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Veterinary treatment of cows with isoxsuprine for a caesarian section may temporarily lead to residues in hair of both cow and calf

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Isoxsuprine is a beta-agonist that can be used for growth promotion in cattle, but it is also used as registered veterinary medicine. To investigate if veterinary treatment of cows could lead to residues of isoxsuprine in the hair of their newborn calves, an animal experiment was performed. Four cows, treated on veterinary indication with isoxsuprine lactate (Duphaspasmin) before a caesarian section, were included in the experiment. Hair samples from cows and from their calves were analyzed. The animals were shaved every week for 16 weeks and levels of isoxsuprine were measured in hair. In the cows, the levels of isoxsuprine were highest (>15 μg/kg) just after administration of the isoxsuprine lactate. After two weeks in two cows, a sort of plateau was reached and then the levels decreased. After approximately 10-15 weeks the levels were around the CCα level of the method used (0.5 μg/kg). In calves, for the first two weeks after birth, no isoxsuprine was found above CCα level in three of the four animals. At about 20–30 days old, a maximum concentration of 4 μg/kg was found. Then the levels dropped again under the CCα level, after 60 days no levels above CCα level were found. In one animal, the levels never reached CCα level. We conclude that veterinary treatment of cows with isoxsuprine may temporarily lead to low levels of isoxsuprine in the hair of their newborn calves which can be measured for a maximum of 60 days after birth. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: isoxsuprine; cattle; beta-agonist; hair analysis

Introduction

Isoxsuprine is a phenylethylamine derivative of epinephrine which has both β-adrenergic agonistic and α-adrenergic antagonistic properties.[1] In human medicine, isoxsuprine is used as a vasodilator[2] and tocolytic.[3] In domestic animals, it has been used to produce uterus relaxation and to perform obstetric procedures.[4,5] In horses, isoxsuprine hydrochloride is used for treatment of navicular syndrome and laminitis.[6] In the Netherlands, isoxsuprine lactate is registered as a veterinary medicine, Duphaspasmin, and used for uterus relaxation (tocolysis) during caesarian section in cows.

Isoxsuprine is a β-agonist and these compounds also have a history of illegal use as repartitioning agent in cattle,[7] leading to more lean muscle, less fat, and reduced feed conversion. In Europe, the use of β-agonists for growth promotion is forbidden under the Council Directive 96/22/EC.[8] In the Netherlands, isoxsuprine is sometimes found in the hair of veal calves during control for illegal growth promoters. It is not clear if this is the result of treatment of the mother or the result of illegal treatment for growth promotion. Residues in the hair of newborn calves were not investigated in the registration process of Duphaspamin as a veterinary medicine. Therefore calves derived from isoxsuprine-lactate-treated cows were followed in time, as well as the cows. The aim of this investigation was to gain an insight into the kinetics of isoxsuprine in hair and to get information on the detection window of isoxsuprine in hair from untreated calves.

Material and Methods

Experimental, veterinary treatment, and sampling

The experiment was performed in cooperation with the Veterinary Faculty of Ghent University (Belgium) on a nearby farm. For this experiment, four cows (black-and-white Holstein Friesians) were used that were veterinary treated with Duphaspasmin, (REG NL 8514, isoxsuprine lactate 11.58 mg/ml, Fort Dodge Animal Health Benelux B.V. Naarden, Netherlands) for a caesarian section. Calves were separated from the cows (three male calves and one female) after birth according to dairy practice and were fed colostrum from the freezer containing no isoxsuprine. Both cows and calves were sampled weekly for hair, starting with the day of birth of the calves. Hair was shaved from the back and the sides of the animals, left and right side alternately each week. The samples consisted of black hair mixed with white hair. The animals were followed for

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16 weeks. First the hair was cut with a scalpel as close as possible to the bare skin. Later a shaving machine was used and only dry hair was sampled.

Analytical methods

Chemicals

The chemicals and solutions used were of analytical reagent grade. Water was purified using a Milli-Q system from Millipore (Bedford, MA, USA). Isoxsuprine was obtained from Sigma (St Louis, MO, USA). The isotope-labelled internal standard clenbuterol-d_6 was obtained from RIVM (Bilthoven, the Netherlands). Stock solutions were prepared in methanol at 1 mg/ml. Bondelut Certify mixed-mode solid-phase extraction (SPE) columns (300 mg) were from Varian (Harbor City, CA, USA).

Hair

Hair samples were cut with a pair of scissors to less than 2 cm. From each sample two test portions of 500 mg were weighed. Both test portions were spiked with the internal standard clenbuterol-d_6 at 10 ng/g. In addition, one of these portions was spiked with isoxsuprine at 5 ng/g.

Blanks and controls were spiked with the internal standard clenbuterol-d_6 at 10 ng/g. The control hair samples were prepared by spiking blank hair with isoxsuprine at 0, 2.5, 5.0, 10, 20, and 50 ng/g. Following the addition of 4 ml NaOH (1.0 M) solution, digestion was carried out in a water bath at 65° for 2 h. Next, 3.5 ml of HCL (1.0 M) and 5 ml of 0.25 M sodium acetate buffer pH 4.8 were added and the test tubes were centrifuged for 10 min at 2000 g. The supernatants were transferred to a clean test tube, adjusted to pH 4.8 if necessary, and centrifuged again for 10 min at 2000 g. The supernatants thus obtained were further subjected to solid phase extraction (SPE) on a mixed-mode column, previously conditioned by methanol and sodium acetate buffer. The vacuum-dried SPE column was washed subsequently with 1 ml of 1M acetic acid, 6 ml of methanol and 2 ml of acetone/chloroform (1 : 1). Finally the contents of the dried SPE column were eluted with 7.5 ml of 1% ammonia in ethyl acetate and the eluate was evaporated to dryness under a stream of nitrogen gas at 40 °C. The residue obtained was redissolved in 300 µL of 0.24% formic acid. 50 µl was injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

LC-MS/MS

The LC-MS/MS system consisted of a Shimadzu (Milford, MA, USA) model LC system equipped with a Waters (Manchester, UK) model Quattro Ultima triple-quadrupole mass spectrometer. The mass spectrometer was operated in the positive electrospray ionization (ESI) mode at a capillary voltage of 2.7 kV, a desolvation temperature of 300°, a source temperature of 120° and a cone voltage of 35 V. The desolvation gas was nitrogen and the collision-induced dissociation (CID) gas was argon. For isoxsuprine, the most abundant multiple reaction monitoring (MRM) transition (used for the quantification) was acquired using the following conditions: precursor ion mass (*m/z*) 302.1, product ion mass (*m/z*) 135.0 with a Collision energy of 20 (eV). A second MRM transition, used for the confirmation of the identity of isoxsuprine, was acquired using the following conditions: precursor ion mass (*m/z*) 302.1, Product ion mass (*m/z*) 150.0 with a Collision energy of 20 (eV).

The analytical column was a 150 mm × 3.2 mm internal diameter 5-µm C18 Alltima column (Alltech, Breda, the Netherlands), kept in a column oven at 40°. The two mobile phases used consisted of 100 : 0.1 water/formic acid (v/v) (solvent A) and 100 : 0.1 acetonitrile/formic acid (*v/v*) (solvent B) and the flow rate was 0.4 ml/min. Following a 1-min isocratic period at 0% solvent B, a linear gradient was started towards 60% solvent B at 8 min, and up to 100% solvent B at 9 min. The liquid chromatograph was connected to the ESI-MS/MS instrument using a 1 : 2 split. The concentrations of isoxsuprine in hair samples were estimated using the isotope dilution method and a standard addition of 5 ng/g isoxsuprine to each individual sample.

Results and Discussion

During the experimental period, the animals remained on the farm where they were born and were shaved weekly. All four calves and their mothers were followed for four months and no health problems were observed in the experimental period in either the cows or the newborn calves.

Weekly samples of hair per animal were received. The cow samples (encoded 3377, 4976, 4982, and 4999) and their respective calves (encoded 7568, 7569, 7570, and 7572) were analyzed for isoxsuprine. The results of both cows and their calves are shown in Figure 1.

On the left, you see the levels isoxsuprine in the hair samples from the cows and on the right, the levels in the hair samples from their calves. The level of isoxsuprine in the hair samples from the cows was highest just after administration of the drug, reaching levels >15 µg/kg, with 50 µg/kg as highest level found. After two weeks, two cows reached a sort of plateau level and after 50 days the levels dropped. In two other cows however, additional peaks were observed at 80–90 days after treatment (animals 4976 and 3377). After approximately 10–15 weeks, all levels were around or below the CCα level of the method used (0.5 µg/kg).

In the hair samples from their calves, in the first two weeks after birth no isoxsuprine was found above CCα level. At about 20–30 days of age a maximum concentration of 2.5–4 µg/kg was found in three of four calves. Then the levels dropped again under the CCα level and after 60 days no levels above CCα level were found anymore. In one animal (7568) the levels of isoxsuprine in hair never reached the CCα level during the whole sampling period.

In this experiment, levels of isoxsuprine in hair samples from cows treated with isoxsuprine lactate for a caesarian section and levels of isoxsuprine in hair samples from their newborn calves were followed for 16 weeks. Isoxsuprine was found in hair of both cow and calf.

The detection of β-agonists in hair is performed since 1994.[9] Analysis of isoxsuprine in hair and urine is described more recently.[10,11] There are several ways in which drugs can enter the hair, such as passive diffusion from the blood stream into the growing cells at the base of the hair follicle, or binding of the drug to melanin or to sulfhydryl-containing amino acids such as cystine in hair. Another possibility is diffusion of the drug along the hair shaft transferred from sweat and sebum.[12]

The excretion of isoxsuprine in the cows' hair was remarkably fast after administration of this drug. This implies that isoxsuprine is excreted via sweat or sebaceous glands, since hair growth would require several weeks to appear above the skin. Another possibility is that hair was contaminated by the injection fluid, but that is

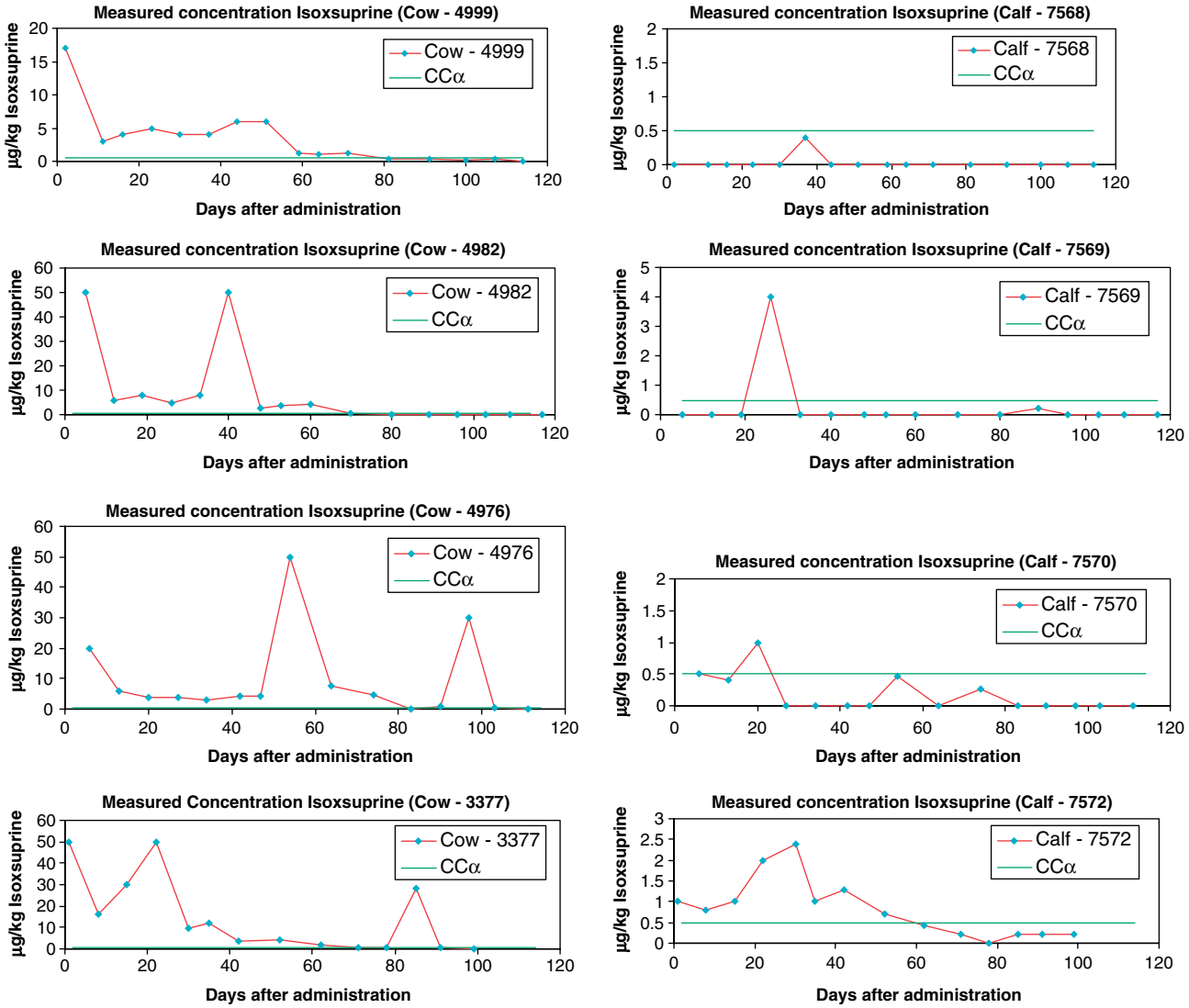

Figure 1. Levels of isoxsuprine in hair: cows left and their calves on the right side.

not likely because the operation site had been shaved already and the hair was also sampled at other sites. The peaks following the first peak might be due to isoxsuprine that reached the hair via the blood stream or bound to pigments in the hair. In horses, prolonged excretion (up to six weeks) of isoxsuprine in urine is found after cessation of treatment. Melanin affinity of isoxsuprine in the horse is suggested as a possible mechanism to explain this finding.[13] A similar mechanism may be present in cows leading to prolonged levels in hair (80–100 days after treatment) as is seen in cows 4976 and 3377. The hair samples in our experiment were not selected for white or black hair and all samples were mixed. In future experiments we aim to differentiate the samples into all black, all white, and mixed hair samples to investigate the possible effect of binding of the drug to melanin.

In the calves, the first isoxsuprine was seen in the hair after 3–4 weeks which may be due to passive diffusion from the blood. Differences between the levels of isoxsuprine in the calves may be explained by the natural variation in physiology between the animals and differences in the times necessary to deliver the calves after treatment of the mother. The calves did not receive isoxsuprine via the milk since they got colostrum from non-treated cows and also in the following period they did not receive milk from treated animals. On this farm, it was common practice to feed newborn calves with colostrum from the freezer and not from their own mother as is usually done. For our study, this meant that the only route of isoxsuprine for the calves was via the mother during the partus. Milk from the treated cows was not analyzed for isoxsuprine.

Since a withdrawal time of 5 days for cows' milk is required after treatment with Duphaspasmin, the milk from treated cows cannot be used for human consumption. In practice this milk is fed to the calves which may lead to additional exposure to isoxsuprine. In conventional dairy farming, most calves are fed milk-replacer after the first three days of life, and if extra milk is added it will be diluted with milk replacer and water. It is not clear if these levels influence the levels of isoxsuprine in hair. Calves intended for veal production are sold after 10 days of birth so it is only in the first weeks of their lives that they may have got additional isoxsuprine, levels of which would be very low. In our calves, the highest levels were seen between 20 and 40 days after birth. Additional isoxsuprine via the milk may possibly cause the excretion period to be a week or two longer. But in that case there is no veterinary indication

for treatment, so this kind of administration of isoxsuprine can be considered as illegal treatment.

No data about uptake of oral isoxsuprine in calves are available. To evaluate the possible contribution of isoxsuprine in milk from treated cows to levels in the calves' hair, another experiment should be performed, one which was beyond the scope of this study. In healthy human volunteers, the absorption of isoxsuprine was assumed to be about 51%,[14] and for horses, oral bioavailability was found to be 2.2% with a high first-pass effect.[6] However, caesarian sections are not very common on Dutch dairy farms although one study mentioned percentages per farm ranging from 0 to 3.6%.[15] Based on this, findings of isoxsuprine in calves' hair should be rare, if found at all.

The New Food and Consumer Product Safety Authority in the Netherlands may control hair samples from veal calves at any age, thus also from animals from 0–10 weeks. During these inspections, hair positive for isoxsuprine has been found in young calves. These findings were the reason this study was performed. The results of our experiment provide important information that may help prevent unnecessary prosecution of farmers due to the inadvertent presence of banned substances in hair samples from their animals.

In the Netherlands, isoxsuprine is the only beta-agonist registered as a veterinary medicine for cows. Planipart REG NL 7068, containing clenbuterol hydrochloride, was also registered as tocolyticum for cows but hasn't been registered since 2009. There are other clenbuterol containing veterinary medicines but these are only registered for horses.

Other veterinary medicines that might lead to residues in hair are esters of steroid hormones, such as estrogens, gestagens, and androgens. But in practice there are no indications to treat pregnant cattle with these hormones.

Conclusion

We can conclude from this experiment that veterinary treatment of cows with isoxsuprine for a caesarian section may temporarily lead to residues in hair of both cow and calf. Low levels of isoxsuprine in calves' hair during the first two months of their lives can be caused by veterinary treatment of their mother with isoxsuprine lactate. Levels higher than 4 μg/kg in calves under two months and levels above CCα (0.5 μg/kg) in older calves may be suspect for illegal treatment for growth promotion. In these cases, further action is required. In case of doubt, records for veterinary treatment of the calf's mother should be consulted as well as information on the use of isoxsuprine contaminated milk to feed the calves.

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