline hair extract was loaded onto the GC cartridge. To remove impurities, cartridges were washed with 2 ml of a solution consisting of 60 parts methanol and 40 parts 2% aqueous ammonium hydroxide solution (60+40, v/v). The elution and measurement of clenbuterol was then carried out using 2 ml methanol and water (9+1, v/v). After drying, the extract was derivatised by MSTFA/NH4I/propanethiol (20 ml/100 mg/40 µl) to form bis-TMS-derivatives of clenbuterol. Measurement was carried out by gas chromotography/high resolution mass spectrometry (GC-HRMS) (Autospec, Micromass)[2] at a resolution of 10,000. Method validation included calculating the values for the limit of detection (LOD), limit of quantification (LOQ), repeatability and interassay precision. Ten calibration samples (containing a clenbuterol concentration of 2 pg/mg) were analysed in one run to determine the repeatability (within-run precision). The results showed mean µ = 1.8 pg/mg; s.d. = 0.3 pg/mg; and repeatability limit (r) = 0.06 (confidence level 95%). These measurements were repeated on 4 different days to evaluate interassay precision, resulting in mean value as µ = 1.7 pg/mg; s.d. = 0.3 pg/mg; and r = 0.03 (confidence level 95%). LOD and LOQ were estimated based on signal to noise ratios. A signal to noise ratio ≥3 is defined as the LOD. The following values were determined: LOD = 0.2 pg/mg; LOQ = 1 pg/mg.

Analysis of blood and urine samples

In blood and urine, clenbuterol is not conjugated and can be isolated without hydrolysation. After solid-phase extraction (XAD), clenbuterol was further purified by liquid/liquid extraction (diethylether, pH 10 or 11), derivatised and finally detected by GC-HRMS as described for the hair samples. All data are expressed as mean values ± s.d. Since clenbuterol does not occur as a natural substance in the untreated horse, any value exceeding the limit of detection was considered to indicate application of clenbuterol.

Results

The average rate of hair growth of mane and tail was 0.56 and 0.57 cm/week, respectively, over the 11 weeks tested. Hair grew at a very uniform rate (Fig 1).

Prior to treatment (Table 1), clenbuterol was not detected in any tail hair samples. Hair segments from control horses were also free of clenbuterol. By Day 5 after initiating treatment, a marked increase of clenbuterol concentration was detected in the basal segment; this peaked at more than 20 pg/mg on the last day of treatment (Day 10). Thereafter, a continuous decline of clenbuterol concentration took place in the basal segment, reaching 0 on Day 90 (11 weeks after the last dose). In the subsequent 20 mm segments, a growth rate-dependent temporal shift of the first detectable and peak concentrations of clenbuterol was observed. The greater the distance from the root, the later the appearance of clenbuterol. Each subsequent 20 mm segment showed a loss of

TABLE 3: Mean ± s.d. clenbuterol concentrations in plasma, urine, mane and tail hair during the first 2 months and on Day 360 of testing

Sample	Day after start of 10-day treatment					
	0*	5	10	30	60	360
Urine (pg/ml)	0	8150 ± 5031	11,413 ± 5428	68 ± 17	0	Not measured
Plasma (pg/ml)	0	58 ± 32	90 ± 67	0	0	Not measured
Tail hair[†] (pg/mg)	0	11.75 ± 4.35	23.25 ± 5.68	19.75 ± 7.89	20.25 ± 8.38	16.75 ± 4.79
Mane hair[†] (pg/mg)	0	11.50 ± 6.61	22.75 ± 12.84	17.00 ± 10.55	18.00 ± 9.56	9.25 ± 6.85

*Day 0 = day prior to treatment; [†]Segment with the highest clenbuterol concentration on that particular day.

detectable clenbuterol, with a delay of approximately 11 weeks as hair growth shifted the contaminated portion further from the root. Between Days 150 and 360, data suggest a considerable increase in the total amount of clenbuterol. This is due to an unusually high but not explainable concentration in a single subject.

The pattern of clenbuterol concentration in mane hair followed the pattern of tail hair concentration with striking similarity (Table 2). All control samples were free of clenbuterol. In hair samples of the treated group, clenbuterol concentration increased in the first segment up to the last day of treatment and declined thereafter. On Day 120, the first segment was devoid of clenbuterol. This was 30 days later than in the comparable tail hair segment.

The considerable deviations shown in Tables 1 and 2 are due not to the analytical method but probably to variations in hair melanin concentration. It was striking that the 3 horses with black tail and mane hair had considerably higher clenbuterol concentrations than the horse with light brown hair. As an example, this is shown in Figure 2 for one black and one light brown horse.

Analysis of plasma and urine samples (Table 3) confirmed the absence of clenbuterol prior to treatment. Following administration, blood and urine concentrations rose faster and to much higher concentrations than observed in hair. However, while urine and blood ceased to contain clenbuterol between Days 20 and 50 after the last administration, tail and mane hair continued to reveal clenbuterol.

Discussion

The results show clearly that the β-agonist clenbuterol is detectable in tail and mane hair over an extended period of at least 360 days. Popot *et al*. (2000) detected clenbuterol in 180–210 mm segments of tail hair from 2 horses 10 months after treatment. Interestingly, in the 2 studies, hair growth rate was nearly identical, 2.24 and 2.28 cm/month in the present study and 2.3 and 2.4 cm/month in the study of Popot *et al.* (2000) for mane and tail hair, respectively. In contrast, urine and blood samples were void of clenbuterol 20–50 days after the end of treatment. Detection of clenbuterol was unambiguous, since samples taken prior to treatment and samples of control horses were always below the limits of detection. Furthermore, as hair increases in length, segments proximal to hair roots became void of clenbuterol while segments containing clenbuterol moved away from the root at the rate of hair growth. It is noteworthy that on Days 90–120 clenbuterol was detectable over 4 consecutive segments only; this increased to 5 segments on Day 150 and eventually over 11 consecutive segments on Day 360. The reason for this is not evident; it could have been due to differences in the growth rate of individual hairs during the anagen phase, but in older hair the catagen phase could also be responsible. The data presented show that it is possible to detect clenbuterol treatment for a much longer period than has previously been reported (Hagedorn *et al.* 1995; Popot *et al.* 2000). This technique may eventually allow conclusions to be drawn as to the duration of treatment and identification of instances where this exceeds normal therapeutic usage. An important application could be the identification of horses treated to enhance growth and development at an early age for show or breeder selection purposes. The technique also permits the detection of drugs that cannot be accounted for by veterinary prescription where documentation of treatment of horses is legally required. Providing evidence of prolonged or unusually high dosage application of clenbuterol in young horses would help to identify illegal use of this drug as an anabolic agent. When clenbuterol has been used therapeutically in young stud stallions over extended periods it can cause respiratory dysfunction, and the animal should not be used for breeding. Potential hereditary predisposition to chronic obstructive pulmonary disease (COPD) would be masked.

Although the present study was limited to a single drug, it is probable that other β-agonists, steroids or other drugs are also incorporated into the hair of the horse and could be analysed in a similar fashion. Dunnett (2002) recently reviewed the wide range of potential applications of hair analysis, but did not provide specific data on clenbuterol. Clenbuterol in hair of horses was first described in a conference report (Popot *et al*. 2000). The determination of clenbuterol in the tail or mane hair of horses will not replace blood or urine analysis, which are well established methods for detection of recent drug use (Lehner *et al.* 2001; Harkins *et al.* 2001). However, hair analysis enables identification of application and duration of application outside the time range covered by blood and/or urine analysis. A horse tail of approximately 60 cm length and growth rate of about 2.4 cm/month would allow analysis of clenbuterol usage over a period of 24 months. Additional advantages of testing hair specimens compared to urine and blood are the ease of collection, transportation and storage of hair samples. At present, one limitation exists; hair analysis yields no data on the quantity of clenbuterol administered. In this study, we did not perform dose response studies. Our results indicate that hair colour may affect the measured concentration of clenbuterol. This has been shown in human hair (Gleixner *et al.* 1996) and was noted by Popot *et al.* (2002) in the horse. A second pilot study performed by the authors, in which clenbuterol was analysed by ELISA, confirmed these observations (A. Schlupp *et al.*, unpublished data). The stabilisation of various substances in hair has been shown to depend on their binding affinity to melanin (Sauer and Anderson 1994). Therefore, it is to be expected that more will be retained in darker than in lighter hair. A practical outcome of this observation is that other drugs can be tested for melanin binding affinity to reveal whether they are likely to be detectable in horse tail hair.

The present report demonstrates a reproducible and sensitive technique by which clenbuterol can be determined in both mane and tail hair of horses, for a period up to 1 year after a 10-day therapeutic treatment. The method can also evaluate the duration of treatment, although the precision of the duration measurement decreases with time, due to differences in the growth rates of individual hairs.

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Manufacturers’ addresses

[1]Boehringer Ingelheim, Ingelheim, Germany.
[2]Waters Corporation, Milford, Massachusetts, USA.
[3]Biolab, Risskov, Denmark.

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