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Hair testing for residue control in food-producing animals – A pilot study on six beta-agonists in cattle

Die Haaranalytik in der Rückstandskontrolle bei Lebensmittel-liefernden Tieren – Eine Pilotstudie mit sechs beta-Agonisten beim Rind

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Summary

A fourteen-year-old Holstein Friesian cow was treated successively with the aniline-type beta-agonists clenbuterol and brombuterol, the hydroxy-phenyl-type ß-agonists ractopamine, salbutamol and terbutaline as well as with the ß-agonist zilpaterol, which belongs to neither of these chemical classes. Each ß-agonist was applied for five consecutive weeks. After a drug-free interval of four weeks the next ß-agonist was administered, and so on.

Black hair and urine samples were collected once a week, white hair samples once a month. Sampling was continued as long as residues could be found, which resulted in a total study period of two years. After cleaning, extraction and SPE an HPLC-MS/MS method validated according to Commission Decision 2002/657/EC was used for analysis.

The residues of the six ß-agonists are detectable in black hair 3 times, 6 times, 4 times, 9 times, 13 times and 7 times longer than in urine, respectively. Differences in black hair concentration result primarily from different black hair/dose ratios. These ratios are less than or equal to one for the hydroxy-phenyl-type ß-agonists and greater than one hundred for the aniline-type ß-agonists. The black hair concentrations for the six ß-agonists are always higher than the white hair concentrations. The black hair/white hair ratios are less than ten for the hydroxy-phenyl-type ß-agonists and greater than ten for the other substances.

Due to its long detection window hair testing might be a valuable supplement to the analysis of traditional matrices like plasma and urine. The method used here is suitable for screening purposes.

Keywords: food, residues, hair testing, ß-agonists, bovine

Zusammenfassung

Eine vierzehnjährige Holstein-Friesian-Kuh wurde nacheinander mit den anilinischen beta-Agonisten Clenbuterol und Brombuterol, den phenolischen ß-Agonisten Ractopamin, Salbutamol und Terbutalin sowie mit dem ß-Agonisten Zilpaterol behandelt, der zu keiner dieser Substanzgruppen gehört. Jeder ß-Agonist wurde über fünf Wochen appliziert. Nach einem behandlungsfreien Intervall von vier Wochen wurde der nächste ß-Agonist verabreicht u.s.w.

Proben von schwarzem Haar und von Urin wurden einmal wöchentlich genommen, Proben von weißem Haar einmal monatlich. Die Probennahme wurde solange fortgesetzt, bis keine Rückstände mehr nachweisbar waren. Dies ergab eine Gesamtstudiendauer von zwei Jahren. Nach Reinigung, Extraktion und SPE wurden die Proben mit einer nach der Entscheidung 2002/657/EG validierten HPLC-MS/MS-Methode analysiert.

Die Rückstände der sechs ß-Agonisten sind in schwarzem Haar 3mal, 6mal, 4mal, 9mal, 13mal bzw. 7mal länger nachweisbar als in Urin. Unterschiede in den Konzentrationen in schwarzem Haar resultieren in erster Linie aus unterschiedlichen Haar-Dosis-Quotienten. Diese Quotienten sind kleiner als oder gleich eins für die phenolischen ß-Agonisten und größer als hundert für die anilinischen ß-Agonisten. Die Rückstandskonzentrationen in schwarzem Haar sind immer größer als in weißem Haar. Die schwarz-weiß-Quotienten sind kleiner als zehn für phenolische ß-Agonisten und größer als zehn für die anderen Substanzen.

Durch das lange Nachweisfenster kann die Haaranalytik eine wertvolle Ergänzung zur Untersuchung traditioneller Matrices wie Plasma und Urin darstellen. Die hier verwendete Methode ist als Screening-Verfahren geeignet.

Schlüsselwörter: Lebensmittel, Rückstände, Haaranalytik, ß-Agonisten, Rind

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Introduction

Many residues accumulate in hair and can be detected ideally until the hair falls out. Human hair testing started in the late seventies (Baumgartner et al., 1979). In the meantime it has been established as a complement to the analysis of traditional matrices and is used worldwide hundreds of thousands of times each year. In farm animals the situation is quite different from that in humans. Although farm animal hair testing started only a decade later (Yeung et al., 1988), it did not gain widespread acceptance and is only rarely used in food safety residue control.

This paper deals with hair testing for beta-agonists. In higher dosages, clenbuterol has marked anabolic effects which were first described in sheep (Baker et al., 1984) and cattle (Ricks et al., 1984) in 1984. In the early nineties it was discovered that clenbuterol residues are detectable in the eyes of cattle (Meyer and Rinke, 1991; Elliott et al., 1995) and chickens (Malucelli et al., 1994) even after long periods of withdrawal.

Soon after hair testing for ß-agonists was introduced for experimental animals. These first studies on rats (Adam et al., 1994) and guinea pigs (Polettini et al., 1995) already revealed that the accumulation in hair is dose-dependent, that the detection window in hair is wider than in traditional matrices and that there are higher concentrations in black hair as compared to lighter hair. At the same time hair testing for ß-agonists was introduced for farm animals, namely cattle (Sauer and Anderson, 1994; Dürsch et al., 1995; Elliott et al., 1995, 1996; Panoyan et al., 1995; Gleixner and Meyer, 1996a, 1996b; Gleixner et al., 1996b; McGrath et al., 1996), broilers (Malucelli et al., 1994) and pigs (Gojmerac et al., 2008). In 1996 the first detection of a ß-agonist in human hair was published (Gleixner et al., 1996a).

Not only these first animal studies on bovine hair but also the later ones exclusively dealt with clenbuterol. Clenbuterol is the ß-agonist with the most pronounced anabolic effect in fattening animals. Although other ß-agonists show similar effects, the respective animal experiments with bovine hair are still missing. Therefore we performed a pilot study on six selected ß-agonists in cattle to evaluate the suitability of the alternative matrix hair compared to that of the traditional matrix urine for food safety residue control. These selected substances belong to different chemical classes in order to cover the main physico-chemical characteristics of the ß-agonists included most frequently in National Residue Control Plans.

Material and methods

Experimental animal and treatment

A fourteen-year-old dry Holstein Friesian cow with a body weight of 750 Kg was treated successively with the aniline-type beta-agonists clenbuterol and brombuterol, the hydroxy-phenyl-type ß-agonists ractopamine, salbutamol and terbutaline as well as with the ß-agonist zilpaterol, which belongs to neither of these chemical classes (Tab. 1). The daily dose was administered orally in two fractions every 12 hours. Each ß-agonist was applied for five consecutive weeks. After a drug-free interval of four weeks the treatment with the next ß-agonist was started, and so on.

Sampling

Each hair sample was obtained from a 10 x 10 cm^2 area of skin using an electrical shearing machine with a 0.5 mm blade. The black and white hair samples were collected once a week and once a month, respectively. To guarantee that each sample area of skin was sheared only once during the study period, the whole body surface of the cow except head, tail and distal legs was used systematically. Samples of spontaneous urine were collected once a week.

Sampling started before the beginning of the animal study and continued as long as residues could be found for a total period of two years.

Analytical technique

Chemicals and apparatus

Clenbuterol-hydrochloride, ractopamine-hydrochloride, salbutamol, salbutamol-*tert*-butyl-d9, terbutaline-hemisulfate and protease type XIV derived from *Streptomyces griseus* were purchased from Sigma-Aldrich-Chemie (Steinheim, Germany). Brombuterol and clenbuterol-d9-hydrochloride were provided by WITEGA Laboratorien Berlin-Adlershof (Berlin, Germany). Zilpaterol-hydrochloride was obtained from Akzo Nobel Intervet International b.v. (Boxmeer, The Netherlands) and ractopamine-d5-hydrochloride was bought from RIKILT (Wageningen, The Netherlands). All solvents were of HPLC grade.

Screen Dau solid-phase extraction columns (500 mg, 6 ml) used for clean-up were obtained from Amchro (Hattersheim, Germany). The hair samples were homogenised in a mixer mill MM400 (Retsch, Haan, Germany), then filtrated and centrifuged using an Amicon Ultra Centrifugal Filters Ultracell 100 K (Millipore, Carrigtwohill, Ireland), and finally analysed on a UPLC-Aquity-MS/MS Xevo™ TQ-S (Waters, En Yvelines Cedex, France).

Chromatographic and mass spectrometric conditions

The UPLC separation was performed on a column Zorbax Eclipse C18 (4.6 x 100, 1.8 µm particle size) at a flow rate of 1.00 ml/min and a temperature of 40 °C. The mobile phase consisted of constituent A (0.1 % formic acid in water) and constituent B (0.1 % formic acid in acetonitrile / 0.1 % formic acid in water = 95:5). A gradient elution program was employed as follows: 0–5 min 95 % A, 10 min

TABLE 1: *Drugs and dosages.*

Beta-agonist	Daily dose (mg/Kg BW)	Veterinary medicine, humane medicine or standard substance used
Aniline type		
Clenbuterol	0.008	Ventipulmin® for horses (Boehringer Ingelheim Vetmedica, Ingelheim, Germany)
Brombuterol	0.008	Brombuterol free-base (Witega Laboratories, Berlin, Germany)
Hydroxy-phenyl type		
Ractopamine	0.4	Ractopamine-hydrochloride (Eli Lilly, Bad Homburg, Germany)
Salbutamol	0.02	Loftan® (GlaxoSmithKline, Munich, Germany)
Terbutaline	0.02	Terbutalin® (Aliud Pharma, Laichingen, Germany)
Miscellaneous		
Zilpaterol	0.15	Zilpaterol-hydrochloride (Akzo-Nobel Intervet Int. bv, Boxmeer, The Netherlands)

BW: body weight

80 % A, 18 min 50 % A, 20 min 5 % A, 21 min 5 % A, 22 min 95 % A and 25 min 95 % A. The injection volume was 10 µl.

The following mass spectrometric conditions were applied: electrospray ionisation, positive polarity, capillary voltage 0.75 kV, source temperature 150 °C, desolvation temperature 550 °C, cone gas 20 l/h, desolvation gas 1200 l/h, and collision gas 0.1 l/h. The mass spectrometer was operated in the multiple-reaction monitoring mode. The specific parameters for each analyte are shown in Table 2.

The CCα values for the six ß-agonists in hair and urine are 0.06/0.04 ng/g for clenbuterol, 0.19/0.07 ng/g for brombuterol, 0.24/0.37 ng/g for ractopamine, 2.31/0.17 ng/g for salbutamol, 0.44/0.37 ng/g for terbutaline, and 0.45/0.5 ng/g for zilpaterol.

TABLE 2: *Analytical parameters for each analyte.*

Analyte	Retention time (min)	Precursor ion	Cone (V)	Production ion	Collision energy (V)	Dwell time (sec)
Terbutaline	2.84	226.10	22	107.12	28	0.033
				125.13	24	0.033
				152.12*	14	0.033
Salbutamol	2.89	240.17	18	148.16*	18	0.033
				166.18	14	0.033
				222.18	10	0.033
Salbutamol-d9	2.83	249.17	18	149.00	18	0.033
Zilpaterol	2.91	262.17	18	185.15*	24	0.033
				202.18	20	0.033
				244.19	12	0.033
Ractopamine	7.88	302.17	20	107.12*	32	0.014
				121.16	22	0.014
				164.15	14	0.014
Ractopamine-d5	7.96	307.1	20	167.20	18	0.014
Clenbuterol	9.08	277.07	18	132.09	24	0.013
				168.05*	28	0.013
				203.1	16	0.013
Clenbuterol-d9	9.00	286.13	20	204.09	14	0.013
Brombuterol	10.54	366.95	20	214.40	32	0.015
				293*	18	0.015
				349.04	10	0.015

* Quantification ions

Hair sample preparation and clean-up

Approximately 1 g of uncut hair was washed twice with 10 ml of water to remove adherent faeces and other dirt, and a third time with 10 ml of 0.05 M HCL in ethanol. After washing the hair was dried at 80 °C in a vacuum drying oven. The internal standards, 5 ml of tris-buffer (pH 8) and 100 µl of protease solution (50 mg/ml in water) were added to the hair sample (500 mg). This mixture was then incubated overnight at 55 °C in a shaking water bath. After incubation, 2 ml of phosphate buffer (pH 6) were added and the pH was adjusted to 6. The samples were then shaken in an ultrasonic bath at room temperature and centrifuged at 4 °C and 4000 rpm using Amicon filter units. After transferring the supernatants to another tube, 200 µl of methanol were added, then the mixture was centrifuged at 4 °C and 4000 rpm. The centrifuged extracts were transferred to SPE cartridges conditioned with 2 ml of methanol, 2 ml of water and 2 ml of phosphate buffer pH 6, respectively. The cartridges were then washed with 1 ml of 1 M acetic acid and evaporated to dryness, followed by washing with 2 ml of methanol and, again, evaporation to dryness. The elution was performed with 6 ml of a mixture consisting of ethyl acetate and ammonia in a ratio of 97 : 3. The samples were evaporated to dryness under a stream of nitrogen at 35 °C. The residues were then dissolved in 200 µl of HPLC mobile phase consisting of 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile in a ratio of 95 : 5.

Evaluation of data

Window of detection

The window of detection for black hair is expressed in weeks and for urine in days from the start of treatment until weeks after the end of treatment. The first sample was always taken one day after the start of treatment. If a hair sample was positive one day after the start of treatment, this was considered as 'contamination' and not used for the determination of the detection window. White hair was sampled at monthly intervals only and so the beginning of the detection window is not known.

B/W Ratio

A black hair/white hair ratio was calculated for all samples that were taken at the same day. Because white hair samples were taken only once per month, this led to one value per month and substance, resulting in 44 pairs of samples for all six substances. The arithmetic mean of theses values is expressed as the B/W ratio for each ß-agonist.

B/W ratio = Black hair concentration (ng/g) / White hair concentration (ng/g)

B/D Ratio

The black hair/dose ratio is a factor to calculate the maximum black hair concentration from the daily dose.

B/D ratio = 10^3 x Black hair concentration (ng/g) / Daily dose (mg/Kg BW)

Results

Window of detection

The windows of detection for black hair, white hair and urine are given in Table 3. In most cases black hair was positive one week after the start of treatment, only in case of terbutaline it took two weeks.

For all ß-agonists but clenbuterol the first urine sample taken 24 hours after the start of treatment was positive.

TABLE 3: *Window of detection.*

	Window of detection			
	Black hair (weeks)	White hair (weeks)	Urine (days – weeks)	Black hair/ urine
Aniline type				
Clenbuterol	1–49	–41	–15	3
Brombuterol	1–51	–30	1–9	6
Hydroxy-phenyl type				
Ractopamine	1–42	–39	1–10	4
Salbutamol	1–27	–23	1–3	9
Terbutaline	2–13	–10	1–1	13
Miscellaneous				
Zilpaterol	1–34	–34	1–5	7

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With clenbuterol this first sample was not positive and the next sample was taken only one week later. Therefore a beginning of the detection window for clenbuterol in urine is not given.

The residues are detectable for a longer period of time in black hair than in white hair. Only for zilpaterol it is 34 weeks for both black and white hair.

The residues of the six ß-agonists are detectable in black hair 3 times, 6 times, 4 times, 9 times, 13 times and 7 times, respectively, longer than in urine.

Black hair concentration and B/D Ratio

The maximum residue concentrations in black hair are less than 500 ng/g for the hydroxy-phenyl-type ß-agonists and greater than 1000 ng/g for the other substances (Table 4). These differences do not result primarily from different dosages but from different black hair/dose ratios. The B/D ratios are less than or equal to one for the hydroxy-phenyl-type ß-agonists and greater than one hundred for the aniline-type ß-agonists. Zilpaterol belonging to neither of these groups has a B/D ratio of 16, which is between these measures.

TABLE 4: *Maximum residue concentrations (ng/g) and ratios.*

	Black hair	White hair	Urine	B/W Ratio	B/D Ratio x10³
Aniline type					
Clenbuterol	3674.5	173.02	154.45	26	459
Brombuterol	1348	66.5	92.01	13	168
Hydroxy-phenyl type					
Ractopamine	410.5	340.3	32741	2	1
Salbutamol	10.17	1.87	438.57	4	0.5
Terbutaline	4.25	1.16	104.1	7	0.2
Miscellaneous					
Zilpaterol	2467.0	204.6	4585	27	16

* B/W Ratio – black hair/white hair ratio, B/D Ratio – black hair/dose ratio

B/W Ratio

The ratios for the six ß-agonists are always greater than one, indicating that the black hair concentrations are higher than the white hair concentrations. The ratios are less than ten for the hydroxy-phenyl-type ß-agonists and greater than ten for the other substances.

Indications of contamination

In all but three cases the black and white hair samples were positive one week after the start of treatment at the earliest. Only in case of brombuterol, ractopamine and zilpaterol the first white hair sample that was taken 24 hours after the start of treatment was positive. These samples were taken from the right shoulder, which is within the reach of the cow's tongue, and are considered 'positive due to contamination'.

The single values for the 44 pairs of samples for the calculation of the B/W ratios are not given here. They are very inhomogeneous, in six cases the concentrations in white hair are even higher than those in black hair. A contamination of hair samples by the cow's tongue, urine or faeces might be an explanation for this inhomogeneity.

Discussion

The most important advantage of human hair testing compared to traditional matrices is its wide window of detection. In this study black hair was positive for clenbuterol, brombuterol, ractopamine, salbutamol and zilpaterol one week, and for terbutaline two weeks after the start of treatment. This is in accordance with other groups who found bovine hair to be positive for clenbuterol after 4–10 days (Gleixner et al., 1996a; Gaillard et al., 1997; Fente et al., 1999).

The end of the detection window for black hair in this study was between 13 and 51 weeks depending on the substance. For clenbuterol, which is the most relevant ß-agonist concerning the illegal use in meat production, it was 49 weeks. Haasnoot et al. (1998) found black bovine hair samples to be positive 33 weeks after clenbuterol treatment. This difference might be due to the different sensitivity of the analytical methods. Whereas Haasnoot et al. (1998) used an ELISA method with a limit of detection (LOD) of 3–4 ng/g, in our study an HPLC-MS/MS method with a CCα of 0.06 ng/g was applied. Hair testing for residual clenbuterol also works in fair porcine hair (Gojmerac et al., 2008). After a single isoxsuprine treatment before a caesarian section, Groot et al. (2012) could detect residues in the hair of newborn calves for up to 8 weeks (CCα 0.5 ng/g).

Two studies on horses were published. Popot et al. (2001) found clenbuterol in tail hair for up to 13 months after the last administration of the drug (LOD 1 ng/g). Schlupp et al. (2004) performed segmental hair analysis. Clenbuterol was detectable as early as day 5 in the first 20 mm segment of tail and mane hair. One year after the end of treatment a 20 mm segment of tail hair 26–28 cm away from the skin surface still contained up to 21 ng/g clenbuterol, which is far above the LOD of 0.2 ng/g. Clenbuterol was not detectable in blood or urine after day 30. Mane and tail hair results were very similar.

In contrast to equine tail and mane hair, the body hair in bovines and other food-producing animals is usually replaced in spring and autumn. The data of these equine studies on the one hand and the bovine studies on the other hand suggest that seasonal hair replacement might be a main factor that restricts the window of detection in food-producing animals. The time and extent of seasonal hair replacement in meat-producing animals might depend on species, breed, sex, age, body region, climate, type of housing and other factors. More research is clearly required in this area.

Besides these limiting factors the application of segmental hair analysis should be considered at least for longer hair. If hair is not analysed segmentally but in total, a diluting effect will occur (Salquebre et al., 2007).

It is well known that black bovine hair can accumulate more clenbuterol than lighter hair, which again accumulates more residues than white hair (Dürsch et al., 1995; Elliott et al., 1996; Gleixner et al., 1996a). In the present study it was demonstrated for the first time that this applies to other ß-agonists, too. The black hair/white hair ratios are less than ten for the hydroxy-phenyl-type ß-agonists ractopamine, salbutamol and terbutaline and greater than ten for the aniline-type beta-agonists clenbuterol and brombuterol as well as for zilpaterol, which belongs to neither of these chemical classes.

The same chemical difference might apply to the black hair/dose ratio. The B/D ratios are less than or equal to one

for the hydroxy-phenyl-type ß-agonists and greater than one hundred for the aniline-type ß-agonists. Zilpaterol belonging to neither of these groups has a B/D ratio of 16, which is between these measures. The amino-group of the aniline-type ß-agonists might explain this difference.

While the detection window is the most important advantage of human hair testing, the contamination issue is its major limitation. Several groups working in the human sector published a complex washing procedure using non-swelling and swelling solvents together with a 'wash criterion' (Baumgartner and Hill, 1992, 1993a, 1993b; Cairns et al., 2004a, 2004b; Hill et al., 2008; Schaffer et al., 2005, 2007). The main purpose of washing and determining the wash criterion is not solely to remove contamination, but to identify a sample that is externally contaminated as distinct from those positive because of ingestion (Hill et al., 2008). Other goups strongly criticise these decontamination procedures (Blank and Kidwell, 1993, 1995; Cone et al., 1991; Kidwell and Blank, 1993; Stout et al., 2006, 2007; Wang and Cone, 1995; Welch et al., 1995). They question the existence of accessible, semi-accessible and non-accessible areas in the hair and state that the entire hair volume is accessible to the external environment. Therefore, no procedure is likely to distinguish external exposure from active use. The Society of Hair Testing has evaluated these data and published 'Guidelines for Drug Testing in Hair' that were reviewed very recently (Cooper et al., 2012). These guidelines are similar to those published by the European Workplace Drug Testing Society (Agius and Kintz, 2010). So it might be sensible to consider them for animal hair testing, as well.

In contrast to human hair, animal hair might be contaminated by urine, faeces or bedding material. In this study the recommendations of the two societies were adapted to these conditions. The hair samples were first roughly cleaned with water and then, in a second step, cleaned with an organic solvent.

Although several multi-methods for ß-agonists in farm animal hair have been published (Gallo et al., 2007; Haasnoot et al., 1998; Nielen et al., 2008; Ramos et al., 2009), there is only one contribution about decontamination (Hernández-Carrasquilla, 2002). In this article the authors study the decontamination of recently contaminated bovine hair.

Because the decontamination issue has not yet been addressed appropriately in food-producing animals, hair testing should be used for screening purposes only but not for confirmation. This is not necessarily a limitation. In human hair testing, e. g. in doping control or workplace drug testing, no other matrix is available that has a comparable window of detection. So the laboratories must confirm their hair test results if they want to proof illegal drug use. In farm animal hair testing the situation is different. A screening test in hair one or two weeks prior to slaughter provides a record of the animal's drug use of the previous months. In chickens, turkeys and pigs for fattening this record comprises the animal's whole life. If positive, retina could be analysed after slaughter. Retina should have an even longer window of detection, it is free of contamination and analytical methods for confirmation are well established.

Conclusions

The alternative matrix 'hair' is a valuable complement to traditional matrices like plasma and urine when testing living bovines for the illegal use of ß-agonists. In addition to the aniline-type substance clenbuterol, which had been dealt with in earlier studies (Cristino et al., 2003; Gaillard et al., 1997; Gleixner et al., 1996a; Pleadin et al., 2009), this is also true for the other five substances used in this study. As the six substances investigated so far represent different chemical types of ß-agonists, the data from this study might also apply to other ß-agonists covered in National Residue Control Plans. Hair analysis has the widest window of detection in living animals ranging from weeks to months. In bovines this time might be restricted mainly by seasonal hair replacement to a realistic detection window of about six months. Black hair is most suitable followed by fair hair and white hair. But even white hair has a longer detection window than urine. As long as the decontamination issue has not been addressed appropriately, farm animal hair testing should be used for screening only. For confirmation it can be combined with urine testing in living animals or preferably with retina testing after slaughter. With the combination of hair and retina testing the advantage of the wide detection window is preserved.

From human hair testing it can be speculated that hair testing might also be applicable to other food-producing mammals and birds as well as to other illegal substances and contaminants. More research is clearly required in this area.

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