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Clenbuterol: longterm detection via hair analysis

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Introduction

Clenbuterol is a ß-agonist, a synthetic derivate of the natural catecholamines, e.g. epinephrin, and was first synthezised in 1972 (Keck et al., 1972). It is a very selective $ß_2$-receptor agonist with negligible $ß_1$-side effects. Due to its chemical structure it is orally active and, moreover, it is protected against the enzymatic metabolisation by monoamino oxidase and catechol-O-methyl transferase. Clenbuterol is registered as a bronchospasmolytic agent; of minor importance is its use as tocolytic agent. From animal experiments it is known, that in doses about tenfold higher than the ones for therapeutic purposes, Clenbuterol has significant anabolic effects (Baker et al., 1984; Ricks et al., 1984). The effects are based on the metabolic actions of Clenbuterol: it decreases lipogenesis, it increases lipolysis and glycogenolysis; simultaneously it attenuates protein turnover by reducing protein degradation. In meat producing animals this leeds to reduced portions of fat and increased portions of lean meat (for review see Hanrahan, 1987). This effect together with an increase of muscle strength is of course of interest in sports, too, and this is why Clenbuterol became involved in doping (Spann & Winter, 1995).

Due to the fact that Clenbuterol is detectable in blood and in urine only for a few days after withdrawal, approaches are necessary to detect Clenbuterol intake beyond these relatively short time intervals. Most of the knowledge accumulated on the pharmacokinetics of Clenbuterol is derived from animal husbandry and the initial hints how to solve the problem indeed came from residue studies in cattle: Meyer & Rinke (1991) have demonstrated that Clenbuterol is accumulated in some tissues especially in liver and in the eye. More detailed studies on the precise localization of Clenbuterol accumulation in the eye have demonstrated that it is retained in the pigmented epithelium (Dürsch et al., 1993). Comparing the Clenbuterol accumulation in eyes of pigmented and of albino rats, a relation to the melanin content of the eye became obvious: In albino rats in which the tyrosinase pathway for the generation of melanin is defect and thus melatonin synthesis is inhibited, no significant Clenbuterol accumulation was observed (Dürsch et al., 1995). Melanin accounts for the pigmentation not only in the retina but also in

hair. Based on these findings and on the known advantages of hair analysis (Baumgartner et al., 1979; 1982) we started to investigate the accumulation of Clenbuterol in hair and the applicability of Clenbuterol hair analysis as a tool for doping control.

Materials and Methods

Animal model: twelve male veal calves with initial body weights of 97 ± 10 kg were used. To study the effect of pigmentation on Clenbuterol accumulation, different breeds with different coat colours were included. The breeds were Holstein Friesian (black and white hair; n = 2), Brown Swiss (cream coloured to dark grey; n = 6), Simmental (yellow/red and white; n = 2) and F_1 cross breeds from Brown Swiss x Simmental (yellow/grey; n = 2). All calves were kept in pens and were fed twice daily with milk replacer. After two weeks of adaptation the calves were fed 0.8 µg Clenbuterol/kg twice daily with milk replacer on days 1 to 10 of the experiment. The dosage corresponded to therapeutic applications. Blood samples were obtained from all calves by jugular venipuncture on days 1, 2, 4, 6, 8, 10, 12 and 18 of the experiment. Plasma was separated by centrifugation and stored at -20°C until analyzed. Throughout the experiment hair samples were collected on d 4 (during Clenbuterol treatment) and on d 11, 18, 25, 35, 41, 55 and 70. Some of the calves had limited spots of white or colored hair and therefore sampling was not possible on each day indicated above from all calves; at the end of the experiment (e.g. when there was no colored hair left) values of a few samples are missing. Two different types of hair samples were obtained from different locations. Samples designated as "old hair" consisted of hair taken at the surface of the skin by shaving the animal with a small animal clippers (Favorita II, AesculapR, Tuttlingen, Germany). Hair samples refered to as "new hair" in the present study were obtained from the same specific areas on the animals which were shaved in regular intervals of 2 weeks, starting before the treatment and continued during the experiment. This "new hair" represents the hair grown during the experiment on the same area.

Human hair samples: For the determination of Clenbuterol in human hair, hair samples from 67 volonteers were obtained. Fifty hair samples were from 50 randomly selected volonteers, representing a cross-section of the local population and were used as negative controls. These 50 volonteers were subdivided according to sex, age, hair color and their manners of nutrition. Nine hair samples were obtained from volonteers who took the known therapeutic daily dosage of Clenbuterol (2 x 5 mL Spasmo-Mucosolvan; = 2 x 5 µg) for 25 days. Six volonteers have used a cough-mixture not further specified in terms of dosage and regimen. Two hair samples were obtained from body builders who were suspected taking Clenbuterol.

Full length hair samples (in average 250 mg of weight) were taken at the surface of the skin on

the back of the head (occiput) of each volonteer. The shape of the samples resembled cylinders of hair with an average diameter of 0.5 cm and the lenght of the hair of a volonteer from its head to the hair tips.

The effect of hair bleaching on Clenbuterol concentrations measurable in hair was tested by incubating 9 hair samples with known Clenbuterol concentrations from volonteers who were treated with Clenbuterol in 35% H_2O_2 for 2 h at room temperature. Extraction was done after rinsing the sample with water and drying at 60°C.

Preparation of Hair Samples: Approximately 1 g of hair was incubated in 100 mL of water for 1 h. Then the hair was put into a plastic syringe for single use where the piston was removed. The hair was washed by filling the syringe with the washing solutions mentioned below and by pressing the piston into the syringe. Hair was rinsed twice with 30 mL of 0.1 % BSA and three times with 30 mL of 0.2 % Tween® 80 to remove potential exogenic Clenbuterol contaminations. The hair was subsequently dried at 60°C and was then pulverized with a Mikro-Dismembrator II (B. Braun, Melsungen, Germany). Specimen were ground in prechilled teflon vessels containing a tungsten carbide bullet by shaking them with maximal frequency. Each sample was treated for four periods of 40 s with 60 s intervals of cooling in liquid nitrogen. The obtained hair powder was stored at -25°C until further analyzed.

Extraction of Clenbuterol from Hair: Fifty milligrams of hair powder, 1 mL of 50 mM aqueous 1,4-Dithiothreitol (DTT), 50 µL of 5 M NaOH, and 2.5 mL of tertiary butylmethyl ether were shaken over night at room temperature. The ether was separated by centrifugation and the extraction was repeated for 1 h with tertiary butylmethyl ether. The ether phases of each sample were combined and evaporated at 60°C in a waterbath. Residues were dissolved in water and appropriate dilutions were assayed as duplicates by enzyme immunoassay.

Enzyme Immunoassay (EIA): As final detection system the Clenbuterol-enyme immunoassay as described by Meyer and Rinke (1991) was used. The antibody against Clenbuterol-diazo-BSA was obtained from rabbits and showed negligible cross reactions (< 0.01%) with epinephrine and norepinephrine and some cross reactivity with other ß-agonists (Salbutamol: 11%, Terbutaline: 9%).

Validation of the Extractions: For a detailed validation of the extraction protocol developed herein hair powder (50 mg at a time) from untreated calves as well as from volunteers who were not taking Clenbuterol was spiked with 1, 2.5, 5, 10, 25, 50, 100, 500, and 1,000 ng of Clenbuterol/g (n = 8, each) and gently shaken at room temperature for 1 h to allow binding of Clenbuterol to the hair material. Hair was spiked at levels of Clenbuterol that could bind to hair material in vivo. Samples were then analyzed according to the method decscribed above. The

mean recovery amounted to 60% and was linear, the coefficient of variation was 12 ± 4% including the variations of the extractions and of the enzyme immunoassay. Calf plasma was spiked with 25, 50, 100, 150, 200, 300, 450, 600, and 800 pg of Clenbuterol/mL. The mean recovery was 74%, the coefficient of variation was 11 ± 2%. Procedural blanks estimated in negative samples were always below the limit of detection (0.3 ng of Clenbuterol/g of hair and 12 pg of Clenbuterol/mL of plasma). The final results were calculated by taking the average recoveries into account.

High Performance Liquid Chromatography (HPLC): Appropriate amounts of the hair samples were extracted as described, the residues of the ether extracts were redissolved in 200 µL of water and 100 µL were applied to an HPLC column (LiChrospher RP-select B, 125 mm x 4 mm, 5 µm, Merck, Germany) by a Sample Injection Valve (Model 210, Beckmann, München, Germany). Isocratic elution was done with 20 mM KH_2PO_4 pH 4.0 / acetonitrile at 80/20 (v/v). The flow rate was 1 mL min-1 at 25 °C. Fractions of 300 µL were collected around the Clenbuterol peak (from minutes 3 to 7 of the run) and were evaporated to dryness at reduced pressure in a Buchler Vortex Evaporator (Buchler Instruments Inc., Fort Lee, NJ, USA). The residue of each fraction was redissolved in 200 µL water and analyzed in duplicate by the Clenbuterol-EIA.

Confirmation of Enzyme Immunoassay Results: Ten hair samples (two negative, two spiked and six positive at different levels) were analyzed by EIA (without HPLC) and by HPLC/EIA. With the combined HPLC/EIA analysis of negative, spiked and positive hair samples the identity of Clenbuterol standard and the extracted Clenbuterol could be confirmed. All positive and spiked samples were used for a comparison of both methods. The ratios between the results of HPLC/EIA and EIA alone were calculated and the mean ratio was 0.9 ± 0.1. Negative samples were below the limit of detection. Samples measured with and without purification by means of HPLC resulted in identical concentrations of Clenbuterol. All immunoreactivity migrated in the HPLC like Clenbuterol. That demonstrated that no other substances in the samples, which might exhibit a cross-reactivity did interfere with the EIA.

Results and Discussion

Methodology: The extraction protocol developed herein met the three main criteria of validity, i.e. it was highly efficient and complete, the recovery from spiked samples was sufficiently high and linear, demonstrating that Clenbuterol was not degraded during the extraction. Third, negative samples were always below the limit of detection of the EIA and thus showed that there was no interference with the immuno assay. For an adequate extraction method for

Clenbuterol from hair, the reducing agent DTT, which breaks the disulphite bonds between the longitudinal chains of the keratin molecules, proved to be the most important factor for a maximally efficient extraction. All other disintegrating agents commonly used in hair analysis, e.g. NaOH or keratolytic acids (Chatt & Katz, 1988) were less effective or were interfering in our final detection system, the EIA. Comparing with other methods reported in the literature on Clenbuterol concentrations in hair (Adam et al., 1994; Polettini et al., 1995), the effectiveness of the DTT based extraction protocol developed herein is further supported since our measurements yielded the highest concentrations reported. As demonstrated by HPLC, the quantification of Clenbuterol from hair extracts by enzyme immunoassay (EIA) reported herein is not impaired by other extract constitutents or Clenbuterol metabolites. All immunoreactivity migrated within a single peak corresponding to Clenbuterol and both methods, the HPLC/EIA as well as the EIA alone yielded consistent results. Our results demonstrate that the EIA is suitable for the screening of large sample numbers to identify positives which than can be confirmed by HPLC or GC-MS.

Clenbuterol accumulation in calf hair of different colour: Fig 1 shows the Clenbuterol concentrations both in plasma and in hair ("old hair") from four treated calves throughout the experiment. Within two days after the onset of the Clenbuterol application, the plasma concentrations reached a plateau varying between 200 and 550 pg/mL until the end of treatment. After 15 days withdrawal Clenbuterol was no longer detectable in plasma. In all hair samples obtained before the onset of the treatment, Clenbuterol was not detectable. In "old" hair, Clenbuterol concentrations reached detectable concentrations as early as day 4 of the Clenbuterol application. Comparing the Clenbuterol plasma and hair concentrations from our calf study, the advantages of the hair analysis are evident from the extended time of detectability in the hair matrix. With regard to urine a similar time limit for Clenbuterol detection as in plasma exists. Clenbuterol incorporated into the hair matrix is fixed there and will not be eliminated as long as the hair is not shed.

The accumulation of Clenbuterol in hair of different phenotypic hair colour is summarized in Figure 2. Maximal concentrations were reached approximately on day 35, i.e. 25 days after Clenbuterol withdrawal. From then on the Clenbuterol decreased but remained still well above the limit of detection at the end of the experiment (day 70) for all hair colours. Clenbuterol accumulation showed a strong colour dependency being particularily high in black hair and lowest in white hair. In the other hair colours Clenbuterol was accumulated in decreasing concentrations according as follows: darkgrey > red > gray> yellow. The dependency of

Clenbuterol accumulation from hair pigmentation is most obvious from the spotted breeds used in the present study: Comparing the Clenbuterol concentrations in white and in coloured hair grown on one individual animal, melanin content seems the only possible explanation since all other paramenters e.g. Clenbuterol blood levels are assumingly identical.

To investigate the time course of Clenbuterol accumulation in hair, regrown hair at spots shaved prior to the treatment ("new hair") was sampled. The results are presented in Figure 3.

In hair grown during the treatment Clenbuterol was well detectable; after cessation of the treatment (day 11) Clenbuterol concentrations in newly grown hair still increased and reached maximal levels in hair grown between day 11 and 25 of the experiment. Beyond this time no further increase was observed but detectable levels were maintained in hair grown 45 to 60 days after the end of the Clenbuterol application.

Clenbuterol analysis in human scalp hair: in the 50 hair samples obtained from 50 randomly selected volonteers, which represent a cross-section of the local population and were used as negative controls, no Clenbuterol was detectable. Table 1 summarizises the Clenbuterol concentrations detected in hair from patients who took Clenbuterol for therapeutic purposes or were suspected for taking Clenbuterol for anabolic purposes. For those volunteers who took Clenbuterol in a known defined scheme (#1 to #9), the hair Clenbuterol concentration was related to the 1 cm hair bundle in which Clenbuterol was incorporated. Considering the length and weight of the hair sample, a mean hair growth velocity of 1 cm per month (Chatt & Katz, 1988) was used as a basis. In hair samples obtained from these volunteers before Clenbuterol intake, Clenbuterol was not detectable. Similar to the results from the calf study, the mean concentrations observed in dark hair (#1- #5) were higher ($p < 0.05$, two-sided Mann-Whitney U-test) than in fair hair (#6 - #9). In those samples obtained from individuals who were taking Clenbuterol in unknown regimen, lower concentrations were found, but this might at least in part be explained by the fact that in those samples the Clenbuterol concentrations had to be related to the entire hair strand. Compared to these persons, the Clenbuterol concentrations found in hair samples from the body builders which are related to the entire hair strand, too are relatively high and might be attributed to the intake of Clenbuterol doses necessary for anabolic purposes.

Table 2 shows the Clenbuterol concentrations measured in sequential sections of a 12 cm hair bundle from 2 volunteers obtained 4 months after the last Clenbuterol intake. Clenbuterol incorporation was maximal in the segment 4 to 6 cm distant to the scalp. In the hair tips grown before Clenbuterol intake no Clenbuterol was detectable. The same was true for hair close to the scalp which grew well after the treatment phase.

To investigate the length of time in which Clenbuterol intake can be detected by hair analysis, hair samples were obtained from the nine volonteers who were treated for 25 days with Clenbuterol at different time intervals after the treatment (table 3). The concentrations are related to the entire hair sample. In dark haired individuals (#1 - #5), Clenbuterol intake was detectable 1 up to 145 days after the last intake whereas in fairer hair samples (#6 - #9) the detectability was limited to 95 days. As demonstrated in table 3, the concentration of Clenbuterol in an entire hair sample which is not subdivided is reduced by the continued hair growth thus leading to a dilution. The detectability might therefore be provided as long as the hair grown during the treatment is not shed; according to our data, Clenbuterol intake can be detected after several months by hair analysis. Bleaching of hair samples in which Clenbuterol was incorporated with 35% H_2O_2 led to a mean decrease of 25 ± 5% of the measurable Clenbuterol concentrations. Thereby the detectability of Clenbuterol intake via hair analysis would nevertheless be provided within comparable time intervals.

Conclusions and Outlook

Taking the results from the calf model study and the human samples together, hair analysis allows to detect the application of Clenbuterol well beyond the time of positive measurements in urine or plasma. Hair analysis thus provides a powerful tool for the control of Clenbuterol doping in sports.

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GLEIXNER A., SAUERWEIN H. & MEYER H.H.D. (1996). Accumulation of the β2-adrenoceptor against clenbuterol in calf hair of different pigmentation. *Archiv für Lebensmittelhygiene* **47,** 131-135.

GLEIXNER A., SAUERWEIN H. & MEYER H.H.D. (1997). Nachweis von Clenbuterol in Kopfhaar: eine Methode zur Trainingsdopingkontrolle. *Deutsche Zeitschrift für Sportmedizin* **48,** 50-55.

Table 1: Clenbuterol concentrations in hair samples from volonteers with admitted or suspected intake of Clenbuterol

Individual number	sex	age (years)	hair colour	ng Clenbuterol/ g hair
#1	male	27	black	126
#2	male	30	black	80
#3	female	27	brown	161
#4	female	30	brown	120
#5	female	60	black	114
#6	female	28	light brown	38
#7	female	60	grey	35
#8	female	60	grey	26
#9	female	27	blond	23
Volunteers #1 to # 9 took 10 µg Clenbuterol/day for 25 days. Clenbuterol concentrations in hair are related to a 1 cm hair bundle grown during the treatment.				
#10	male	30	black	8
#11	male	2	blond	4
#12	male	5	blond	3
#13	male	6	blond	3
#14	male	35	blond	3
#15	female	45	brown	5
Vounteers #10 to #15 were treated with Clenbuterol in an unknown regimen (dosage, treatment time and interval until sampling; the given hair Clenbuterol concentration is related to the entire hair sample obtained.				
#16	male	35	brown	92
#17	male	40	brown	50
Vounteers #16 and #17 were body builders suspected for Clenbuterol intake; the given hair Clenbuterol concentration is related to the entire hair sample obtained.				

Table 2: Clenbuterol concentrations in sequential sections of a 12 cm hair sample from 2 volunteers obtained 4 months after the last Clenbuterol intake

scalp distance (cm)	**0-2**	**2-4**	**4-6**	**6-8**	**8-10**	**10-12**
female, brown hair	<	2	16	8	<	<
female, blond hair	<	3	9	4	<	<

< below the limit of detection (0.3 ng/g)

Table 3: Detectability of Clenbuterol in hair samples from 9 volunteers (#1 - #9)obtained at different times after the last Clenbuterol intake

	dark hair					**fair hair**			
days beyond last treatment day	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**
35	12	9	13	19	11	4	4	3	2
95	5	4	-	-	4	2	2	-	0.5
125	-	-	4	-	-	<	<	-	<
145	2	1	-	2	2	-	-	-	-

< below the limit of detection (0.3 ng/g)
- no sample obtained

Legends

Figure 1: Mean Clenbuterol concentrations in yellow and red "old hair" and in plasma from four calves (2 Simmental and 2 cross breeds)

Figure 2: Mean Clenbuterol concentrations in calf hair of different pigmentation; "old hair" was sampled from different spots at the days indicated

Figure 3: Mean Clenbuterol concentrations in calf hair of different pigmentation; "new hair" was sampled from defined spots which had been shaved prior to the treatment. After the time interval indicated the regrown hair was sampled.

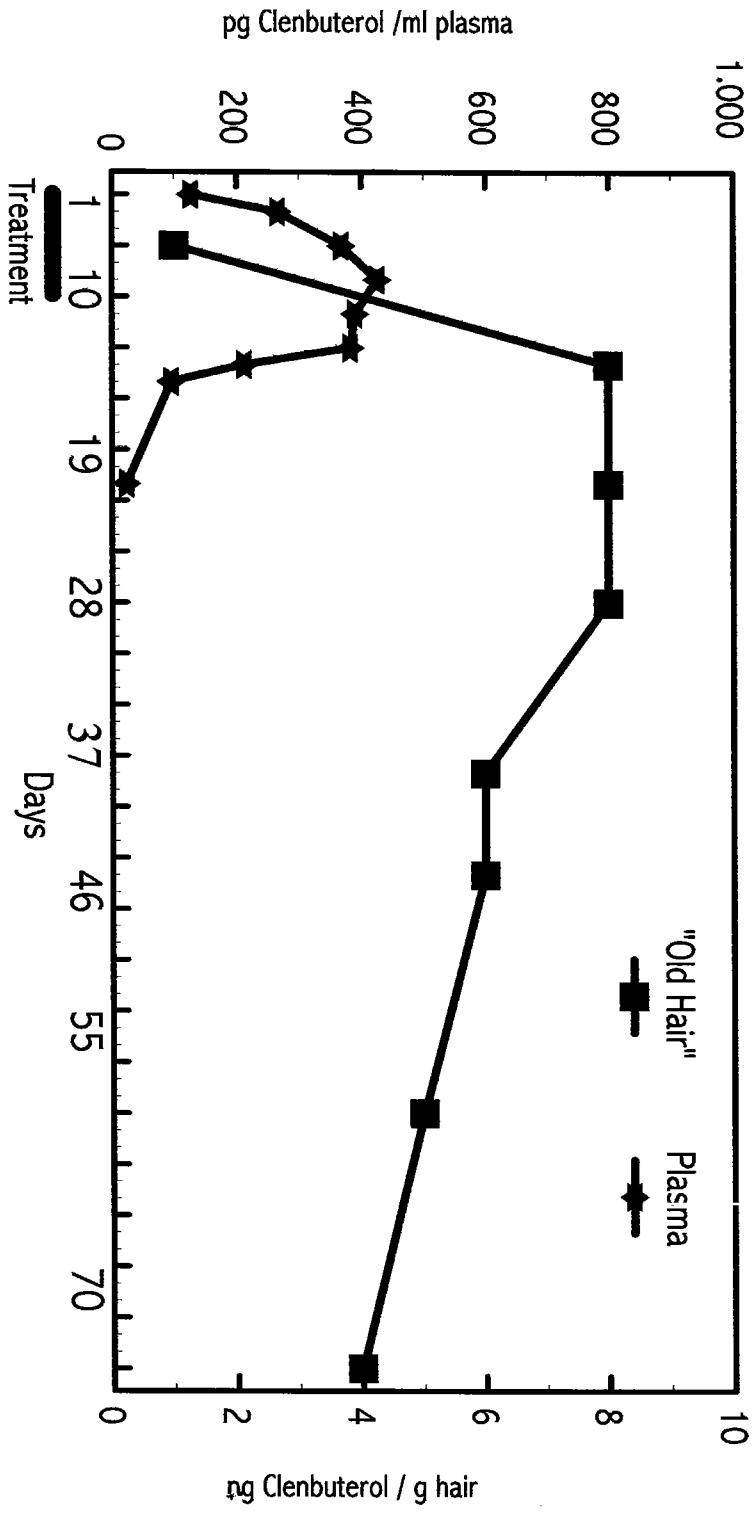
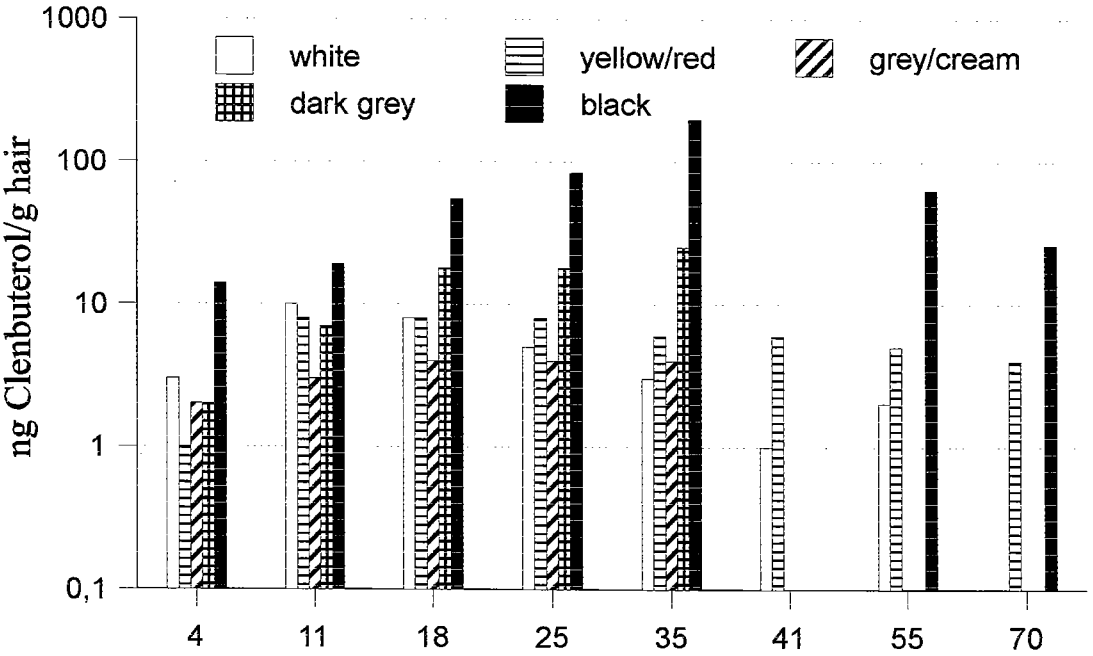
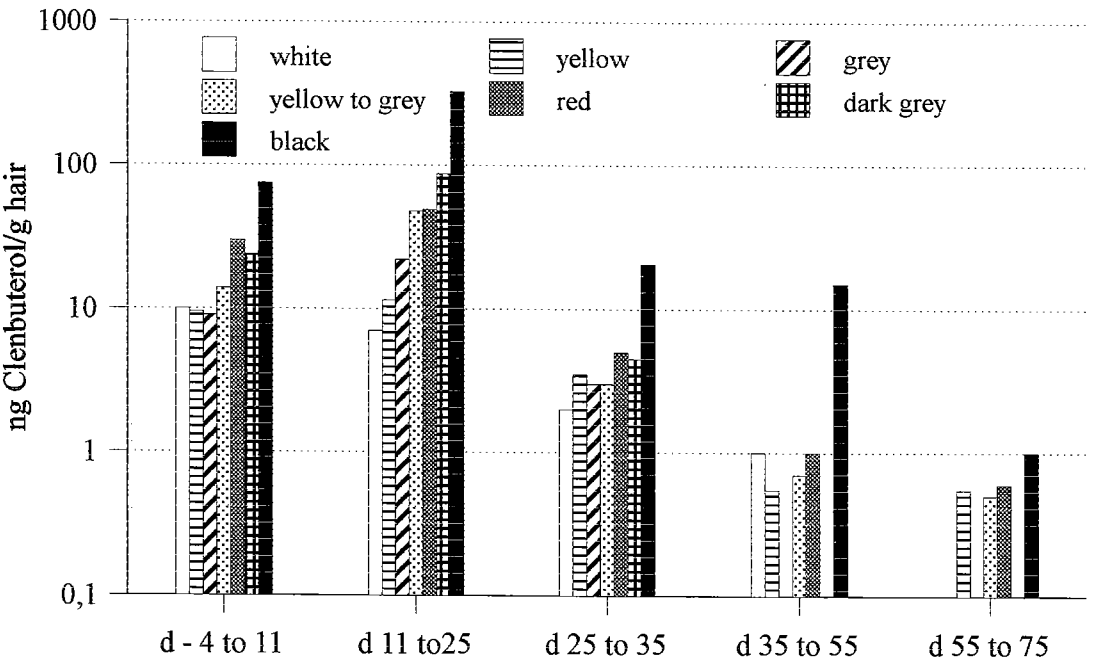

pg Clenbuterol /ml plasma
0
200
400
600
800
1.000
Treatment
1
10
19
28
37
46
55
70
Days
"Old Hair"
Plasma
0
2
4
6
8
10
ng Clenbuterol / g hair

Figure 2



days of the experiment; d 1- 10: Clenbuterol treatment (0.8 µg/kg twice daily)

Figure 3



days of the experiment; d 1- 10: Clenbuterol treatment (0.8 µg/kg twice daily)