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EXPERT OPINION

The challenges in hair analysis from the perspective of an analytical chemist

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Introduction

Head hair has nowadays become an attractive and commonly used biosample. Still, it is considered as non-conventional or alternative biological matrix, used to implement the information coming from other (so called conventional) bioanalytical samples, such as blood serum/plasma and urine, which only reflect exposure for a short period upon administration. Hair sampling is a non-invasive sampling procedure compared with bioanalytical methods for common biosamples.

However hair has also certain difficulties in handing, sample preparation and data interpretation. These are briefly discussed herein.

Hair as an alternative biosample

Testing for drugs of abuse as well as for pharmaceutical compounds in hair is a unique information source in toxicology, forensic science, as well as workplace drug exposure. It is well known that head hair can provide an historical record additionally with short term information on recent use, since it grows at an average rate of 1 cm per month and preserves the drug use history during the past period of growth. Once incorporated into hair, the drugs are considered to be stable for at least one year. Thus hair is an attractive non-conventional or alternative biological matrix used to implement the information coming from common bioanalytical samples, such as blood serum/plasma and urine, which only reflect exposure for a short period upon administration.

Hair provides not only a longer detection window compared to conventional biofluids, but the possibility of drug retrospective analysis as well. It offers the advantages of possibility to obtain new subsamples, which can not be easily adulterated and are usually available in post mortem cases of high decomposition conditions. Moreover hair sampling is non- invasive compared to needle sampling or tissue biopsy.

The concentration of the drugs in hair depends on the dosage of drug (obviously, the higher the concentration, the longer the detection time; on the distance from the root, on the polarity of drug, on the phase of hair growth-whether anagen or telogen one, the eventual growth rate that is affected by therapeutic drugs age, gender, race and depends even on seasonal fluctuations.

Disadvantages in hair analysis, which have to be considered for the correct interpretation of the results, include environmental contamination, variations with regards to hair color, cosmetic treatment etc. as well as sample preparation approaches. These will be briefly discussed in the following sections [1-3].

Analytical concerns

The main challenges in hair analysis involve the ability to determine (qualitatively and quantitatively) drugs and metabolites in hair that arise from ingestion, taking care that any other derived from passive exposure or exogenous application are excluded.

An issue that may arise is due to the cosmetic treatment

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of an individual, which can cause deterioration or even distortion of the findings. Therefore cosmetic history of a person must be taken into account, while interpreting hair analysis results. In this regard analytical chemists have to deal with a series of concerns, when developing a bionalytical method for hair samples.

More specifically, analytical chemists deal with a great variety of matrices. These must be treated in some way before they can be introduced in the analytical instrument.

Complexity of samples varies depending on matrix composition and nature. Endogenous interferences are not the only issues that need attention by bioanalysts. The state of sample, whether liquid, gaseous or solid, determines to a great extend the choice of the sample preparation technique. As it is well known any sample preparation technique must be fast, simple, precise and accurate and most of all representative for the sample composition. The analytes must be intact and the measurement must reflect the real image of the content.

All methods when developed are validated in that concern. However, when dealing with solid samples, this procedure has many traps that may lead to false positive or false negative results.

In contrast to liquid samples for which protein precipitation and extraction/isolation of analytes are routinely adequate procedures, tissue preparation techniques may are more complicated including mechanical treatment, digestion and extraction based approaches. Not to ignore the fact that liquids can be easily homogenized, while solid samples cannot.

Mechanical techniques are necessary to disrupt cellular structure so that analytes can be extracted from low-water-content tissues such as hair. Digestion may be necessary to release analytes that are bound to proteins or other groups. Acidic digestion for instance, with strong acid (e.g., 12 M hydrochloric acid) may be applied as routine procedure, or alkaline digestion using NaOH. Enzymatic digestion can also be applied using a variety of enzymes with different digestive properties and efficiencies.

Extraction of target analytes is following using several traditional or modern sample preparation approaches. These include Accelerated Solvent Extraction, Microwave Assisted Solvent Extraction, ultrasound assisted extraction, Headspace Solid Phase Microextraction, Stir Bar Sorptive Extraction, Matrix Solid Phase Dispersion, Supercritical Fluid Extraction.

The lack of reference material for most analytes in appropriate or comparable matrices adds another problem to be answered, together with the heterogeneity of the analyte within the tissue matrix.

Although internal standards could be used, they are generally added as a solution to the homogenate, digest or extract, that is an intermediate stage rather than from the beginning. Unfortunately, till now no method is able to disperse the internal standard into the tissue matrix by physical means so that extraction efficiency of an internal standard in the matrix can be accurately determined. Similarly spiking samples with known concentrations in order to estimate recovery rates cannot reflect true behaviour of matrix effect.

Several other parameters such as hair matrix type, the chemical structure of the target analyte, are the crucial features that affect the final extraction yield in any isolation.

To this direction, The Society of Hair Testing (SoHT), that was founded in 1995, has been doing excellent work aiming to define, update and harmonize provisions and procedures related to hair analysis [4,5].

Sampling

Hair can be collected using non invasive techniques following certain instructions. The sample should be cut from the posterior vertex region of the head, as close as possible to the scalp. The reason is that this area is of least variation in growth rate.

If this procedure is not feasible, then the source of the sampling should be described in detail.

Sufficient quantity at least 50 mg should be taken in order to allow initial screening, confirmatory analysis, as well as re-analysis, if necessary.

In order to prevent degradation, loss of analyte, or contamination from other sources, the sample and any aliquots or extracts must be handled and stored with certain precautions. Dry hair should be stored in the dark at room temperature.

Documents for the Chain-of-custody describing the collection procedure, the handling and storage of the hair specimen from the time the donor gives it to the collector until it is destroyed should be intensively kept [4-6].

Washing

External contamination can cause confusion with actual drug use, as well as laboratory contamination.

Decontamination should be performed prior to sample preparation using detergents or organic solvents like methylene chloride or acetone to remove lipophilic contaminants followed by aqueous solvents or methanol. However the use of the latter should be minimized or avoided in prolonged time period as it can extract analytes and thus cause analytes' loss. The number of clean up steps depends on the extent of contamination.

In some cases e.g. in autopsy, additional pre-treatment of

the hair in the laboratory may be necessary. After washing, the hair segment is dried, and then pulverized or cut into small pieces. The washings should be stored at 4°C for later analysis. More specifically hair washings must be also analysed along with the extracted part to evaluate the impact of environmental exposure. It has been proposed that the ratio washing/hair extract can be used. In case this ratio is less than 0.1, it suggests a drug abuse situation, whereas a higher ratio than 0.1, but less than 0.5, suggests a possible drug abuse and when it is higher than 0.5, then external contamination is the main source [6,7].

Extraction

There are two options when a solid sample is handled prior to its analysis: either the entire sample is of interest and must be dissolved or only a part of the solids is of interest and the analytes must be selectively isolated. If the sample matrix is insoluble in commonly used solvents, the analytes of interest can be removed or leached out. Hair belongs to the latter case, therefore techniques such as filtration, soxhlet extraction, supercritical fluid extraction, ultrasonication or solid-liquid extraction can be applied.

Hair consists of a special matrix. Its composition is proteins (65–95%, mainly keratin), water (15–35%), lipids (1–9%) and minerals (up to 1%). Thus the drugs can be enclosed tightly in the hair shaft and to a certain extent maybe bound to proteins, melanin or lipids. The latter play a crucial role in the drug binding and pharmacokinetics. The binding to lipids is influenced by hydrophobicity of the target compounds.

There are several factors that influence the final extraction yield, such as the hair matrix type, structure of the drug, method and duration of extraction, and solvent [4,5,8].

Method validation

Sample collection and leaching of analytes by digestion or removal of drugs from hair matrix are causing serious troubles to the analytical chemists, but these are not the only ones, when developing a method for hair analysis. Validation issues especially with regards to recovery are very important.

First results can be obtained by screening techniques like Immunoassay (ELISA). But positive samples need to be confirmed with a confirmatory validated method, such as Gas chromatography-Mass Spectrometry (GC-MS) or Liquid chromatography-Mass Spectrometry LC-MS.

Due to the fact that limited certified reference materials are available, participation of hair testing laboratories in proficiency testing programs is often highly recommended at least twice a year.

Internal Quality Control for hair is more difficult compared to other biosamples, since spiked control samples cannot substitute for the actual hair of the drug user.

The Society of Hair Testing (SoHT) has published internationally accepted guidelines providing step-by-step guidance for hair analysis, regarding sample collection, storage, preparation, pre-treatment, analysis, and the use of cut-offs. It recommends that the confirmation technique should include the detection of a parent drug and metabolite(s) so that the presence of drugs due to passive exposure can be differentiated from those by ingestion. It is also recommended by SoHT that segmental analysis should be carried out when the analytes are endogenously present such as cortisol for example. In this case, controls may be prepared using an alternative medium, for example, synthetic melanin.

Besides SoHT, the "Substance Abuse and Mental Health Services Administration (SAMHSA)", have also published guidelines for hair analysis and recommendation according confirmatory cutoffs to distinguish positive from negative samples [4,5,9].

Quality controls and validation criteria

One technique is to expose drug-free hair to aqueous solutions of drugs at high concentrations, for several days and then thoroughly wash the hair before drying and analysis. These spiked samples can be used for precision studies, as routine QCs, and as internal degradation controls, using various hair types.

One low and one high concentration control must be measured at the beginning and at the end of each batch of samples, which should include at least 5% controls.

The low level control concentration is suggested to be close to the confirmation cut-off concentration, and not greater than twice the confirmation cut-off level.

For self-made spiked controls, the upper and lower limits should be specified according to acceptable statistical criteria. Additionally, the uncertainty of measurement should be calculated.

The following acceptance criteria are recommended:

- Bias <20%.
- For intra-assay and inter-assay imprecision <20-30%.
- The number of false negative samples should not exceed 2%.
- For Selectivity: measurement of 6 blank samples and 2 zero samples (blanks containing the internal standard)
- For Linearity at least five replicate measurement at five different, if possible equidistant spiked concen-

trations
- Verification of linearity with an adequate statistical test in order to eliminate outliers.
- The lower limit of quantification (LLOQ) must be lower than the given cut-off [4,5,9,10].

Examples of Specific Drug Classes

The determination of certain drug classes such as antipsychotics, drugs of abuse, antidepressants etc is of paramount importance in forensics. Not only the initial drugs but their metabolites are of interest in hair analysis. Obviously each class may present its own particular concerns. For example possible ingestion of legal drugs may produce positive results for methamphetamine and amphetamine.

In the case of ethanol the direct determination is not feasible due to its volatility. Therefore the minor ethanol metabolites ethyl glucuronide (EtG) and/or fatty acid ethyl esters (FAEE) such as ethyl myristate, ethyl palmitate, ethyl oleate and ethyl stearate should be measured as a direct alcohol consumption marker.

The identification of the specific metabolites is recommended in certain cases:
- For the confirmation of cocaine consumption, benzoylecgonine and cocaethylene should be identified.
- For the confirmation of heroin consumption 6-acetylmorphine and morphine are suggested as the metabolites to be identified.
- Carboxy-THC should be identified for the confirmation of cannabis consumption;
- MDA is suggested to be identified for the confirmation of ecstasy (MDMA) consumption.

Moreover the use of metabolite-to-parent drug ratios is also recommended in some cases. For example in the case of cocaine, the ratio benzoylecgonine/cocaine should be ›0.05, since benzoylecgonine is not always present, hydrolysis controls should be used. For heroin, the ratio 6-acetylmorphine/morphine should be greater than 1.3 (after correction for hydrolysis). However one should keep in mind that metabolites are usually more polar and as such have less affinity to hair matrix.

False negative results however should be checked using complementary analysis results of blood or urine [11-14].

Conclusion

Forensic testing for drugs of abuse in hair has been introduced as a useful tool in order to determine recent past drug use and moreover for the examination of long-term drug history by means of segmental analysis. Hair can be collected using non invasive techniques and is considered as a complementary sample in documenting human exposure to drugs in the fields of clinical and forensic toxicology and workplace drug testing makes the accurate analysis being of paramount importance in the bioanalytical field.

However its analysis addresses several concerns for the analytical chemists and this is why it is often not possible to correlate the concentration of drugs found in hair with consumption pattern.

Challenges in order to provide accurate, precise and representative results are resolved by various approaches, so that this matrix is increasingly used in bioanalytical routine.

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