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External contamination of bovine hair with β_2-agonist compounds: evaluation of decontamination strategies

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Abstract

Hair analysis has shown great potential in the control of illegal use of veterinary drugs such as β_2-agonists. However, it has been shown that hair can be externally contaminated with drugs which can lead to false positive results. Exposure of bovine hair to aqueous solutions of β_2-agonist compounds results in incorporation of these drugs into the hair. Standard hair washing procedures found in the literature: detergent (Tween-20), phosphate buffer or organic solvents (dichloromethane or methanol) cannot eliminate this external contamination. β_2-Agonists can be extracted from hair very efficiently with 0.1 *M* HCl, the extraction kinetics of externally and endogenously accumulated clenbuterol at room temperature are different which makes it feasible to discriminate between them. Treatment of hair samples with a 0.1 *M* HCl solution for 2 h at room temperature results in a ratio of clenbuterol content in the wash solution to clenbuterol content in the washed hair equal to or less than 0.25 for samples from treated cattle; whereas this ratio is equal to or higher than 0.70 for externally contaminated samples. The design of the study was intended to resemble the plausible scenario of hair being sampled a short time after external contamination. A similar study to detect external contamination for hair sampled a long time after exposure is in progress. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

β_2-Agonist compounds are used in animal rearing as growth promoters, the overall effect being an increase in muscle mass with concomitant decrease in fat mass [1]. Given their pharmacological activity, residues of these compounds in edible tissues are potentially toxic and in consequence its use has been banned in European Union (EU) countries. However, they are still used illegally as our experience and that of other european laboratories show.

Hair has been shown to accumulate a great diversity of drugs under chronic exposure conditions and β_2-agonists have been found in hair of animals treated with these agents [2,3]. Hair is a very useful sample for monitoring chronic exposure to drugs since the exposure history is preserved which represents a clear advantage over other samples, such as urine, in which detection of illegal drugs can be avoided by discontinuing the treatment an appropriate time before sample collection. In consequence, analysis of β_2-agonist (especially clenbuterol) in

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bovine hair has received considerable attention in the last few years [3–7].

Regardless of its high potential, hair analysis is not free from problems, one of the most important being the possibility of false positive results arising from the presence of drugs in hair due to passive (exogenous) exposure and not to chronic drug intake [8]. This important issue has been present in forensic analysis literature during the last decade and is still the object of fierce scientific debate. The work presented here shows the potential of bovine hair of being contaminated externally by aqueous solutions of β_2-agonist compounds and evaluates different strategies to distinguish between endogenous and externally applied drug.

2. Experimental

2.1. Chemicals and reagents

Tulobuterol hydrochloride, terbutaline hemisulfate, clenbuterol hydrochloride, salbutamol hemisulfate, ritodrine hydrochloride and fenoterol hydrobromide were purchased from Sigma (St. Louis, MO, USA). Cimaterol was obtained from ICN Pharmaceuticals (Costa Mesa, CA, USA). Stock solutions (1 mg ml^{-1}) in methanol of each standard were kept at −20°C in amber vials. For the hair contamination experiments a diluted mixed working standard at 20 μg ml^{-1} for all analytes in methanol was prepared from these stock solutions and kept at −20°C.

N - Methyl - *N* - (trimethylsilyl)trifluoroacetamide (MSTFA) and chlormethyldimethylchlorosilane (CMDCMS) were obtained from Aldrich (Gillingham, UK). Tween-20 was provided by Sigma. All additional chemicals used were analytical grade from Merck (Darmstadt, Germany). Purified water was produced with a Milli-Q system from Millipore (Bedford, MA, USA). For sample clean up, 500 mg Bond Elut Certify columns from Varian (Harbour City, CA, USA) and 500 mg Sep-Pack Plus C_{18} cartridges from Waters (Milford, MA, USA) were used.

2.2. Apparatus

Gas chromatography (GC) was performed in a Hewlett-Packard GC 5890 system (Palo Alto, CA, USA) fitted with a Hewlett-Packard 7673 autosampler. Instrument control, data acquisition and data processing were carried out with G1370 Chemstation software, also from Hewlett-Packard. The column used was a HP 5MS (30 m×0.25 mm I.D., 0.25 μm film thickness) from Hewlett-Packard fitted with a 5 m×0.25 mm I.D. deactivated fused-silica tube from Supelco (Bellafonte, PA, USA). A Hewlett-Packard 5972 quadrupole mass-selective detector was used for detection.

2.3. Sample treatment

2.3.1. Hair pre-treatment and contamination

Previous to any treatment, blank and hair samples from treated cattle were rinsed three times with tap water and three times with purified water and dried at 80°C in an oven for 30 min. This prerinse was necessary to remove blood and other extraneous matter from the samples. The dried hair samples were stored at 4°C in closed glass containers until used. A contamination solution was prepared just before use by diluting with water or bovine urine the mixed standard to obtain a final concentration of 100 ng ml^{-1}. Hair was contaminated by exposure to this solution for 2 h at room temperature. Once this time has elapsed, the contamination solution was filtered off, the hair was rinsed two times with purified water to eliminate completely the contamination solution and dried in an oven at 80°C for 30 min. The dried hair was kept at 4°C in closed glass containers.

2.3.2. Decontamination experiments using standard procedures

Samples (0.5 g) of hair contaminated using aqueous solutions of β_2-agonist compounds were soaked at room temperature for 10 min in the required decontamination solution. This solution was then discarded and the samples were further rinsed three times with purified water and subjected to analysis. When dichloromethane was used as the decontamination agent, the hair was briefly dried in an oven at 80°C to eliminate completely the solvent and further processed as above.

2.3.3. Washing of contaminated hair with 0.1 M HCl

Samples (0.5 g) of hair contaminated using aqueous solutions of β_2-agonist compounds were soaked

in closed glass containers with 20 ml of a 0.1 *M* HCl solution; in the experiments of wash time course the solution was poured off at the required time and fresh acid solution was added; whereas in the experiments at a fixed wash time the acid wash was poured off after completion of the time. All acid washes were kept in closed glass containers until analysis. The hair remaining after the wash was processed as indicated below.

2.3.4. Analysis of β_2-agonists

A 15-ml volume of a 0.1 *M* HCl solution was added to 0.5 g hair samples and kept at 60°C overnight (ca. 18 h), after cooling the extraction liquid was poured off. A 10-ml volume of 0.1 *M* phosphate buffer, pH 6.0 was added to these extracts and the solutions were adjusted to, pH 6.0 with either HCl or NaOH solutions and subjected to solid-phase extraction (SPE) in Bond Elut Certify columns. After activation of the SPE column with 2 ml of methanol, 2 ml of water and 2 ml of a 0.1 *M* potassium dihydrogenphosphate, pH 6.0 buffer; the extract was percolated, the column was rinsed with 1 ml of 1 *M* acetic acid and dried at full vacuum (ca. 760 mmHg, negative pressure) for 5 min (1 mmHg=133.322 Pa). The column was then washed with 6 ml ethyl acetate and dried again at full vacuum for 2 min. Final elution was performed with 6 ml ethyl acetate–32% ammonium hydroxide (98:3).

Extracts were evaporated to dryness at 40°C under nitrogen sparging, derivatized with 50 μl MSTFA at 60°C for 15 min and injected into the GC–MS system after cooling.

The solutions obtained after washing hair with 0.1 *M* HCl were processed as above after addition of 10 ml of 0.1 *M* phosphate buffer, pH 6.0.

2.3.5. Analysis of clenbuterol

The hair samples were incubated as above, both extracts and wash solutions were adjusted to, pH 10.0 and purified with SPE in C_{18} cartridges. After activation of the cartridges with 5 ml of methanol and 5 ml of purified water, the extract was percolated. The cartridge was washed with 5 ml of purified water and 5 ml of methanol–water (50:50, v/v) and finally eluted with 5 ml of methanol. After evaporation under nitrogen sparging in a water bath at 40°C, the dried extract was taken up in 2 ml of 0.01 *M* HCl and washed twice with 2 ml of *n*-hexane. A 100-μl volume of 2 *M* NaOH was added to the contents of the tube and extracted with two 1.5-ml volumes of *tert*.-butyl methyl ether. The pooled ether extracts were evaporated to dryness under nitrogen sparging at room temperature and derivatized.

The solution obtained after washing hair with 0.1 *M* HCl was adjusted to pH 10.0 and processed as above.

A cyclic dimethylsilyl derivative of clenbuterol (DMS-clenbuterol) was prepared [9]: to 4 ml of *n*-hexane, 200 μl of diethylamine and 320 μl of CMDMCS were added, mixed and centrifuged at 28 000 *g* for 5 min. A 100-μl volume of the supernatant obtained was added to the dried extracts and kept at 60°C for 40 min. After this time, the reaction vial was cooled to room temperature, its contents were evaporated to dryness at room temperature with a gentle nitrogen stream and resuspended with 50 μl of toluene for injection into the GC–MS apparatus.

2.4. Gas chromatography–mass spectrometry

2.4.1. β_2-Agonists

GC conditions were as follows: initial temperature, 100°C for 1 min; raised at 10°C min^{-1} to 280°C, 5°C min^{-1} to 300°C; this temperature was held for 5 min. A 2-μl volume was injected into the GC–MS system in the splitless mode. The injector port temperature was 260°C, the transfer line from the GC system to the mass-selective detector was held at 275°C. Helium was used as the carrier gas at 1.0 ml min^{-1}. The mass-selective detector was operated in the electron impact mode, using selected ion monitoring (SIM). The nature of the derivatives obtained, their retention times and ions monitored are shown in Table 1.

2.4.2. Clenbuterol

The chromatographic conditions used were: initial temperature 70°C for 1 min; raised at 18°C min^{-1} to 200°C, further raised to 290°C at 6°C min^{-1} and finally raised to 300°C using a 35°C min^{-1} ramp; this temperature was held for 5 min. Injector port and transfer line temperatures were held at 260 and 275°C, respectively. A 2-μl aliquot was injected into the GC–MS system in the splitless mode. The mass-selective detector was operated in the SIM mode

Table 1
Identity, retention times and ions monitored of the trimethylsilyl (TMS) derivatives of β_2-agonists, ions are in the order higher to lower abundance

Compound	No. TMS groups	Retention time (min)	Ions monitored (m/z)				
Tulobuterol	1	10.3	194*	196	213	284	
Terbutalina	3	14.3	356*	336	251	426	370
Clenbuterol	1	14.7	262*	243	212		
Salbutamol	3	15.1	369*	262	350	440	
Cimaterol	2	15.5	291*	275	176	258	348
Ritodrine	3	20.8	236	193*	267	179	
Fenoterol	4	21.6	322*	236	356	412	
DMS-clenbuterol	–	16.3	331*	289	128	187	346

Also included are the ions corresponding to the dimethylsilyl derivative of clenbuterol (DMS-clenbuterol). * Denotes ion used for quantitation.

with a unique acquisition group, the ions used and the retention time of DMS-clenbuterol are reported in Table 1.

3. Results and discussion

Accumulation of drugs in hair has been known since the 1950s and has been an active field in forensic chemistry since the 1980s. Most of the published studies have dealt with drugs of abuse (e.g., cocaine or heroin) in humans. Hair is extremely useful for assessing chronic exposure to drugs since they are accumulated in the hair matrix and are preserved for a long time (even thousands of years [10]). Due to this property, hair shows a great potential in those fields where chronic exposure to relatively low concentration of drugs during long periods of time must be evaluated: e.g., doping control [11] or exposure to pharmaceuticals or drugs of abuse [12] in humans. The monitoring of the use of growth promoting drugs in cattle rearing is a quite similar situation regarding concentration and exposure times and in consequence, hair analysis has received considerable attention, especially for β_2-agonist drugs [3–7].

The obvious assumption made when analysing hair samples is that all the drug found has been incorporated endogenously; unfortunately, hair can be contaminated quite easily from external sources which can lead to false positive results. Although several processes can theoretically depose drugs on hair, e.g., dust, aerosols or saliva, it has been proposed that the final mechanism of incorporation into hair is by bathing in aqueous media through hair wetting [13]. In the case of cattle plausible scenarios for external contamination are contact with contaminated feed followed by wetting with water or saliva and wetting with contaminated water or saliva (or urine) of treated animals. External contamination with urine of treated animals is the most plausible scenario since animals from different farms are usually grouped during transport or in the slaughter house previous to slaughtering. This real-life situation, which is mimicked in the experimental set-up used, would result in contaminated hair being sampled a short time after contamination.

3.1. External contamination of hair with β_2-agonist compounds

Samples of bovine hair were exposed to an aqueous solution of β_2-agonists or to bovine urine spiked with these compound at realistic drug concentrations. A normal growth-promoting dose of clenbuterol is 10–50 $\mu g\ kg^{-1}$ body mass, which can result in a concentration of clenbuterol in urine of up to 500 $ng\ ml^{-1}$ [14]. Higher doses must be used in the treatment with β_2-agonist compounds with OH groups in the aromatic ring due to deactivation by conjugation in the liver and the intestine [15]. Although these β_2-agonist compounds are secreted mainly as conjugates, the dose required results also in high parent drug concentration in urine, and, in fact, up to 100 $ng\ ml^{-1}$ salbutamol in urine of treated animals has been reported [16].

Table 2
Content of β_2-agonist compounds in hair after soaking in different contamination solutions for 2 h at room temperature

Compound	Water	Urine
Tulobuterol	473	156
Terbutaline	338	36
Clenbuterol	656	146
Salbutamol	279	25
Cimaterol	581	62
Ritodrine	1311	133
Fenoterol	850	69

The concentration of drug in the contaminated hair is expressed as ng g^{-1} hair.

As shown in Table 2, this treatment results in incorporation of the drugs into the hair, it being more efficient from water than from urine, probably due to competition from other compounds present in urine; this is supported by the fact that the chromatograms obtained in the analysis of these samples show more matrix peaks. This result is consistent with the reported in vivo contamination of bovine hair after spraying with bovine urine spiked with clenbuterol [17].

3.2. Evaluation of standard decontamination procedures

A variety of decontamination procedures have been used in the literature to eliminate externally applied drugs or to discriminate between endogenously and exogenously accumulated drug [13,18], usual approaches are washing with organic solvents, detergents and/or buffers. The effectiveness of these schemes is still the object of fierce scientific debate [13,19–21], but the scientific consensus, nevertheless, is that assessment of external contamination and/or its elimination is a difficult task. This fact has been shown recently in a interlaboratory study of analysis of drugs of abuse in hair: in some cases laboratories reported as contaminated hair from drug users and in other cases samples that had been contaminated externally were reported as endogenous [22].

Hair externally contaminated with β_2-agonists by exposure to an aqueous solution of these compounds was subjected to representative decontamination procedures found in the literature. The content of drugs remaining in the hair after each treatment was determined. As shown in Table 3, no washing treatment eliminates completely the external contamination, which implies a high risk of false positive results when analysing contaminated hair using these standard decontamination procedures.

3.3. Washing of hair with 0.1 M HCl

As has been previously shown [7], treatment of hair with 0.1 *M* HCl is very efficient in extracting β_2-agonist compounds from hair. Since standard decontamination procedures are unable to remove completely from hair these externally applied compounds, this treatment seemed a good alternative.

Externally contaminated hair samples were treated with 0.1 *M* HCl solutions both at room temperature and at 60°C. In order to gather information of the kinetics of the washing/extraction process, the acid washes were poured off at designated times and replaced with fresh ones, these acid solutions were analysed to ascertain their β_2-agonists content. Finally, the remaining hair was extracted overnight (ca. 18

Table 3
Externally incorporated β_2-agonist drugs remaining in hair after standard decontamination procedures

Compound	Decontamination procedure			
	0.2% Tween-20	0.1 *M* Phosphate buffer, pH 6.0	Dichloromethane	Methanol
Tulobuterol	64.3±6.5	48.0±6.3	49.6±9.3	60.9±8.7
Terbutaline	89.5±11.6	96.6±7.9	83.8±11.3	96.2±4.5
Clenbuterol	74.7±2.5	73.0±9.3	73.0±10.9	74.0±3.8
Salbutamol	64.7±9.0	68.9±6.6	68.9±12.6	68.9±3.3
Cimaterol	65.9±7.0	69.0±9.0	62.2±7.2	60.2±6.9
Ritodrine	71.8±2.5	68.4±7.1	68.4±5.2	73.0±3.3
Fenoterol	78.3±18.9	67.8±15.0	67.8±4.2	78.0±2.5

The results are reported as percentages over content of unwashed contaminated hair. Data are means±S.E.M. ($n=3$).

h) with 0.1 *M* HCl at 60°C, this is the standard procedure in our laboratory for the analysis of these compounds in hair; the content of β_2-agonists found in this final extract was taken to be the remaining drug present in hair. The data summarised in Table 4 shows that washing at room temperature does not eliminate completely contaminating drug, which can be removed using relatively harsh conditions: 60°C for 4–6 h depending on the drug. As mentioned above, these temperature and acid concentration correspond to the standard conditions used in this laboratory to extract β_2-agonist compounds from hair samples (albeit in this case the acid extraction is performed for a much longer time). This fact raises the concern of extracting during the wash at 60°C enough endogenously accumulated drug from the hair to generate a false negative result.

To test this possibility, the wash kinetics of hair samples containing internally accumulated clenbuterol were determined. The method for determination of clenbuterol was used in these experiments since its limit of detection is lower than the method for analysis of all β_2-agonists. The degree of drug elimination in the wash was expressed as the ratio: drug content in the wash divided by drug content remaining in washed hair. The results obtained in these experiments and the data from externally contaminated samples are presented in Fig. 1. Washing at 60°C extracts endogenously deposited clenbuterol with a certain risk of generating a false negative result, moreover, the wash kinetics of contaminated hair and samples from treated animals do not allow for a clear discrimination between both. The situation is different when performing the acid wash at room temperature: externally applied clenbuterol is reduced in about 60% but in this case, the solubilisation kinetics of both types of samples are clearly different, which offers an opportunity to discriminate between them.

Hair samples are very heterogeneous in terms of porosity, due mainly to their different diameter and degree of damage which has a strong influence on the accessibility of external contamination to the hair matrix and on the effectiveness of the acid wash. Thus it was necessary to perform additional wash experiments using different contaminated and positive samples to verify these preliminary findings, the range of clenbuterol content in the samples from treated cattle was 20–400 ng g^{-1} of hair and in the contaminated samples was 60–1200 ng g^{-1}. Since performing a full wash kinetics analysis for each sample is not attractive as a routine basis, a wash time of 2 h was used. As Fig. 2 shows, washing at room temperature results in wash ratios equal to or

Table 4
Content of externally incorporated β_2-agonist in hair after washing with 0.1 *M* HCl, at 60°C and room temperature

Compound	Temperature	Wash time (h)			
		1	2	4	6
Tulobuterol	Room	59–78	42–61	36–47	28–38
	60°C	16–28	10–13	5–6	<4–6
Terbutaline	Room	44–52	38–31	18–20	12–14
	60°C	14–17	<8–9	nd	nd
Clenbuterol	Room	58–67	43–46	32–36	26–30
	60°C	23–36	9–18	3–4	nd
Salbutamol	Room	72–48	37–28	16–17	12–14
	60°C	14–20	<4–10	nd	nd
Cimaterol	Room	66–72	48–52	37–41	33–35
	60°C	14–21	7–11	nd	nd
Ritodrine	Room	51–60	38–41	30–34	29–30
	60°C	20–28	10–17	2–3	<1–2
Fenoterol	Room	51–58	42–44	35–37	32–33
	60°C	14–25	7–18	<2–4	nd

Figures are the range of drug concentration expressed as a percentage over content of unwashed contaminated hair. nd, Not detected.

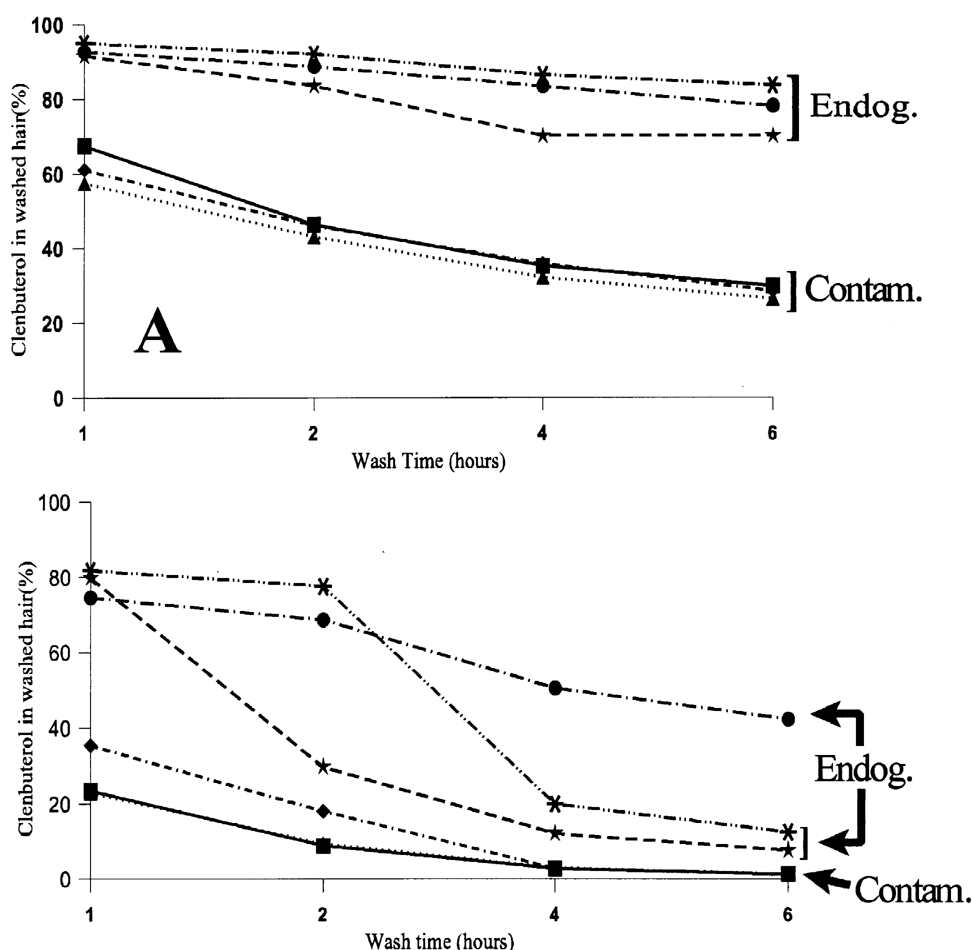

Fig. 1. Wash kinetics of clenbuterol from contaminated hair and from hair of treated animals at room temperature (A) and 60°C (B). The content of clenbuterol remaining in the washed hair is expressed as a percentage over the clenbuterol content of unwashed hair.

less than 0.25 for samples from treated cattle whereas the ratios for contaminated samples are equal to or higher than 0.70. On the other hand, washing at 60°C cannot distinguish clearly between both types of samples, although the ratios of the contaminated samples tend to be higher. It is worthwhile to point out that this strategy detects external contamination in the most plausible real-life scenario: hair sampled a short time after an external contamination. Evaluation of external contamination a long time (e.g., a month) after exposure would be much more difficult, especially if the hair has been washed (e.g., by rain) [17]; this is also a potential problem with hair analysis in humans [20].

Since the behaviour under HCl wash conditions is similar for the other β_2-agonist compounds studied a similar result can be expected for them, however this must remain as working hypothesis until samples from treated cattle are studied.

4. Conclusions

Analysis of β_2-agonists in hair is receiving increasing attention as a means of enforcing the EU ban on these drugs as growth promoters in animal rearing. However, it has been shown that hair can be contaminated externally with different drugs with

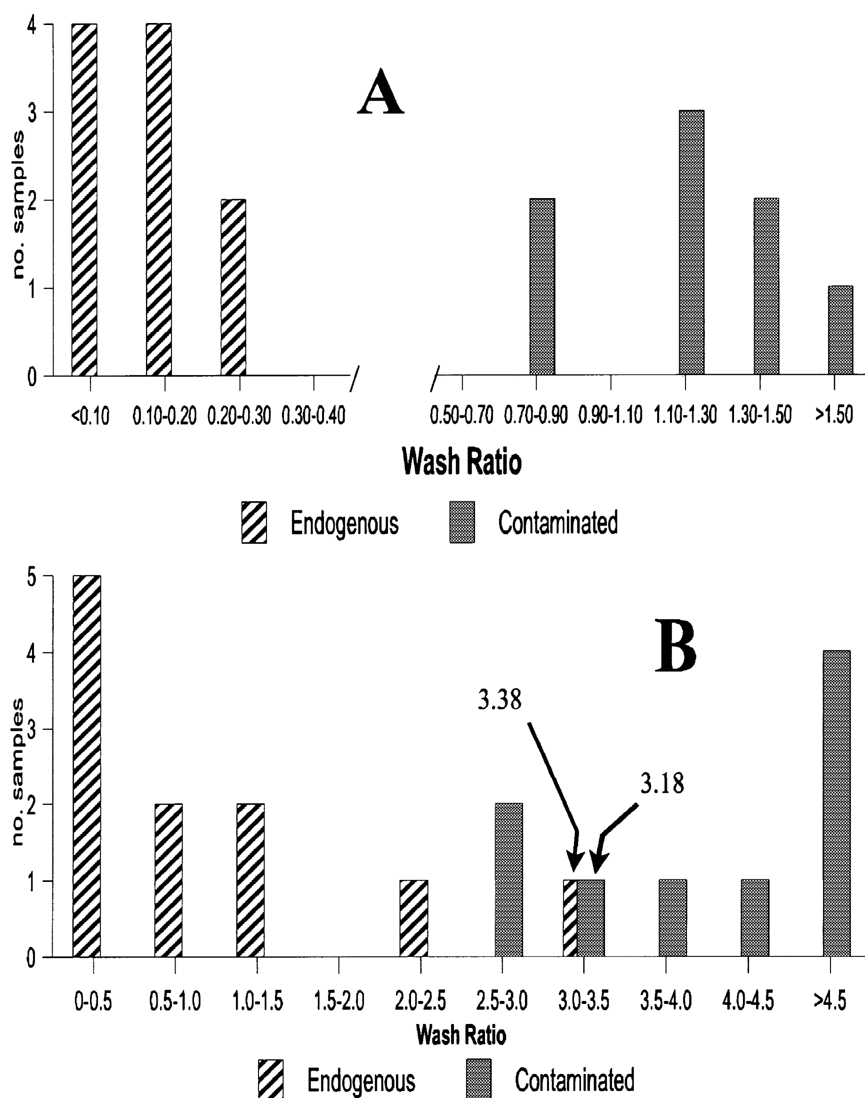

Fig. 2. Distribution of values of wash ratio for clenbuterol-containing hair samples washed with 0.1 *M* HCl at room temperature (A) and 60°C (B) for 2 h. Wash ratio is calculated as the content of clenbuterol in wash solution divided by the content of clenbuterol remaining in hair.

concomitant risk of false positive results. The results presented here show that bovine hair can be contaminated externally quite easily by soaking in aqueous solutions of β_2-agonists compounds. Once contaminated, standard wash procedures (e.g., detergents, aqueous buffers, organic solvents) cannot eliminate the externally accumulated drugs from the hair which implies a considerable risk of false positive results. Hair samples externally contaminated with clenbuterol can be discriminated from samples from treated cattle by washing with a 0.1 *M* HCl solution for 2 h at room temperature and analysing both the wash solution and the remaining hair. For samples from treated cattle the ratio of clenbuterol in the

wash to the clenbuterol content remaining in the hair is equal to or less than 0.25 whereas this ratio is equal to or higher than 0.70 for contaminated hair.

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