AORC Guidelines

Recommendations for the screening and confirmatory analysis of animal hair samples

Background

1. A Drug Testing in Hair Committee was first proposed at the 2016 ICRAV meeting in Montevideo and its formation was formally requested by the then AORC President (Charlie Russo) following the 2017 meeting in Doha.

2. The Committee members were confirmed in late 2017 and currently (January 2022) comprise Bob Gray (UK, Chairman), Paul Zahra (Australia), Ludovic Bailly-Chouriberry (France), Benjamin Moeller (USA) and Wai Him Kwok (Hong Kong), giving the Committee representation from each of the International AORC Sections.

3. It was agreed that the first task of the Committee was to put together a document for laboratories which present a set of recommendations for drug testing in animal hair, similar to those published by the Society of Hair Testing (SoHT) for the analysis of human hair samples but adapted to the needs of equine hair testing.

4. The document should also be suitable to provide guidance on the analysis of hair samples from other animal species, such as greyhounds and camels.

Scope

5. The purpose of this document is to provide laboratories undertaking animal sport doping control with a set of guidelines to assist with the establishment and on-going delivery of hair testing services. The document should act as a useful guide to laboratories in ensuring that they understand the requirements of a robust and high-quality hair testing service.

Introduction

6. Hair is a strong, stable tissue that is largely unaffected by adulterants and is simple to collect, transport and store, as such it has a number of potential advantages over the traditional matrices of urine and blood.

7. Hair is able to provide a much-extended detection window than blood or urine, with coverage of many months being possible dependent upon sample length and growth rates.

8. The results obtained from animal hair samples are qualitative, i.e. they provide evidence of exposure to a drug but cannot be used to assess the extent of that exposure.

9. It is possible to detect a broad range of drugs in hair samples, but the generally accepted practice is to target those drugs which are prohibited at all times. For equine hair this means a focus upon those substances featured in Article 6E, Paragraph 5 of the IFHA International Agreement on Breeding, Racing and Wagering [1].

10. It is generally accepted that drugs can be incorporated into hair via a variety of routes (the multi-compartmental model, after Henderson [2]). These routes of incorporation and external deposition may all contribute to the detection of drugs in hair samples and should be considered when interpreting results.

11. The rate of incorporation of a drug into hair is dependent upon the physiochemical properties of the molecule and therefore hair analysis cannot be used to provide an indication as to the dose or frequency of an administration. In some cases incorporation of an administered drug into hair may be very low or even zero and therefore not all drug exposures can be detected in hair.

12. Segmental analysis of hair samples can be used in an attempt to provide additional information on the potential timing of an administration. However, caution should be employed when interpreting the results of such analysis as there are a number of factors which can affect the incorporation of drugs into hair and deposition onto the outside of the hair.

Sample collection

13. Sample collection should be performed by individuals familiar with the process and with an understanding of the requirements of the laboratory. In this way a sample that is appropriate for analysis should be obtained, ensuring that issues with the quality of samples are minimised.

14. Care should be taken to ensure that hair samples are dry before collection. Storage of wet samples can lead to bacterial growth and potential issues with degradation and/or conversion, complicating analysis and interpretation of results.

15. Samples can be collected in a number of ways and the approach taken should be based upon discussion between the laboratory and the appropriate regulatory body.

16. For the equine, mane hair is the preferred sample, although tail hair can also be used if required.

17. Collection of mane hair is usually performed by either cutting, using scissors or a concealed blade, or by the standard grooming procedure of 'mane pulling', using a mane comb.

18. A sample of sufficient size to complete the required analysis should be collected – this will vary and should be agreed between the laboratory and the regulatory body, dependent upon the testing requirements. It is recommended that a minimum sample size be agreed with the regulatory body prior to collection to ensure sufficient material for testing.

19. If required by the regulatory body, a B-sample should be collected at the same time as the A-sample and using the same technique. The B-sample should be stored in a separate security bag or container and labelled accordingly.

20. If samples are to be collected by cutting, then care should be taken to ensure that the cut is made as close to the skin as possible to ensure that a full incorporation profile is maintained. An appropriately sized bunch of hair should be selected and secured with a suitable clamp/band and the sample cut below this, as close as possible to the skin.

21. If using a mane comb (mane pulling) then the sample should be collected from the full length of the mane, from poll to withers, using standard grooming procedures. After pulling, the sample should be secured at the proximal end using a suitable clamp/band.

22. In both cases (mane pulling and cutting), the collector should ensure that the collected sample remains aligned and that the proximal (newest growth) end is clearly identified.

23. Collection of coat hair samples from other animal species (e.g. canine and camel) is often performed by shaving the coat hair using electric clippers or cutting with scissors. In this case the collector should ensure that the clipper blades and/or scissors are thoroughly cleaned between animals using appropriate solvent wipes. For the canine, coat hair from the base of the tail is preferred, as it is easier to handle and has shown to contain the highest concentration of drugs [3].

24. Once collected, the dry sample should be placed within a security bag or container, the bag/container sealed and the appropriate paperwork completed. In some instances an additional storage device may be used (e.g. a hair collection envelope) and should be placed within the security bag or container itself.

25. Samples should be stored dry, at room temperature or lower and protected from light.

26. Blood and/or urine samples may also be collected at the same time as a hair sample in order to provide as complete an analytical picture as possible.

Sample Collection Kits

27. Sample collection kits are usually supplied by the testing laboratory and will vary depending upon the requirements of the laboratory and the relevant regulatory body. Sample collection kits could contain, but are not limited to:

    - Security bags or containers with appropriate labelling to ensure chain of custody compliance
    - Additional storage device. e.g. hair collection envelope
    - Submission paperwork with appropriate labelling/annotation to link paperwork and bagged sample(s), including linking of A and B samples if required.
    - Collection device – e.g. mane comb, scissors, concealed cutter
    - Clamps/bands to enable bunching of hair, maintain alignment and identification of the proximal end (e.g. cable tie, rubber bands)
    - Disposable gloves
    - Disinfectant wipes

Sample Preparation – General

28. Upon receipt at the laboratory hair samples should be treated in the same way as other samples and booked into the Laboratory Information Management System (LIMS) or equivalent and assigned a unique identifier. At this stage, the hair samples should be inspected to ensure that they are suitable for analysis.

29. The preparation of hair samples involves a number of steps, usually including washing, segmentation (dependent upon requirements), disruption (physical and/or chemical) and extraction.

30. Washing steps are included in order to minimise the potential for any finding to be as a result of external contamination of the hair sample from an environmental source. Washing also assists with the removal of substances which may interfere with the analysis, such as sweat, sebum and hair care products.

31. Hair, unlike urine and blood, cannot be aliquoted out by volume and it is therefore important to ensure that the portion tested is representative of the whole sample.

32. To ensure sample homogeneity hair samples should be prepared by either cutting into short (1-3 mm) sections or grinding to a fine powder prior to extraction. A weighed portion of the prepared hair is then selected for analysis.

33. As drugs are internally incorporated into the hair structure, physical disruption also has the benefit of improving drug recovery as it presents a far greater surface area for the extraction solvents to act upon.

34. Laboratories should agree with the relevant regulatory body on a standard approach to the length of sample analysed (not relevant for greyhound and camel coat hair). As equine mane (and tail) hair can vary quite considerably in length, a standardised approach is preferred, for example, all mane samples should be shortened to 10 cm length, or all samples are split into two segments of 7.5 cm length.

35. If additional information is required then shorter segments can be prepared, for example, 5 x 2 cm segments. However, the regulator should be made aware of the additional time and cost incurred with segmental analysis and also the requirement to supply a larger than usual volume of hair.

36. Equine mane hair is generally considered to grow at a rate of approximately 2 cm per month, but this can vary considerably among individual hair strands and between different breeds and according to the time of year. Additional variation to growth rates of individual hairs is also present as a result of each hair being at a different stage of the growth cycle, with some hairs actively growing (anagen) whilst others are in a dormant state (catagen and telogen).

37. Analysis of coat hair samples (e.g. from greyhound or camel) is usually performed on the full length of the sample, with no segmentation or shortening of the sample.

Screening analysis of hair samples

38. **NB** – Sample preparation, extraction and analysis will vary depending upon a number of factors and hence the following is generic guidance for the treatment of hair samples. Laboratories should develop and validate their own approaches for the analysis of hair samples within this framework and according to the requirements of the regulatory body and the drug coverage required.

39. A portion of the A-sample should be split from the main sample and removed from the security bag or container. The remaining A-sample is stored in the bag or container for future analysis if required.

40. The suitability of the sample for analysis should be assessed and any discrepancies noted and reported to the regulatory body.

41. Sample alignment is assessed and if necessary adjusted to ensure that all proximal ends (cut or pulled) are aligned. At this stage the sample length can be adjusted according to the requirements of the regulatory body and the sample cut into the required segments.

42. For screening analysis, a simple wash procedure to remove gross environmental contamination may be employed. Wash procedures should be developed based upon the analytical requirements but generally include the use of a detergent to remove lipophilic material. A suitable wash protocol for screening analysis could be rinsing with a weak solution (e.g. 0.1-0.5 %) of sodium dodecyl sulphate (SDS) followed by rinsing with laboratory grade water to remove excess detergent residues. Additional washes with organic solvents may be employed to facilitate drying. The wash solutions can be discarded at this stage unless there is a requirement for them to be analysed.

43. Following washing the sample should be completely dried prior to further processing. A suitable procedure would be to place the samples in a laboratory oven at 37°C overnight though other procedures may be employed.

44. The washed and dried sample should be physically disrupted by either cutting into short sections (1-3mm) or, ideally, ground to a fine powder using a suitable ball mill or similar mechanical device. A temperature-controlled unit can be used to avoid thermal degradation of heat-labile drugs. Care should be taken to avoid potential cross-contamination of samples at this stage.

45. A portion of the prepared hair sample should be weighed into an appropriate container ready for pre-treatment and extraction. The weight of sample required will depend upon the analytical requirements and should be assessed by the laboratory.

46. It is recommended that appropriate internal standards are added to the sample at this point by pipetting a small volume of spiking solution directly onto the hair sample.

47. Sample pre-treatment is often used to improve the recovery of internally incorporated drugs from the hair matrix. Appropriate pre-treatments may include, but are not limited to; incubation in buffer solution, hydrolysis with an acidic or basic solution and incubation in organic solvents. Care should be taken to ensure that the pre-treatment employed does not adversely affect the integrity of the targeted substances during sample processing.

48. After appropriate pre-treatment the sample is extracted using a technique such as solid phase extraction (SPE) or liquid liquid extraction (LLE). Multiple extraction steps may be employed if required to produce an aliquot suitable for instrumental analysis.

49. Samples should be extracted and analysed according to the laboratories normal procedures for sample analysis. It is recommended that the analysis includes extracted standards and blank samples in order to demonstrate the successful nature of the procedure.

50. If available, an appropriate incurred hair sample, known to contain a drug (or drugs) which is (are) representative of those included in the method is highly recommended as a quality control measure (QC sample). Such samples are generally in short supply and in many cases may not be available, but they provide compelling evidence as to the success of the analytical procedure. For example, the detection of testosterone (endogenous) in hair samples collected from colts (intact males) can be used to demonstrate the successful nature of a method for the analysis of anabolic steroids and associated compounds.

51. Samples should be analysed using appropriate instrumental methods, developed by the laboratory to be fit for purpose.

Confirmatory analysis of hair samples

52. **NB** – Laboratories should employ the same analytical approach for confirmatory analysis of hair as they would for routine samples such as urine and plasma/serum.

53. In general, the analytical approach to confirmatory analysis of hair samples may be the same as that employed for screening but with additional measures put in place to meet confirmatory requirements

54. Samples are subjected to a wash procedure that has been designed to minimise the potential for a positive finding to arise as a result of external contamination. The procedure can be tailored to suit the drug(s) of interest but designed in such a way as to minimise extraction of the internally incorporated drug(s) from the hair matrix whilst maximising removal of externally deposited contamination.

55. In general, confirmatory wash protocols may be similar to those employed during screening analysis. In some cases the use of additional sequential washes with organic solvents to remove lipophilic material is appropriate. The use of water and other protic solvents is possible but should be approached with caution as they can swell the hair and begin to extract internally incorporated drugs. Laboratories should develop and employ wash protocols that are appropriate for the drug(s) being confirmed.

56. Confirmatory hair samples should be treated in the same way as more common matrices such as urine and plasma/serum and extracted and analysed alongside appropriate control samples. These samples typically include, but are not limited to; an extracted matrix blank (biological blank), an extracted non-matrix blank (system blank) and appropriate spiked matrix samples. These samples should all be prepared, extracted and analysed in the same way as the confirmatory hair sample.

57. The extracted non-matrix blank (system blank) may be composed of a portion of all of the solvents used during the preparation of the confirmatory hair sample, with the addition of portions of washes from scissors or any other tools used during sample preparation.

58. If available, the inclusion of a known incurred sample containing either the drug(s) being confirmed or a drug that is considered to be representative of that being confirmed is highly recommended. The successful detection of drug(s) in this sample demonstrates the suitability of the preparation, extraction and analysis protocol.

59. Samples should be analysed using appropriate instrumental methods, developed and validated by the laboratory to meet the requirements.

60. In certain cases it is appropriate to retain the final wash solution and extract and analyse it alongside the confirmatory hair sample. If required, the results from the final wash solution and the confirmatory hair sample can be compared using an appropriate wash criteria, such as that proposed by Tsanaclis *et al* for the positive identification of the ingestion of drugs in human hair [4]. Laboratories should determine and apply their own wash criteria.

Quality assurance and quality control

61. The implementation of a thorough and rigorous quality control system is a fundamental principle for all testing laboratories and accreditation to the International Standard ISO/IEC 17025 is a requirement of most regulatory bodies.

62. The ILAC G7:04/2021 document [5] provides guidance to equine sport testing laboratories on the implementation of a suitable quality system and guidance on screening and confirmatory procedures. However, currently it does not include any information specific to the analysis of hair samples.

63. It is recognised that hair is a new matrix for many animal sport testing laboratories and in most cases its analysis will not have been previously assessed by the local ISO/IEC accrediting organisation. As such, following discussion with laboratories around the world, there has been a mixed response and varied requirements in terms of validation and accreditation of methods from the accreditation bodies. Laboratories should therefore develop and validate confirmatory approaches for the detection of drugs in animal hair that meet the requirements of their local accreditation body.

64. A significant challenge to the validation and subsequent accreditation of animal hair analysis is the limited availability of appropriate incurred material for use as quality control samples in order to demonstrate the suitability of the method. Laboratories should endeavour to obtain suitable incurred material wherever possible (e.g. past-positives and post-administration samples) for use as internal quality control samples.

65. An inherent part of an on-going laboratory quality assurance program is regular participation in proficiency testing (PT) schemes. Whilst no commercial equine hair PT schemes are currently available, the annual AORC PT test set includes the option to receive a hair PT sample. This sample is a pseudo-incurred sample, prepared by prolonged soaking with solutions of the drug(s) followed by extensive washing to remove externally deposited drug whilst retaining internally incorporated drug. As such it presents a suitable alternative to a genuine incurred sample and is considered a valuable test of a laboratory's hair testing procedures.

66. In addition to participation in externally provided PT schemes (as and when available) it is recommended that laboratories implement their own in-house internal quality control processes, such as the use of double- and single-blind QC samples, submitted to the screening process on a regular basis.

References

1) https://www.ifhaonline.org/resources/ifAgreement.pdfAccessed 4th January 2022.

2) Henderson, G.L. Mechanisms of drug incorporation into hair. *Forensic Science International.* (**1993**), 63, 1-3, p. 19-29.

3) Leela, J., Zahra, P., Vine, J.H. and Whittem, T. Determination of testosterone esters in the hair of male dogs using liquid chromatography-high resolution mass spectrometry. *Drug Testing and Analysis* (**2018**), 10(3), p. 460-473.

4) Tsanaclis, L., Nutt, J., Bagley, K., Bevan, S. and Wicks, J. Differentiation between consumption and external contamination when testing for cocaine and cannabis in hair samples. *Drug Testing and Analysis* (**2014**), 6(1), p. 37-41.

5) https://ilac.org/latest_ilac_news/revised-ilac-g7-published/ Accessed 4th January 2022.