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Long-term detection of clenbuterol in human scalp hair by gas chromatography–high-resolution mass spectrometry

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Abstract

A method for the detection of clenbuterol in human scalp hair by gas chromatography–high-resolution mass spectrometry (GC–HRMS) is described. The sample preparation involved chemical digestion of the protein structure, which was achieved by incubating the hair with 1 *M* KOH at 70°C. A single extraction step with *tert*.-butyl methyl ether provided approximately 90% of the analyte, which was dried and derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) to yield clenbuterol *N*,*O*-bis-trimethylsilyl (TMS). Hair was collected from four pregnant women who were therapeutically treated with Spiropent® (clenbuterol–HCl) and from the infant of one female patient. Hair samples were taken during the application time and two to six months after completion of clenbuterol administration. The detection limit of the method was approximately 4 ng clenbuterol/g hair when 25 mg hair material were processed and 2 ng/g for 50 mg hair samples (corresponds to 4 pg per injection). The method allows clenbuterol to be measured retrospectively for up to at least six months. The levels of clenbuterol determined in hair ranged from 2 to 236 ng/g. No clenbuterol was found in the hair of the infant, which was taken five and a half months after delivery. To improve sample preparation, an additional purification step via immuno affinity chromatography (IAC) was integrated. The IAC purified extracts showed reduced biological background interference and an improved limit of detection (0.8 ng/g). © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Clenbuterol

1. Introduction

Clenbuterol is a β_2-agonist and has relaxing effects on smooth muscle. The normal indication of clenbuterol therapy in the treatment of asthma bronchiale and obstructive bronchitis is based on its action as a bronchodilator [1,2]. The stimulation of adrenergic β_2-receptors is also relevant for tocolysis. The oral therapy of premature labor with clenbuterol and other β-mimetic agents is a controversial application field and is discussed in references [3,4]. Other effects of β_2-agonists are reflected in their stimulatory activities on the central nervous system and respiration [5]. When the applied dosage of clenbuterol is increased by an order of magnitude, clenbuterol enhances protein synthesis and activates the rate of lipolysis [6,7]. These effects are the reason for the misuse of clenbuterol in cattle fattening to increase meat mass and reduce the fat moiety [8,9]. More recently clenbuterol has also found application in horse racing as well as in human sports. Especially in those forms of sports where

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increased lean body mass and muscle strength can enhance performance e.g. in body-building, weight-lifting and athletics, clenbuterol is an effective doping agent [10,11].

The short half-life of clenbuterol limits the time of its detection in biological fluids [12]. In an excretion study with humans, Ayotte [11] found that after a single dose of 100 μg clenbuterol hydrochloride, the drug could be identified in urine samples for up to 24 h.

Hair offers a medium for drug detection with prolonged retrospectivity, in contrast to other biological matrices such as urine or blood [13–15]. Detection of clenbuterol in human and animal hair using enzyme-linked immunosorbent assay (ELISA) techniques has been described [16,17].

The main disadvantage of immunochemical techniques is a higher potential of false positive samples reported, because an antibody can usually not discriminate between structurally related compounds. To reduce these crossreactants, washing steps and the separation of clenbuterol by high-performance liquid chromatography (HPLC) have been efforted [16,18,19].

The gas chromatography–high-resolution mass spectrometry (GC–HRMS) method is not affected by these interfering substances, because the use of HRMS with a resolution of 3000 provides high selectivity for clenbuterol. The spectral data of characteristic ions demonstrate unambiguously the presence of clenbuterol. This is illustrated in Fig. 1. The sensitivity is comparable to that found with immunochemical detection and lies in the low ng/g range.

It has become well established that drugs are accumulated in hair at different rates and in different amounts [20,21].

Critical factors in the transport process into hair are the physico-chemical properties of the substance itself, such as its molecular mass, basicity and hydrophobicity [22]. It has been shown that a combination of basicity and a certain portion of hydrophobicity provides ideal conditions for incorporation into hair [23,24]. Melanin, the pigment responsible for the coloration of hair and other tissues, plays a key role during transport from the blood stream into hair [25–27].

It is assumed that melanin functions as a carrier in the passage of a drug through the membrane of the hair papilla and that it also fixes the drug by ionic interaction in the hair shaft [28,29]. Substances with a basic group, such as clenbuterol, are able to bind protons and exhibit a positive excess charge. These molecules have high affinity for melanin, which has an excess of negative charges at a physiological pH of 7 due to its acidic character [23,24,28,29].

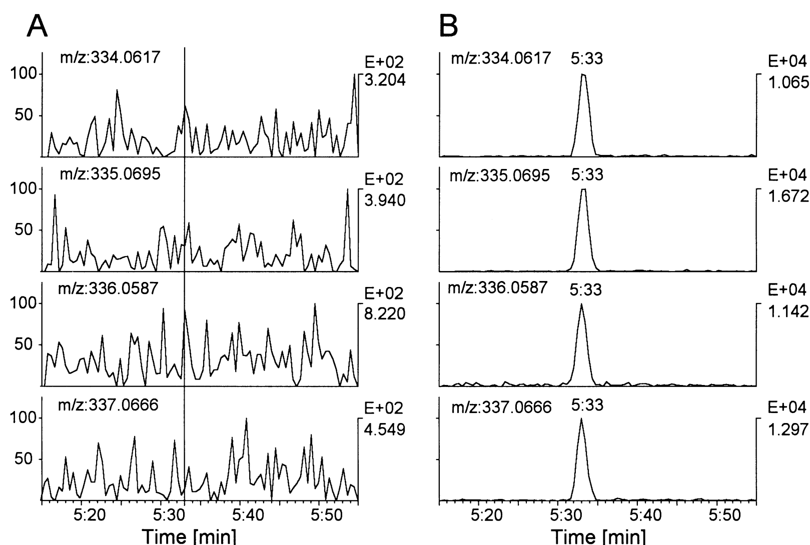

Fig. 1. Selected ion monitoring of clenbuterol *N*,*O*-bis-TMS in human hair by GC/HRMS.

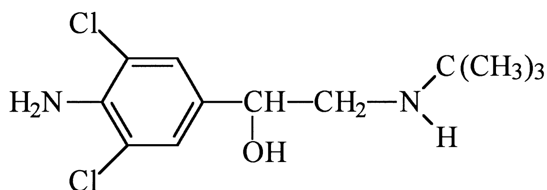

Fig. 2. Molecular structure of clenbuterol.

The structure of clenbuterol (Fig. 2) fulfils these criteria: It has a hydrophobic moiety and a basic character based on the two nitrogen functions. Therefore, it seems a promising substance to be accumulated in hair and to be detected using sensitive analysis methods, even if low doses are applied.

By means of a study with pregnant women who were using clenbuterol as a tocolyticum under a controlled regimen, the performance and validity of the developed method was estimated. In the course of sample treatment, a liquid–liquid extraction was performed after the basic digestion of the hair material. Further purification was necessary to improve the detection limit. Therefore, an immunoaffinity chromatography (IAC) step using clenbuterol-selective antibodies was applied after the normal sample preparation procedure [30,31]. Reduction of the background interference and the improved detection limit of clenbuterol with this isolation technique was investigated. We also focused on future potentials as well as limitations of hair analysis in the context of doping control.

2. Experimental

2.1. Chemicals

Clenbuterol hydrochloride was purchased from Boehringer, Ingelheim, Germany. Clenbuterol-D6 was obtained from the National Institute of Public Health and Environment, BA Bilthoven, The Netherlands. The clenbuterol-selective immunoaffinity chromatography gel was developed and prepared by C.E.R. Laboratoire D'Hormonologie (Marloie, Belgium). Norandrosterone and *N*-methyl-*N*-trimethylsilyltrifluoracetamide (MSTFA) were synthesized in our laboratory.

A stock solution of ammonium iodide and ethanethiol trimethylsilyl (TMS; 100 mg and 300 μl, respectively) in 5 ml of MSTFA was prepared and stored in the dark. Separate stock solutions of norandrosterone and clenbuterol (10 mg in 10 ml of methanol each) were prepared and stored in the dark at −20°C. *tert*.-Butyl methyl ether (TBME) was obtained from Kraemer and Martin (St Augustin, Germany) and was distilled before use. KOH was obtained from Merck (Darmstadt, Germany). All of the solvents and reagents were of analytical grade.

2.2. Profile of patients and clenbuterol administration

Four females who had therapeutically taken Spiropent® (Thomae, Biberach, Germany) as a tocolyticum were tested for clenbuterol residues in hair. The therapeutic doses of clenbuterol–HCl were either 3×20 μg or 2×20 μg per day. The duration of application was five weeks for female A, three months for females B and D and 12 days for female C (Table 1).

Two females had naturally dark brown, one blond and one black hair.

2.3. Hair sampling

Hair samples were collected as indicated in Table 1. Full length hair bundles were taken at the surface of the skin on the back of the head (occiput), leaving hair tufts of 5–10 mm on the surface of the scalp. The hair bundles (diameter ca. 5 mm) were fixed with rubber bands and stored at −20°C until analysis.

2.4. Hair sample preparation

The hair strands were cut into 20 mm segments. From each segment, either 2×25 mg (duplicate determinations) or 1×50 mg of hair material were cut into small pieces with scissors and subsequently digested in 2 ml of 1 *M* KOH at 70°C for 2 h so that complete solubilisation of the hair structure was achieved. After the addition of 3 ml of H_2O, the aqueous phase was extracted with 5 ml of TBME. After decanting the organic layer, 1 ng of norandrosterone was added as a standard and the organic solvent was evaporated in vacuo.

Table 1
Profile of patients who used clenbuterol therapeutically

	Patient A	Patient B	Patient C	Patient D
Race	Caucasian	Caucasian	Caucasian	Caucasian
Age	31 years	40 years	24 years	33 years
Body weight	53 kg	68 kg (50 kg)[a]	66 kg	71.5 kg
Coloration of hair	Dark brown	Brown (synthetically bleached)	Dark blond	Black
Duration of application	5 weeks	3 months	12 days	3 months
Dosage	3×20 μg/day	3×20 μg/day	3×20 μg/day	3×20 μg/day
Hair collection	2 and 6 months after cessation of clenbuterol application	3 months after beginning and 5.5 months after cessation of clenbuterol application	5 months after cessation of of clenbuterol application	2.5 months after cessation of clenbuterol application

[a] Weight measured 5.5 months after delivery.

The dried residue was either derivatized (Section 2.6) for GC–HRMS analysis, or subjected to further purification via immunoaffinity chromatography (Section 2.5). To control the digestion, another procedure with the addition of dithiothreitol (DTT) was tested with hair from a drug user. A 150-mg amount of hair from a drug user (volunteer A) was divided into 25 mg portions. Three portions were digested according to the procedure described and analysed (set A). To three portions, 0.05 m*M* DTT was added and the samples were prepared according to the above-mentioned procedure. These results were compared to the data obtained with set A.

2.5. Isolation of clenbuterol by immunoaffinity chromatography

For the preparation of IAC gels, antibodies were raised in rabbits and were directed towards the conjugate of human serum albumin (HSA) and a clenbuterol derivative that had been diazotised at the aromatic amino group. After isolation, the antibodies were immobilised on a chemically modified agarose gel and were poured into columns.

A 1-ml volume of immuno affinity gel (binding capacity of clenbuterol, 60 ng) was added to an Econo column (Biorad Laboratories, Munich, Germany) and the column was stored at 4°C in phosphate-buffered saline (PBS, 120 mmol NaCl, 2.7 mmol KCl, 10 mmol $Na_2HPO_4 \cdot 12\ H_2O$, adjusted to pH 7.5 with 2 *M* HCl) containing 0.05% of NaN_3.

The following scheme was used for sample isolation: (1) the column was washed with 5 ml of PBS. (2) The dry residue of the hair extract was dissolved in 0.1 ml of methanol, diluted with 5 ml of PBS and applied to the column.

(3) The column was washed with 10 ml of H_2O and then with 10 ml of 15% methanol in water (15 g of methanol were diluted with water to a final volume of 100 ml). (4) Clenbuterol was eluted from the column with 3 ml of an ethanol–glacial acetic acid solution (80:20, v/v). This eluate fraction was evaporated to dryness in vacuo and derivatized according to methods described in Section 2.6. (5) Afterwards the column was washed with 5 ml of the eluting solvent, equilibrated with 15 ml of PBS, and stored. No solvent delivery system was used to apply the solutions, and the flow-rates were adjusted by the hydrostatic pressure of the fluids.

2.6. Derivatisation for GC–MS analysis

TMS ether derivatives were formed by dissolving the dry residue in 50 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA)–ammonium iodide–TMS ethanethiol (100:0.2:0.6, v/w/v) and heating for 15 min at 60°C.

2.7. GC–HRMS determination

High-resolution mass spectrometry (HRMS) analysis was performed with a reversed geometry double focusing mass spectrometer (Finnigan MAT 95, Bremen, Germany) coupled to an HP 5890 gas chromatograph (Hewlett-Packard, Waldbronn, Germany).

A HP Ultra 1 (crosslinked methyl silicone capillary column; length, 17 m; I.D., 0.20 mm; film

thickness, 0.11 μm) was employed with helium as the carrier gas at a flow-rate of 0.7 ml/min at 180°C. A 2-μl aliquot of sample was injected onto the GC column in the split mode (1:20). The column temperature was programmed from 150°C, at 10°C/min, to 250°C and then at 40°C/min to a final temperature of 310°C; final time, 3 min.

To analyze the IAC-purified hair extracts, the same GC temperature program was used but injection was made in the splitless mode.

The injection port and transfer line were heated to 300°C. Ions were formed by 65 eV electron impact (EI) ionization. The ion source was held at 240°C. High-resolution selected ion monitoring was performed by electric field scanning using a reference gas (perfluorophenanthrene) for mass locking and mass calibration. The electron multiplier voltage was set to 1.9 kV (for 10^6 gain) and the conversion dynode to 17 kV.

At a mass resolution of 3000, the following ions from clenbuterol were monitored: m/z 300.1006, 334.0617, 335.0695, 336.0587, 337.0666 and 338.0558. For the detection of the standard, norandrosterone, the ions m/z 405.2645 and 420.2879 were monitored.

2.8. Determination of stability and recovery of clenbuterol after chemical digestion of hair

2.8.1. Stability of clenbuterol in alkaline medium

To assess the stability of clenbuterol under alkaline conditions, samples (five replicates) in 2 ml of 1 *M* KOH were spiked with 1.0 ng of clenbuterol–HCl and incubated for 0, 1 and 24 h at 70°C. After extraction with 5 ml of *tert*.-butyl methyl ether, the recovery of clenbuterol was determined from each incubation experiment using 1.0 ng of clenbuterol-D6 as the standard.

2.8.2. Extraction yield

Four 25 mg portions of drug-free hair were fortified with 10, 20, 40 and 50 μl of a 0.1 ppm methanolic solution of clenbuterol hydrochloride (corresponding to 1, 2, 4 and 5 ng/25 mg hair, respectively). The spiked hair samples were digested and extracted three times with 5 ml of *tert*.-butyl methyl ether for 15 min. To each extract, 1 ng of norandrosterone was added. A mixture of norandrosterone and clenbuterol–HCl in methanol (each with 0.1 ng/ml) was prepared and 50 μl were analysed according to the methods described in Sections 2.6 and 2.7 and used as external standard.

The clenbuterol concentrations of the extracts were calculated from the peak height responses of ions m/z 335.0695 for clenbuterol and m/z 420.2879 for norandrosterone in relation to the respective responses of the external standard.

2.9. Calibration curve and sensitivity

To confirm the linearity within the concentration range under study (4–200 ng/g), a calibration curve was regressed through the data points that were obtained after analysing spiked hair samples. The data points represent the peak height ratios of ions 335.0695 (clenbuterol) and 420.2879 (internal standard norandrosterone) and were determined in duplicate.

From the hair samples spiked with decreasing amounts of clenbuterol, the detection limit of the method was also deduced. In the IAC procedure, the hair samples (125 mg) were spiked with 100 pg of clenbuterol (0.8 ng clenbuterol/g hair) as described above and subjected to GC–HRMS analysis in the splitless injection mode. The criterium for the detection limit was a signal-to-noise ratio (S/N) of three for the ion traces 334.0617, 335.0695 and 337.0666.

3. Results

3.1. Selectivity and sensitivity

Detection and identification of clenbuterol is based on HRMS analysis. Fig. 3 shows the EI mass spectrum of clenbuterol *N*,*O*-bis-TMS. For the detection in HRMS, the ions 300.1006, 334.0617, 335.0695, 336.0587, 337.0666 and 338.0558 were monitored, because they provided signals with low background noise and high intensity (Fig. 1). With regard to the selectivity of the employed HRMS method, it was concluded that a washing step was not necessary in contrast to other analysis methods such as ELISA.

HRMS analysis of the hair extracts showed linear

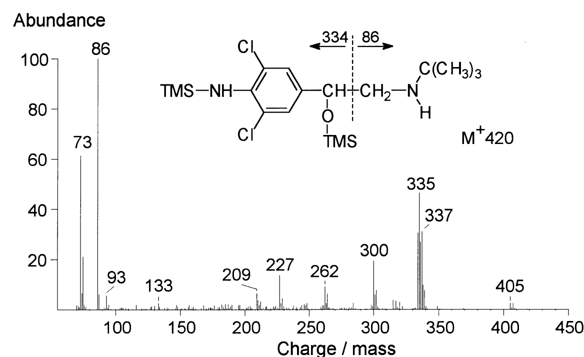

Fig. 3. Molecular structure and EI mass spectrum of clenbuterol *N*,*O*-bis-TMS.

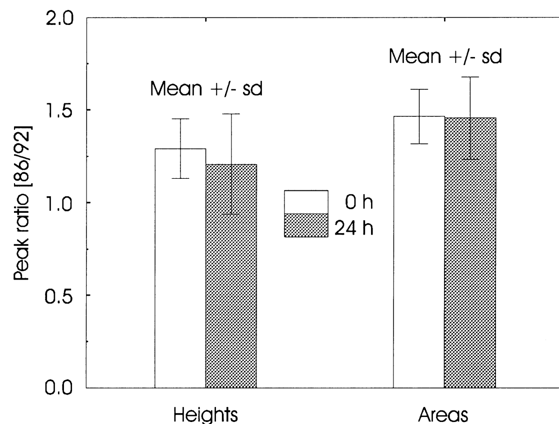

Fig. 4. Stability of clenbuterol during alkaline treatment with 1 *M* KOH at 70°C.

behaviour within the concentration range from 4 to 200 ng/g hair. Calibration graph data:

$y = 0.1475x - 0.0015$, correlation coefficient: 0.990.

To examine the detection limit of the method, hair samples spiked with decreasing amounts of clenbuterol were analysed according to the sample preparation scheme.

Following the sample preparation procedure without IAC purification, the analysis of 25 mg hair samples spiked with 100 pg of clenbuterol–HCl (which corresponds to 0.2 pg on column, split mode) yielded a S/N of four for the ion 335 and a S/N of three for the ions 334 and 337.

3.2. Chemical digestion and extraction

Fig. 4 shows the mean values of five independent determinations of clenbuterol extracts after incubation in alkali (for detailed conditions, see Section 2.8.1). No significant differences in recoveries of clenbuterol after 0, 1 and 24 h alkali treatment were observed, indicating that loss of analyte due to chemical degradation is negligible. From the literature [32], it is known that some investigators include an incubation step with DTT in the process of hair destruction in order to break the disulfide bonds of the keratin protein and to achieve quantitative liberation of the matrix-bound drug. In the current study, preliminary experiments revealed that the digestion of hair from a clenbuterol user, where the drug had been physiologically incorporated into the hair, with and without the addition of 0.05 m*M* DTT in an alkaline bath, resulted in equal recoveries of clenbuterol. From these results, it can be concluded that hydrolysis under the described conditions is sufficient and that all of the clenbuterol is released from the hair matrix.

After the digestion, a single extraction step with 5 ml of TBME was applied to separate clenbuterol from the aqueous hair hydrolysate. Extraction yields of about 90% were obtained (Table 2). At least two extraction steps were necessary to yield 100% recovery.

3.3. Immunoaffinity chromatography

To improve sample clean-up, spiked hair extracts (100 pg clenbuterol/125 mg hair) were applied to the IAC system. Comparison of hair samples prepared according to the normal sampling procedure with samples reanalysed after IAC indicates at least a five-fold improvement in the detection limit (0.8 ng clenbuterol/g hair; 4 pg on column, splitless). Signal-to-noise ratios were three, seven and four, calculated for the ion traces 334, 335 and 337, respectively. Without IAC purification it was not possible to do the injection splitless because of the interfering biological noise.

The crossreactions of the antibody raised against the diazotised conjugate of clenbuterol used in this study are:

Table 2
Extraction yields of clenbuterol from spiked hair samples[a]

Sample no.	Amount added (ng)	Recovery (%) after		
		1. Extraction	2. Extraction	3. Extraction
1	1.0	88	12	b.LOQ
2	2.0	87	13	b.LOQ
3	4.0	88	12	b.LOQ
4	5.0	90	8	2

[a] Basis for calculating the percentage: the sum of recoveries of three successive extractions of each sample was set to 100%. Standard (norandrosterone) was added after discarding the aqueous phase.
b.LOQ: below limit of quantitation.

— Clenbuterol 100.0%
— Terbutaline 9.0%
— Cimaterol 2.7%
— Salbutamol 7.7%
— Pirbuterol 0.1%
— Isoproteranol 0.1%

The results were obtained according to the method of Abraham [33].

3.4. Analysis of clenbuterol in hair

Table 3 summarizes the results of clenbuterol determination in hair from the patients tested.

A 25-mg amount of hair from each 20 mm hair segment was prepared and analysed in duplicate, except for female C (1×50 mg).

3.4.1. Female A

Hair samples of female A were collected two and six months after the administration of clenbuterol. The peak value of clenbuterol in hair collected two months after therapy was detected in the second hair unit (30–50 mm). A considerable amount (36 ng/g) was also found in the first segment.

The data in Table 3 show that, even six months after the cessation of administration, clenbuterol could be detected. The entire amount of clenbuterol could now be found in the hair section from 70–110 mm.

Table 3
Concentration of clenbuterol in hair samples from the females tested[a]

Female A				Female B				Female C		Female D	
Hair sampling 2 months after completion of administration		Hair sampling 6 months after completion of administration		Hair sampling 3 months after beginning of administration		Hair sampling 5.5 months after completion of administration		Hair sampling 5 months after completion of administration		Hair sampling 2.5 months after completion of administration	
Hair segment (mm)	Concen-tration (ng/g)	Hair segment (mm)	Concen-tration (ng/g)	Hair segment (mm)	Concen-tration (ng/g)	Hair segment (mm)	Concen-tration (ng/g)	Hair segment (mm)	Concen-tration (ng/g)	Hair segment (mm)	Concen-tration (ng/g)
10–30	28, 41	10–30	0, 0	10–30	236, 212	10–30	0, 0	10–30	0, −	10–30	24, 44
30–50	100, 90	30–50	0, 0	30–50	30, 32	30–50	7, 6	30–50	b. LOQ	30–50	139, 139
50–70	4, 4	50–70	b. LOQ	50–70	4, 6	50–70	31, 47	50–70	3, −	50–70	29, −
70–90	0, 0	70–90	16, 17	70–90	4, 5	70–90	10, 5	70–90	2, −		
90– 110	0, 0	90– 110	17, 13					90– 110	b. LOQ		
110–130	0, 0	110–130	b. LOQ					110–130	b. LOQ		
		Tips of hairs	0, 0					130–150	b. LOQ		
								Tips of hairs	b. LOQ		

[a] Two values are given for each hair segment, except for those of female C.
b. LOQ: below limit of quantitation.

3.4.2. Female B

Clenbuterol determination in the hair collected during clenbuterol intake revealed that the mean amount (97%) was incorporated in hair sections from 10 to 50 mm.

The low concentration (4 ng/g) in hair segments nos. 3 and 4 can be explained by the fact that a small part of this section grew during the time of clenbuterol therapy and/or that hair strands were shifted against each other during hair collection and sample preparation, which could lead to contamination of vicinal blank hair segments. The study of hair collected 5.5 months after stopping treatment with clenbuterol confirmed the presence of clenbuterol and demonstrates the retrospectivity of this method. The majority of clenbuterol was assimilated in hair segment no. 3 (50–70 mm). No clenbuterol could be detected in a hair sample taken from the infant of this female taken 5.5 months after delivery.

3.4.3. Female C

Again, the majority of clenbuterol was assimilated within a certain fragment (50–90 mm), proving that this hair segment had grown during administration of the drug.

3.4.4. Female D

The hair of female D who had taken clenbuterol for three months had significant incorporation of clenbuterol in all hair segments that were tested. A peak concentration of 139 ng/g was estimated for the second segment (30–50 mm), indicating that this hair portion must have grown during a phase in which a high serum level led to enhanced tansfer of clenbuterol into the hair.

4. Conclusion and discussion

The sensitivity and selectivity of the described GC–HRMS method enables the detection and identification of clenbuterol in human scalp hair. The employed sample preparation procedure is easy to handle and allows detection of 4 ng clenbuterol/g hair by HRMS. Additional immunoaffinity purification of clenbuterol results in lower detection limits and allows identification of clenbuterol in hair even at a concentration of 0.8 ng/g hair.

The total amount of clenbuterol quantified in hair seems to decrease over time, which is indicated by the declining clenbuterol concentration in the hair from female A that was collected two months (134 ng/g) and six months (31 ng/g) after ending clenbuterol therapy.

The same effect can be observed for the hair from female B, which was taken during drug administration, with a total concentration of 276 ng/g compared to 52 ng/g in hair samples collected 5.5 months after the end of clenbuterol application. This result is in agreement with that of Gleixner et al. [34] who found a similar decline in the concentration of clenbuterol in human hair over prolonged time periods.

In the context of the general limitations of hair analysis, the role of individual pigmentation must be taken into account. The incorporation rate into blond or grey hair is poorer than that of dark colored hair. The frequency of hair cutting and hair cuts are other critical factors. Irregular hair growth can cause a concentrating or diluting effect, depending on the rate of growth.

This effect can be enhanced by the potential diffusion of clenbuterol in an axial direction in the hair shaft. A further problem is caused by the above-mentioned continuous decline in the concentration of clenbuterol with time, which limits the prolongation of detectability.

In this context, a washout effect through multiple washing processes over expanded time intervals (half a year) is probable. The possible long-term instability of clenbuterol rather than metabolism in the hair could also contribute to this decreasing concentration phenomenon. Nevertheless, complementary information arising from hair samples to that obtained from urine analysis provides the possibility of discriminating between acute administration, indicated by respiratory tract obstruction, from chronic use, which is necessary to obtain an anabolic effect. From this point of view, a facility has been made available to clarify unequivocally the abuse of clenbuterol by athletes or animals whose urine samples suggest the presence of clenbuterol. In such cases, the athlete can be asked to provide a hair sample for analysis. The fact that the therapeutic doses used in this study are comparable to those used by athletes who want to achieve anabolic effects and the non-invasive availa-

bility of hair samples support this strategy. Keeping the characteristics of a certain substance in mind that favor incorporation into hair, the applicability of hair analysis to other banned substances has to be investigated.

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