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Evaluation of the usefulness of skin derivatives as an alternative biological material in toxicological analysis*

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Summary

Currently, dominant diagnostic methods in veterinary medicine rely on blood and urine analysis, which come with specific limitations regarding the toxicokinetics of elements and the invasiveness of sample collection. Alternative diagnostic materials (hair, nails) could overcome these limitations. Nowadays, they are not widely used in routine toxicological analysis, mainly due to issues with exogenous contamination and limited sample mass. Furthermore, optimized procedures for determining xenobiotics in these materials are lacking. The main objective of the project was to develop procedures for the determination of Cd and Pb in alternative diagnostic materials (combed hair and mane) collected from horses, and to evaluate their effectiveness in comparison to traditional diagnostic materials such as serum. Hair samples were decontaminated and homogenized with a cryomill before their mineralization. The Cd and Pb were determined in samples after their mineralization by electrothermal atomic absorption spectrometry. The recovery was estimated using certified reference materials. The application of hair as an alternative diagnostic material improved the precision and accuracy of toxicological analysis of Cd and Pb. Further studies should be performed regarding other alternative materials, animal species and heavy metals.

Keywords: alternative materials, toxicology, horses, heavy metals

At present most methods of toxicological veterinary diagnostics are based on the analysis of the basic research materials (blood and urine). They are appropriate for the analysis of xenobiotics characterized by their short toxicokinetic profile. In contrast, basic research materials are susceptible to rapid degradation and contamination; therefore, they must be transported to the diagnostic laboratory promptly and stored under specific conditions. Additionally, the sampling of these kinds of samples is invasive and harmful to animals. The sampling volume of the materials can often be limited and insufficient for the reliable toxicological analysis (5, 7, 10, 14, 18, 20).

These problems can be overcome by the application of alternative research materials for veterinary toxicological diagnosis. They can provide the non-invasive and painless sampling, which is related to patient care. Moreover, the sampling of alternative matrices can be performed without any specialized tool and limitations regarding the sample mass/volume. Their transport and storage do not require special conditions. Furthermore, the application of alternative matrices can provide a low measurement uncertainty of determined xenobiotics. This is connected with the short-term changes in the concentration of determined substances (5, 7, 10, 14, 18, 20). Saliva is an example of an alternative research material produced by the salivary glands. It contains various substances that reflect recent drug use. The possibility of providing rapid results makes it especially useful for detecting drugs consumed shortly before testing. Another important matrix is meconium, the first stool passed

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by a newborn, which consists of materials ingested during fetal life. Meconium offers a longer detection window than urine and is particularly significant for indicating drug exposure during pregnancy (6). Hair is another widely used alternative matrix. As keratinized fibers that grow from hair follicles, hair can incorporate drugs over time, providing a historical record of drug use. Substances can be detected in hair for months or even years after use, making hair analysis a valuable tool for long-term monitoring. The sampled hair length is a crucial parameter related to the time of exposure to the xenobiotic (1, 4, 8, 9, 11, 12, 15, 22-24). Breast milk is another significant matrix, as it can contain drugs transferred from a lactating mother. This matrix is essential for assessing drug exposure in infants and gaining insights into maternal drug use (17). In post-mortem forensic toxicology, vitreous humour – the gel-like substance found in the eye – plays a vital role. It is less affected by decomposition compared to other fluids, making it particularly useful for determining drug levels at the time of death. In addition to these more established matrices, other emerging alternatives are gaining attention. Bile, a digestive fluid, provides insights into drug metabolism and excretion. Nails, similar to hair, can retain a record of drug exposure over extended periods. Bone marrow is being explored for its potential to detect long-term drug exposure, while umbilical cord tissue has proven helpful in assessing prenatal drug exposure, reflecting maternal drug use during pregnancy (6).

Although the alternative research materials possess many advantages, they are not without flaws. Exogenous pollution of these materials, which are hard to remove (e.g., air pollution, cosmetic and pharmaceutical residues, varnishes), can seriously affect analytical results. Sometimes the mass of the alternative sample is insufficient for a reliable toxicological analysis. Moreover, the alternative materials often do not meet the criterion of representativeness, and their accurate homogenization is hard and time-consuming. Moreover, the lack of optimized and validated analytical procedures for the determination of xenobiotics in this kind of material hinders their application for routine veterinary diagnostics and forensic veterinary (5, 7, 10, 14, 18, 20).

This article explores the potential of skin derivatives as an alternative diagnostic matrix, evaluating their effectiveness in comparison to more traditional materials like blood and urine, and examining the challenges and opportunities for their integration into veterinary toxicology of Pb and Cd.

Material and methods

Six privately owned horses, all female, of the Małopolska breed were included in the study. Hair samples were taken in winter. They consisted of the hair combed from around the animals' abdominal area and the hair cut from their manes. Blood serum samples were delivered to the laboratory of the Department of Pharmacology, Toxicology and Environmental Protection of the Faculty of Veterinary Medicine, University of Life Sciences in Lublin by the veterinarian responsible for the herd. Employees and students did not participate in collecting serum samples for research. Blood and hair sampling was preceded by a general veterinary check-up. In summer, apart from the time spent exercising, the horses stayed on pastures and in the paddock, while in the wintertime, they spent 2-3 hours per day in the paddock. All the horses were fed on standard feeds consisting of hay, barley, and green forage.

After thawing, blood serum samples were homogenized using a Vortex 3 shaker. Next, for each tested serum sample, three independent samples of 1.0 ml were collected and subjected to the mineralization process. In the case of combed hair and mane, the first preparation step was the removal of the external contamination from their surface. A four-step washing procedure was performed according to the following order: 1) washing with an aqueous Triton-X-100 solution (0.01 wt.%), 2) one rinse with deionized water, 3) one successive wash with acetone, and 4) a final rinse with deionized water. Next, washed, combed hair and mane samples were dried at room temperature until constant weight. The tested hair materials were subjected to homogenization. For this purpose, various available methods and homogenizers were used: cutting with titanium scissors, classic mills with tungsten carbide balls, mills with titanium blades and a cryogenic mill. The latter method was the most appropriate. Three sub-samples of a given research material were prepared for mineralization, each containing 0.5 g of material. Mineralization of all studied samples was performed in the microwave oven Multiwave 2000 (Anton Paar, Austria) using 6 ml of 65% nitric acid (suprapure) per sample. The mineralization conditions were listed in Table 1. After the mineralization process, samples were cooled to room temperature, and then accumulated nitrogen oxides were removed from the vessels. The obtained solutions were transferred quantitatively to measuring flasks (5 ml for serum samples and 25 ml for hair samples) and filled to the mark with deionized water.

Tab. 1. Mineralization conditions (pressure ramp: 0.3 bar s^{-1}, max. pressure: 60 bar, max. temp.: 240°C, drive: continuous rotation)

Step	Power [W]	Ramp [min.]	Hold [min.]	Fan
1	750	20	10	1
2	1200	10	10	1
3	0	–	15	3

The concentrations of Cd and Pb in the mineralized samples were determined by the use of an atomic absorption spectrometer, SpectrAA 220Z, (Varian, Australia), equipped with an electrothermal atomizer, a Zeeman background correction system, and Cd and Pb hollow cathode lamps (Varian, Australia). A palladium and magnesium nitrates solution (Pd: 0.3 g L^{-1}, Mg: 0.9 g L^{-1}) (Merck, Germany) was used as a chemical modifier for the analysis of Cd and Pb. The basic instrument parameters for electrothermal atomic absorption spectrometry technique used during Cd

and Pb determinations were listed in Table 2. The main analytical parameters were estimated: recovery, precision, quantification limit and linearity range. The recovery was studied using the highest-quality certified reference materials: blood (Seronorm® Trace Elements Whole Blood L-2 (13)) and hair (GBW 07601 (GSH-1) (19)).

Tab. 2. Basic instrumental parameters during Cd and Pb determinations by electrothermal atomic absorption spectrometry technique

Analyte	λ [nm]	I_{HCL} [mA]	$T_{pyr.}$ [°C]	$T_{at.}$ [°C]	Slit width [nm]	$V_{mod.}$ [µL]	V_{sample} [µL]
Pb	283.3	5.0	800	2100	0.5	5	20
Cd	228.8	4.0	600	1600			

Explanations: λ – analytical wavelength; I_{HCL} – hollow cathode lamp current; $T_{pyr.}$ – pyrolysis temperature; $T_{at.}$ – atomization temperature; $V_{mod.}$ – volume of modifier; V_{sample} – volume of sample

Tab. 3. The main analytical parameters of developed analytical procedures for Pb and Cd determination in serum and hair samples by electrothermal atomic absorption spectrometry (LOQ – limit of quantification)

Analyte	Sample matrix	Precision [%]	LOQ	Linearity range	Recovery [%]
Pb	hair	1.2	0.02 µg g^{-1}	0.25-2.00 µg g^{-1}	109
	serum	7.5	2.0 µg l^{-1}	25-200 µg l^{-1}	103
Cd	hair	4.3	0.002 µg g^{-1}	0.01-0.05 µg g^{-1}	91.6
	serum	12.9	0.2 µg l^{-1}	1.0-5.0 µg l^{-1}	98.4

Results and discussion

In order to obtain reliable results, each sample should be homogenized appropriately. The heterogeneity of the sample can lead to low repeatability and high relative standard deviation (RSD), increasing the uncertainty of the analyte concentration. In the case of hair samples, the homogenization step was performed in various ways: cutting with titanium scissors, using classic mills with tungsten carbide balls, mills with titanium blades and a cryogenic mill. The application of titanium scissors resulted in no satisfactory homogenization of the studied samples. In turn, hair shredding by classic mills with tungsten carbide blades and mills with titanium blades caused significant heating of the material due to friction and sample burning. The best results were achieved with the cryogenic mill. Hair samples under a low temperature of the liquid nitrogen became brittle, and no heating was observed. Thus, the cryogenic mill ensured effective homogenization of hair samples (RSD values mostly below 10%).

In Table 3 the main analytical parameters for Pb and Cd determination procedures from serum and hair samples were presented. The limit of quantification for Pb and Cd in hair samples was 0.02 µg g^{-1} and 0.002 µg g^{-1}, respectively. In the case of serum samples, this parameter was 2.0 µg l^{-1} (Pb) and 0.2 µg l^{-1} (Cd). The recovery of determined analytes in the studied samples was between 91.6% and 109%. The linearity ranges for the calibration curves for Pb and Cd were 5-40 µg l^{-1} and 0.1-1.0 µg l^{-1}, respectively. Precision for developed procedures of Pb and Cd determination was lower for hair samples (1.2-4.3%) than for serum samples (7.5-12.9%). It could be related to the better homogeneity of hair by the cryogenic mill and a simpler sample matrix(lower Fe concentration).

In Tables 4 and 5, results of Pb and Cd concentration in horse serum and hair were presented. It was observed that hair samples had higher Pb and Cd levels than serum samples (even 57 times higher). It was related to the heavy metal accumulation in hair tissue, the main component of which was keratin. This protein contains abundant -SH groups that strongly bind heavy metals, such as Pb and Cd. Moreover, hair is a long-term toxicologic biomarker, which presents the poisoning exposure for a few months. Both the hair sampling method (cutting distance from the scalp) and the time of sampling (hair growth) are important parameters which affect the obtained results. Hair can also be a useful diagnostic material in veterinary toxicology due to its high stability and ease of storage(16). In our case, Pb serum levels in the studied horses ranged from 21.3 µg l^{-1} to 35.8 µg l^{-1}. It was much below the maximum acceptable Pb level in equine blood (250 µg l^{-1}) (21). Similar observations were noted from hair samples, where Pb was in the range of 0.60-2.48 mg kg^{-1} (healthy racing horses: 0.71-1.24 mg kg^{-1} (2)). In contrast, the Cd level in horse serum samples was

Tab. 4. Pb levels in serum, mane and combed hair (mean of 5 repetitions, SD)

Sample No.	Pb [µg·l^{-1}]	Pb [mg·kg^{-1}]	
	Serum	Mane	Combed hair
	Mean ± SD	Mean ± SD	Mean ± SD
1	35.30 ± 2.90	2.01 ± 0.02	2.48 ± 0.02
2	24.85 ± 3.58	0.67 ± 0.01	1.05 ± 0.04
3	33.90 ± 2.55	1.63 ± 0.04	2.40 ± 0.06
4	21.28 ± 3.19	1.10 ± 0.02	1.32 ± 0.04
5	35.84 ± 5.54	0.60 ± 0.06	0.90 ± 0.07
6	32.17 ± 3.42	1.25 ± 0.07	1.63 ± 0.08

Tab. 5. Cd levels in serum, mane and combed hair (mean of 5 repetitions, SD)

Sample No.	Cd [µg·l^{-1}]	Cd [mg·kg^{-1}]	
	Serum	Mane	Combed hair
	Mean ± SD	Mean ± SD	Mean ± SD
1	1.61 ± 0.16	0.06 ± 0.01	0.09 ± 0.01
2	1.68 ± 0.22	0.11 ± 0.01	0.12 ± 0.01
3	4.68 ± 0.78	0.06 ± 0.01	0.09 ± 0.01
4	2.64 ± 0.35	0.05 ± 0.01	0.08 ± 0.01
5	1.37 ± 0.47	0.04 ± 0.01	0.05 ± 0.01
6	1.77 ± 0.23	0.04 ± 0.01	0.06 ± 0.01

below 6 μg l^{-1}. In agreement with the literature, Cd concentration in equine serum was similar in polluted and unpolluted areas. It followed from the body's efficient mechanisms for filtering and excreting this heavy metal, which readily accumulates in the liver, kidneys, and hair. And so, this is not a reliable diagnostic parameter in veterinary toxicology (3, 16). Hair could be a better material for these purposes. For studied horses, the Cd level in hair ranged from 0.035 to 0.117 mg kg^{-1}, which is similar to levels reported for healthy horses (0.04-0.20 mg kg^{-1} (2)). Furthermore, the Pb and Cd analysis from alternative materials (combed hair and mane) was related with the higher precision (RSD values in the range 0.7-9.3% (Pb) and 4.3-13.2% (Cd)) than in the case of serum (RSD values in the range 7.5-15.5% (Pb) and 9.8-34.1% (Cd)). Lower RSD values during analysis of Pb and Cd in combed hair and mane could be due to better sample homogenization by the cryomill. Additionally, higher RSD values during Cd determination in the studied samples were due to the relatively low concentrations of this element. The correlation between Pb and Cd concentrations in serum and combed hair/mane was negligible ($R^2 < 0.2$), in contrast to mane and combed hair, where the correlation between obtained results was very high (R^2: 0.86-0.94). Further studies regarding the use of alternative materials in the veterinary toxicological analysis of Cd and Pb should be performed with a higher number of samples and various animal species with different levels of analyzed elements in the studied samples.

The use of alternative materials, such as hair, nails, and feathers, is pivotal in veterinary toxicology, especially in effective screening studies. These matrices allow for non-invasive sample collection, are easy to store and transport, and offer cost-effective analysis compared to traditional diagnostic materials such as blood and urine. Importantly, they enable retrospective monitoring of heavy metal exposure due to the presence of sulfur-containing proteins that bind these elements. This makes them an invaluable tool for assessing long-term toxicological profiles in both companion and wild animals, while also reducing measurement uncertainty relative to bodily fluids. Proper processing of these materials is critical to maintaining their integrity and ensuring the reliability of results. Challenges such as exogenous contamination and limited sample mass underscore the necessity for optimized analytical procedures.

In our study, we successfully developed and validated methods for determining Cd and Pb in mane and combed hair of horses, demonstrating their efficacy compared with conventional methods (serum). These findings highlight the potential of such materials to fill a critical gap in routine toxicological diagnostics. The broader adoption of alternative diagnostic materials has far-reaching implications for animal health and welfare. By enabling accurate and efficient detection of heavy metal concentrations, these methods can significantly improve the quality of toxicological data, providing a deeper understanding of environmental exposure and its impact on animal populations. This, in turn, supports more effective herd management, targeted treatment strategies, and enhanced veterinary care. Ultimately, the integration of alternative matrices into routine diagnostics represents a step forward in advancing both scientific accuracy and practical applications in veterinary toxicology.

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