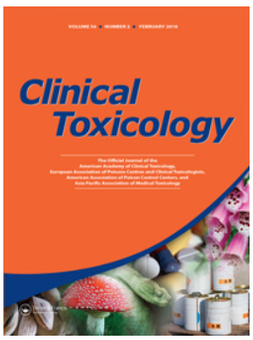
The interpretation of hair analysis for drugs and drug metabolites



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REVIEW

The interpretation of hair analysis for drugs and drug metabolites

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ABSTRACT

Introduction: Head hair analysis for drugs and drug metabolites has been used widely with the aim of detecting exposure in the weeks or months prior to sample collection. However, inappropriate interpretation of results has likely led to serious miscarriages of justice, especially in child custody cases.

Objective: The aim of this review is to assess critically what can, and perhaps more importantly, what cannot be claimed as regards the interpretation of hair test results in a given set of circumstances in order to inform future testing.

Methods: We searched the PubMed database for papers published 2010–2016 using the terms "hair" and "drug" and "decontamination", the terms "hair" and "drug" and "contamination", the terms "hair" and "drug-facilitated crime", the terms "hair" and "ethyl glucuronide", and the terms "hair", "drug testing" and "analysis". Study of the reference lists of the 46 relevant papers identified 25 further relevant citations, giving a total of 71 citations.

Hair samples: Drugs, drug metabolites and/or decomposition products may arise not only from deliberate drug administration, but also via deposition from a contaminated atmosphere if drug(s) have been smoked or otherwise vaporized in a confined area, transfer from contaminated surfaces via food/fingers, etc., and transfer from sweat and other secretions after a single large exposure, which could include anesthesia. Excretion in sweat of endogenous analytes such as γ-hydroxybutyric acid is a potential confounder if its use is to be investigated. Cosmetic procedures such as bleaching or heat treatment of hair may remove analytes prior to sample collection. Hair color and texture, the area of the head the sample is taken from, the growth rate of individual hairs, and how the sample has been stored, may also affect the interpretation of results.

Toxicological analysis: Immunoassay results alone do not provide reliable evidence on which to base judicial decisions. Gas or liquid chromatography with mass spectrometric detection (GC- or LC-MS), if used with due caution, can give accurate analyte identification and high sensitivity, but many problems remain. Firstly, it is not possible to prepare assay calibrators or quality control material except by soaking "blank" hair in solutions of appropriate analytes, drying, and then subjecting the dried material to an analysis. The fact that solvents can be used to add analytes to hair points to the fact that analytes can arrive not only *on*, but also *in* hair from exogenous sources. A range of solvent-washing procedures have been advocated to "decontaminate" hair by removing adsorbed analytes, but these carry the risk of transporting adsorbed analytes into the medulla of the hair therefore confounding the whole procedure. This is especially true if segmental analysis is being undertaken in order to provide a "time course" of drug exposure.

Proposed clinical applications of hair analysis: There have been a number of reports where drugs seemingly administered during the perpetration of a crime have been detected in head hair. However, detailed evaluation of these reports is difficult without full understanding of the possible effects of any "decontamination" procedures used and of other variables such as hair color or cosmetic hair treatment. Similarly, in child custody cases and where the aim is to demonstrate abstinence from drug or alcohol use, the issues of possible exogenous sources of analyte, and of the large variations in analyte concentrations reported in known users, continue to confound the interpretation of results in individual cases.

Conclusions: Interpretation of results of head hair analysis must take into account all the available circumstantial and other evidence especially as regards the methodology employed and the possibility of surface contamination of the hair prior to collection.

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Introduction

In the late 1950s advances in analytical chemistry facilitated arsenic analysis on strands of head hair said to be taken from Napoleon I after his death on St Helena [1], initiating a long-running controversy as to what these results actually meant. It is now clear that the results were those to be expected in someone living in the late eighteenth to early nineteenth century since hair analysis *per se* provides no evidence of the source of the analyte, of dose, let alone of biological effect. Inorganic arsenic species were used widely as

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rodenticides, insecticides, food and (wall)paper dyes, and also as medicines in Napoleon's time. Indeed, subsequent analyses have found arsenic in hair sampled when he was a child, when on Elba, in hair from Empress Josephine, and from his son Napoleon II, King of Rome [2]. But even this latter, thoughtful, survey did not mention the widespread use of arsenic trioxide as a specimen preservative, thought by many to be the most likely origin of the arsenic measured [3].

These same issues of source (when and how the analyte got into the sample), of dose (of compound giving rise to the analyte, assuming systemic exposure), and of biological effect have bedeviled, and continue to bedevil, use of head hair (and indeed of other keratinaceous samples) in order to attempt to give a historical record of drug or alcohol (self-) administration in individuals. In addition, there are issues of preparation of assay calibration, internal quality control (IQC), and external quality assurance (EQA) material, of sample "decontamination" (i.e. attempted removal of analyte from the hair surface), analyte stability, and laboratory accreditation.

Moeller et al. [4], whilst claiming that "Because hair analysis can be used for the determination of drug use months after drug consumption, hair analysis data can often act as important and even decisive evidence in the Courtroom" cautioned that "more research is needed for more detailed interpretations, especially those concerning sectional analysis or the possibility of detecting the low concentrations perhaps stemming from passive contamination".

There have been hundreds of papers written advocating use of head hair analysis for monitoring drug and alcohol exposure since that time, and evidence has been given in thousands of legal cases worldwide. This despite clear cautions that passive (incidental) systemic exposure of adults and of children to cocaine, for example, is readily detectable on analysis of head hair [5–8]. Perhaps more importantly, the validity of so-called "decontamination" procedures, washing procedures used in laboratories ostensibly to remove analytes adsorbed onto the hair surface, have long been questioned [9]. Recent work has shown that such procedures may also serve to move analyte into the hair matrix, in effect confounding claims that segmental analysis gives an accurate record of a person's drug use over time [10]. As an example, studies have shown that synthetic cannabinoids, and indeed their metabolites, can be detected in hair segments seemingly dating from before the compounds in question were available on the drug market [11–13].

Unfortunately, it has become clear that data in child protection cases involving hair analysis for markers of illicit drug and alcohol misuse, respectively, has either not always been presented in a way that enabled the Courts to give proper weight to the evidence, or has been erroneous, with incalculable consequences for the families involved [14–17].

Hair testing is expensive, is usually undertaken for forensic purposes, and interpretation of the results is often left to those who have provided the testing, who may not have been provided with full information as to the circumstances of a particular case. Another issue is the possible effect of publication bias, i.e. papers claiming successes for hair analysis are more likely to be published than examples where hair analysis provided either no useful information, or indeed contradictory information.

Clearly, the attraction of head hair analysis is the potential to obtain a record of exposure to an exogenous compound with time, be it drug, alcohol, pesticide, growth promoter, toxic metal ion, or other analyte when the compound of interest is likely to have disappeared from conventional samples such as blood, oral fluid, and urine. Use of axillary or pubic hair has been viewed less favorably than use of head hair because of presumed much more irregular growth and the fact that hair from such sites is often shorter than hair from the crown of the head. Be this as it may, in situations where simple detection of a xenobiotic is sufficient for evidential purposes, hair analysis has proved invaluable. The detection of steroid residues in hair is a powerful tool to demonstrate administration of prohibited growth promoters in meat production animals, for example. Analysis of the ester form of administered steroids is an unambiguous approach to prove the illegal use of natural hormones [18].

But even here there may be issues as to the limit of detection, which the courts interpret as the limit of reliable quantification, and possible cross-contamination from other animals in stock pens, for example. A further consideration is that it is not possible to estimate dose (i.e., the magnitude and duration of exposure) because of the absence of comparative data, not only data on dose of administered drug, duration of administration, and hair analyte concentration, but also on the analyte in hair from different anatomical sites [19], and on the influence of age, sex, diet, hair color, exposure to UV light, etc [20,21].

In human forensic toxicology, as with the detection of illicit drug administration to animals, the simple fact of detection of a drug or a drug metabolite in hair may be strong evidence. This is true especially if segmental analysis of head hair has demonstrated the presence of analyte in a segment that corroborates a complainant's statement, e.g., with no analyte present in hair corresponding to periods before and after the alleged crime and other possible sources of exposure have been excluded [22].

However, all the caveats noted above in connection with use of hair analysis in meat production animals still apply. In addition, there are issues such as differences in the growth rate of individual hairs, in head hair growth rate depending on the site of hair collection, ethnicity, and inter-individual variability in drug/metabolite incorporation, and indeed of sample availability, women tending to have longer hair than men, who may be bald [20,21]. Moreover, there is always the possibility of self-administration of drug, or of incidental exposure, in the workplace for example [23]. Traces of drugs may arise during preparation of a substance for smoking, or as a result of the smoking process itself, and may be present on surfaces such as carpets, tables, etc.

Clearly potential routes of exposure of individuals living in such an environment include inhalation of airborne particles, ingestion of particles picked up inadvertently from surfaces, absorption of drug picked up inadvertently from surfaces though the skin via sweat, and as regards hair, either direct deposition on hair, or absorption into hair via sweat [6,24].

Hair damaged as a result of cosmetic treatment may be at increased risk of environmental contamination [25].

An attempt may be made to differentiate incidental exposure from drug use via the use of "cut-offs", but these too are guidelines based on anecdote rather than evidence for the reasons listed above. There are also issues of the possible effects of cosmetic hair treatments such as perming and bleaching, differential binding of drugs and/or metabolites in hair of different composition or colour, and differences in drug and metabolite concentrations (and differences in the ratio of parent drug: metabolite concentration), in hair from different anatomical sites [19]. Indeed, the possibility of drug metabolism occurring either during transfer of drug into, or within, growing hair has been raised [26]. All-in-all, quantitative measurements are of extremely limited value in assessing the magnitude and duration of drug exposure.

In the case of drugs such as γ-hydroxybutyrate (and its precursor γ-butyrolactone) that occur naturally in the body there is also the problem of differentiating endogenous production from exogenous administration in the absence of evidence-based control data [27,28]. Similar considerations apply to attempting to define "cut-off" concentrations for markers such as ethyl glucuronide, ethyl sulfate, and fatty acid ethyl esters that are claimed to indicate ethanol ingestion.

Objective

The aim of this review is to assess critically what can, and perhaps more importantly, what cannot be claimed as regards the interpretation of hair test results in a given set of circumstances in order to inform future testing.

Methods

We searched the PubMed database for papers published 2010–2016 using the terms "hair" and "drug" and "decontamination" (36 citations were found, 10 of which were cited in the Introduction and seven were discarded as they referred to decontamination of exposed individuals, exposure in animals, etc.). A further search using the terms "hair" and "drug" and "contamination" (92 citations) yielded 6 further unique relevant citations, a search using the terms "hair" and "drug-facilitated crime" (13 citations) gave 5 additional citations, a search using the terms "hair", and "ethyl glucuronide" gave 9 further citations out of 111 scanned, and finally a search using the terms "hair", "drug testing" and "analysis" gave 7 further citations (out of 190 scanned). Study of the reference lists of the papers identified 25 further relevant citations published before 2010. These searches gave a total of 71 citations, which were selected primarily as regards their relevance to the clinical interpretation of results.

Hair samples

Segmentation

In cutting a hair sample into segments prior to analysis, it is assumed that head hair on average grows at a rate of approximately 1 cm per month, that analyte present in each segment gives a record of drug or alcohol use during the time represented by the segment, that the hair has been aligned perfectly in the hair tuft sent for analysis, and that the hair sample has been stored dry since collection.

However, different individuals may have different rates of hair growth, individual hairs grow at different rates, i.e., the time resolution of a hair segment in a hair tuft decreases with increasing distance from the root, hence it is possible that systemic exposure to drugs or alcohol at times other than that indicated by the segment analyzed could influence the result, diffusion of analyte via sweat or sebum into the hair may have occurred at any time prior to sample collection, cosmetic procedures or exposure to UV light, for example, may have caused loss of analyte, and analyte may have been transferred from the surface of the hair into the matrix of the hair by the "decontamination" procedure used [10].

Decontamination

Washing a hair specimen after collection ("decontamination") is advocated to remove residues of hair care products (wax, shampoo, hair spray) as well as sweat, sebum, and dust, and analytes that may have been deposited on the surface of the hair prior to collection ("environmental contamination") before the actual analysis. Pragst and Balikova [29] recommended segmentation prior to "decontamination", and measurement of analyte(s) in an initial wash solvent to give a measurement of the degree of external contamination of the sample in the event of positive findings. Of course, this leads to much extra analytical work, including validation of the procedure(s) used to quantify analytes in the wash solvent used, and risks altering the results by either enhancing, or reducing the quantities of analyte measured in the actual analysis of the "decontaminated" hair.

Although it is considered mandatory to include a "decontamination" step, there is no consensus on the actual procedure to be followed [30]. Washing with methanol [31], dichloromethane [32–34], dichloromethane and water and perhaps also methanol [35–37], 2-propanol [38], 2-propanol and phosphate buffer [39], ethanol and phosphate buffer [40,41], and room temperature ionic liquids [42] are just some examples of the procedures advocated. Today, the general recommendation of the Society of Hair Testing is a decontamination strategy that includes an initial organic solvent wash to remove oils, followed by aqueous washes [43]. However, even using the most comprehensive and complex decontamination procedures, measureable concentrations of cocaine may still be detected in wash solutions [5,9,44].

The reason for these observations remained unclear until recently, when it was shown that the washing procedures employed may also serve to move surface contaminants into the hair matrix [10,45]. Measurement of analyte concentration in the wash(es) and comparison with the results obtained after digestion of the hair matrix [46,47] is only a partial solution to this problem. Indeed, the conclusion of recent

attempts to discriminate between external contamination and systemic exposure is that there is no way of differentiating unequivocally between these alternatives. Even if systemic exposure is suggested by the presence of metabolites, there remains the possibility of inhalation of particles or absorption via sweat from surfaces after a substance has been smoked, for example. Furthermore, although the effects of laboratory hair-washing procedures on the distribution of (incorporated) drugs in hair remain unknown, decontamination procedures prior to hair analysis are considered to be indispensable in order to exclude external contamination as much as possible [48].

However, insights into the effect of these decontamination protocols on the concentrations and distribution of drugs incorporated in hair are essential in order to be able to draw correct forensic conclusions. Indeed, it has been suggested that use of washing solvents likely lead to swelling of the hair and thus promote the incorporation of analyte on the hair surface into the hair itself [49]. Not only are the implications of these considerations for case work clear, but also the validity of much published work where hair analysis has been claimed to give a record of systemic exposure to drugs at some point prior to sample collection must now also be questioned.

Toxicological analysis

Traditionally analytes in hair are detected and measured after pre-treatment ("decontamination") using some sort of extraction/digestion procedure, usually after cutting into 1–3 mm lengths, followed in some cases by mechanical action such as grinding or pulverizing. A wide range of extraction procedures have been advocated including extraction with methanol, aqueous acids, or buffer solutions, treatment with urea and thioglycolate, supercritical fluid extraction, enzymic digestion, digestion with aqueous sodium hydroxide, and "micropulverized extraction" in which hair is simultaneously ground and extracted in a ball mill [29,30,50].

Except perhaps after methanol and supercritical fluid extraction extraction, or use of methanol:acetonitrile/2 mmol/L aqueous ammonium formate (25 + 25 + 50, v/v/v) as extraction solvent [51–54], some form of extract purification procedure is usually used after the initial extraction prior to gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) analysis. Even so all the caveats associated with LC-MS, notably the risks of ion suppression and/or ion enhancement, still apply. Validation of the myriad of procedures used for all types of hair samples and for all the analytes that may be encountered is clearly a daunting task.

In an attempt to reduce the impact of surface contamination on the results and minimise the amount of hair used in the analysis, new analytical methods have been evaluated. Matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) has been used to detect cocaine and its metabolites after pulverizing and extracting hair [55–57]. More recently, mass spectrometric imaging (MSI) techniques have been used, for example in the analysis of ketamine in a single scalp hair [58]. Porta et al. [59] reported the analysis of cocaine and its metabolites in a single hair using MALDI-triple quadrupole linear ion trap MS. By longitudinal scanning of a single intact hair, information concerning drug disposition throughout the hair was obtained. The measurement of cocaine and cannabinoids in single intact hairs using a MALDI-LTQ Orbitrap XL instrument [60] has also been reported, and Poetzsch et al. [61] monitored the distribution of tilidine in intact hair samples from children thought to have been poisoned with this drug by MALDI-MS/MS imaging.

Despite these analytical advances, sensitivity is poor and the surface contamination and incidental systemic exposure issues remain. Moreover, a decrease in signal intensity may arise due to the application of the MALDI-matrix, for example when poor matrix crystallization has occurred. Severe ion suppression might even lead to analytes being missed completely [62]. Note that there are no uniform guidelines for studying matrix effects in MALDI-MS [57]. In addition, the fact that unintentional, non-malicious ingestion of drug can occur may still confound the interpretation of results.

One issue in using MALDI-MS of intact hair strands is the extraction efficiency of the procedure. As drugs are thought to be trapped inside the matrix (core) of the hair as the hair is formed in the hair follicle, it is difficult to know if the drug is completely extracted out of the hair by the MALDI procedure and if the detected drug originates from either surface contamination, or from within the hair itself. Duvivier et al. [63] used direct analysis in real time (DART) ambient ionization orbitrap MS to detect tetrahydrocannabinol in an entire lock of hair without prior decontamination. Hairs were attached to stainless steel mesh screens and immediately scanned. However, hairs were scanned before and after washing with dichloromethane in an attempt to remove surface contamination hence the possibility of interference from this source remained.

Assay calibration and quality assurance

In hair analysis, three alternative modes of producing specimens for quality control (QC) purposes have been used: adding known amounts of analyte in solution to a known amount of intact analyte-free digested hair ("spiking hair") and allowing the solvent to evaporate; soaking analyte-free hair in a solution of analyte to allow analyte incorporation into the hair matrix (referred to as "fortifying" and confusingly also as "spiking" hair); and homogenization of "decontaminated" authentic hair specimens containing the analyte(s) of interest.

All of these approaches have their problems. The first procedure does not model incorporation of analyte into hair *in vivo* and although extraction recovery and accuracy measurements can be performed, material prepared in this way cannot accurately reflect sample analyses. The analyte-free hair is always washed before being exposed to the analyte(s), whereas case specimens are often washed ("decontaminated") after the hair has been collected, but before segmentation/digestion and analysis of the hair

digest. Therefore, a different concentration in the case specimen may be obtained as a result of a change in concentration caused by the washing procedure. There is also the issue that the analyte-free hair may not bind or incorporate analytes in the same way as the case specimen.

In the second procedure the analyte is typically incubated with the washed hair for several days, but the problem of the effect of "decontamination" remains and indeed this approach of itself demonstrates that analyte may enter hair by means other than being incorporated into the hair in the hair follicle.

In the third approach, that is use of an authentic analyte-containing hair sample, there are issues of the quantity of sample available, and of course it is not possible to know the absolute concentration of analyte in the hair specimen given that the effect of "decontamination" may vary depending on the procedure used, and there may be different analyte concentrations in different portions of the same hair sample.

In the case of ethyl glucuronide, for example, although quality control samples prepared using the first method gave better within- and between-assay precision, the values obtained for quality control samples prepared using fortified or authentic hair specimens are likely to be more representative of the results from case specimens [64].

"Cut-off" values

As noted above, "cut-off" values have been proposed in an attempt to differentiate drug (self-) administration from incidental exposure. For cocaine, a cut-off value of 1 ng/mg (1 part cocaine per million parts of hair) has been suggested [65]. However, the use of "cut-off" values is unreliable because external contamination can occur at any concentration [43]. Another proposed method is the detection of metabolites of a drug. However, metabolites can occur through incidental ingestion of parent drug via contaminated surfaces, for example, as well as by deliberate (self-)administration. Some metabolites are also decomposition products of drugs, as exemplified by benzoylecgonine (from cocaine).

The use of the expression "cut-off" comes from the use of immunoassays where the only actual result is a signal given a numeric value. The "cut-off" as then applied was simply a pragmatic attempt to limit the number of false positive signals, whilst not missing true positive results. Immunoassays, whilst simple to perform, are of course prone to interference, which can cause false negatives as well as false positives. Immunoassay results alone should never be used to report any toxicology results without corroboration ("confirmation", that is independently and definitively corroborated using a "physical" analytical method such as mass spectrometry), although unfortunately in the case of hair analyses this has not always been done [17].

Nowadays, most laboratories go straight to either GC-MS or LC-MS, "confirmation" methodology. Of course, all these methods have limits of detection, which are usually lower than the lowest limit of quantitation. Setting an actual limit of detection is somewhat arbitrary, a commonly used guideline in chromatography being 3 × the signal-to-noise ratio of the background. Things are not so simple with MS, since qualitative as well as quantitative information is at hand, and sensitivity varies depending on the competence of the operator, the cleanliness of the source, the amount of sample available, the reproducibility of the method, etc. Moreover, the Courts will always ask "how much is present?" as well as "what is it?"

The only guidance that is not open to misinterpretation is that concentrations below the lowest limit of quantitation should be reported as "not detected". Method validation guidelines and laboratory accreditation procedures focus on lowest limit of quantitation and not on the limit of detection, in part because the limit of detection can vary as explained above. As analytical journals also only require method validation down to the lowest limit of quantitation, why should the Courts be expected to accept different standards of assay performance than laboratory accreditation bodies? A further important point is that the limit of detection and the lowest limit of quantitation are ascertained by extracting "spiked" hair, i.e., hair to which analytes have been added *in vitro*. Analyte added in this way may be much easier to extract than analytes incorporated in hair *in vivo*.

In hair analysis the term "cut-off" has a different meaning to its use with immunoassays. The Society of Hair Testing has produced "cut-off" values for a range of analytes in an attempt to differentiate deliberate drug or alcohol ingestion from the possibility of incidental exposure or endogenous production (Table 1). However, these "cut-offs" are not analytical "cut-offs", and analyte concentrations below these values (and at or above the lowest limit of quantitation or limit of detection depending on the laboratory) may still indicate deliberate administration, whilst concentrations above these values could simply result from incidental exposure, for example.

Some laboratories performing hair testing report positive identifications to analytical limits routinely, thus risking giving a false impression of unequivocal drug use by the individual sampled, whilst others using either the Society of Hair Testing, or "in-house" cut-off values may report the same analytical result as "negative" or "not detected", thus risking not supplying valuable information in the context of a particular case. Hair analyte concentrations below "cut-off" values could be due to the use of small quantities of drugs, although the minimal detectable dose that can be detected in hair is unknown. Moreover, inter-individual differences in hair colour, ethnicity, rate of hair growth, etc. and the possible influence of cosmetic hair treatment such as thermal hair straightening, dying, or bleaching in destroying analyte or giving rise to false-positives should be taken into consideration when interpreting results, if this information is known [10,68–74].

The underlying problem in all this is that, as with any toxicological analysis, the interpretation of results is absolutely dependent on the circumstances of the case. Simply saying test for drugs/alcohol metabolites is not enough, but unfortunately the way these (expensive) tests have often been marketed serves to give the impression that interpretation is straightforward, whilst in reality the opposite is often true. Commonly, positive results using either Society of Hair

Table 1. Society of hair testing: Recommended cut-offs for substances and metabolites in hair to identify use [66,67].

Screening			Confirmation
Group	Cut-off (ng/mg)	Target analyte	Cut-off (ng/mg)
Amfetamines	0.2	Amfetamine	0.2
		Metamfetamine	0.2
		Methylenedioxyamfetamine (MDA)	0.2
		Methylenedioxymetamfetamine (MDMA)	0.2
Cannabinoids	0.1	Tetrahydrocannabinol (THC)	0.05
		Tetrahydrocannabinol carboxylic acid (THC-COOH)	0.0002
Cocaine	0.5	Cocaine	0.5
		Benzoylecgonine (BZE), ecgonine methyl ester (EME), cocaethylene (CE), norcocaine (*N*-desmethylcocaine, NC)	0.05
Ethanol	–	Ethyl glucuronide abstinence, segment 0–3 cm	0.007
		Ethyl glucuronide "chronic excessive use"	0.03
		Ethyl palmitate abstinence, segment 0–3 cm	0.012
		Ethyl palmitate "chronic excessive use"	0.35
Opiates	0.2	Morphine	0.2
		Codeine	0.2
		6-Acetylmorphine (6-AM)	0.2
Methadone	0.2	Methadone	0.2
		2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	0.05
Buprenorphine	0.01	Buprenorphine	0.01
		Norbuprenorphine (*N*-desalkylbuprenorphine)	0.01

Testing, or analytical cut-offs are reported as "compatible with the hypothesis of ingestion of x, y, or z drug" in the assumption that a positive finding is expected without pointing out that incidental exposure may be an equally plausible explanation for the result (a variant on the "prosecutor's fallacy").

The common practice of reporting results to four or even five significant figures (using pg/mg rather than ng/mg, for example) serves to inflate the evidential value of the data in the eyes of those who do not understand the uncertainties associated with the measurements. For example, it has been shown that hair that has been pulverized gives up to a three-fold higher concentration of ethyl glucuronide than that which has been prepared by cutting [75], let alone the uncertainties brought about by the "decontamination" procedures.

Proposed clinical applications of hair analysis

Pragst and Balikova [29] have pointed out that answers to the following questions are usually expected after performing a hair analysis:

I. Had the individual either used, or been given drugs?
II. If yes, which drugs were used/administered?
III. When were the drugs used/administered and was it single, occasional, regular, or excessive use/administration?

The answers, however, generally require expert and critical examination of the case history, and thorough knowledge of the range of factors that can influence interpretation of the results.

In view of the risk of moving analyte either out of, or into the hair matrix during pre-analytical processes, it must be pointed out that all quantitative data reported in the literature are approximate values as to the analyte concentrations actually within the hair at the time of sample collection. There are also issues of incidental exposure that may have given rise to systemic absorption of an analyte or analyte precursor during synthesis of the hair, or afterwards leading to diffusion of analyte into the hair from sweat, etc., and the possible effects of hair structure, colour, and of shampooing and other cosmetic treatments to consider. For example, 10-fold differences between the concentrations of basic drugs in pigmented vs. non-pigmented hair with the same systemic exposure have been reported [29].

Drug-facilitated crime

Several instances have been documented where drugs seemingly administered during the perpetration of a crime have been detected in head hair [76]. Detailed discussion of such reports is almost impossible, given current understanding of the effects that "decontamination" procedures and incidental exposure from contaminated environments may have on distribution of analytes into the hair shaft, and the influence of other variables such as hair colour or cosmetic hair treatments may have had on the results.

In one case detection of sildenafil in a proximal hair sample from a young female was reported to be in accord with the victim's claim [77]. Use of γ-hydroxybutyrate and/or lorazepam had been suspected (lorazepam was found in the household), but hair analysis gave no clear support for γ-hydroxybutyrate administration (maximal γ-hydroxybutyrate concentration in a segment 0.9–1.2 cm from the root 3.6 ng/mg hair). Neither γ-hydroxybutyrate, nor sildenafil was found in the household. As regards the analysis, dichloromethane "decontamination" was employed prior to segmentation, but analysis of the dichloromethane extracts was not reported. The failure to report norsildenafil coupled with the extremely low sildenafil concentration measured in the proximal hair segment (0.038 ng/mg, i.e., 38 parts sildenafil per thousand million parts of hair) suggests the likelihood that the sildenafil was an incidental finding.

Analysis of scalp hair from two patients who took sildenafil orally at regular intervals gave results of sildenafil 19.8 ng/mg and norsildenafil 55.9 ng/mg, and sildenadfil 1.7 ng/mg and norsildenafil 5.6 ng/mg, respectively [78], data incorrectly

reported as 19.8 ng/mg and 55.9 ng/mg sildenafil by Kintz et al. [77]. Dumestre-Toulet et al. [79] measured sildenafil in unsegmented hair at a concentration of 0.177 ng/mg from a subject found dead in a hotel room, but again norsildenafil was not measured and again incidental exposure is a possible explanation for the result.

Hair analysis has been reported as showing chronic administration of sertraline and quetiapine, but not methylphenidate, to a 4-year-old previously healthy boy who was admitted after suspected accidental ingestion of methylphenidate, sertraline and quetiapine prescribed to his 8-year-old brother [80]. Sertraline and quetiapine and their metabolites were identified in plasma and in urine and the child recovered uneventfully. Quetiapine was found in the first four of six consecutive 2 cm hair segments (mean concentration 1.00 ± 0.94 ng/mg hair) and sertraline and norsertraline were found in all segments (mean concentrations 2.65 ± 0.94 ng/mg and 1.50 ± 0.94 ng/mg hair, respectively. The highest concentrations were found in the segments nearest the root. Methylphenidate and ritalinic acid were not detected in any segment. The hair segments were washed with dichloromethane and with methanol prior to the analysis. Given that there was a large systemic exposure to quetiapine and sertraline, the assertion that the hair analysis showed chronic drug administration to the child would seem speculative at best.

In a further case, Allibe et al. [81] concluded that a single overdose of amitriptyline could not be excluded as the reason for the presence of amitriptyline and nortriptyline in hair segments from a 6-month-old girl.

Child custody

In one case it was accepted that when a 20-month-old child tested positive for cannabis on hair analysis and was classified as a "medium to moderate user", the child had in fact simply been environmentally exposed to the drug by virtue of passive smoking [16].

A recent review divided 52 English-language reports of hair concentrations of drugs in children living in a drug-using environment into three categories:

I. Neonates where hair was sampled at, or shortly after, birth;
II. Children believed to have been exposed passively to drugs in their environment;
III. Children exposed as a result of either accidental ingestion, or deliberate administration by a caregiver [82].

There were limited comparative data in all cases. On average, cocaine, codeine, 6-AM, and morphine showed higher concentrations in hair in category (i) as compared to children exposed passively (category ii). However, there was considerable overlap in concentrations. Hair metamfetamine concentrations showed no significant difference between the two categories, although only one study reported hair concentrations in category (i). There was no difference in concentrations for those cases exposed passively (ii) or actively (iii) for codeine and methadone. There were insufficient data for other drugs and other comparisons.

Data comparison was confounded by the variability in extraction techniques employed as well as a by the variety of washing techniques used; some studies did not employ any "decontamination" technique whatsoever. Simply detecting drugs such as methadone or carbamazepine in the hair of children if they are living in a home where these drugs are available does not of itself prove deliberate drug administration to the children [83–85]. There may be a contribution of *in utero* exposure to hair drug concentrations in young children [86].

Monitoring abstinence to drugs and/or ethanol

Cocaine concentrations measured in head hair from known cocaine users can vary widely (from 0.03 to 227 ng/mg in one study [87]). Even though hair analysis is advocated widely for retrospective monitoring of cocaine intake, differentiating between incorporated cocaine and external cocaine contamination remains problematic. Hair porosity varies and so does incorporation of cocaine from the hair surface [10,88].

A recent study [89] has highlighted the complexities of testing minor cocaine metabolites for definitive proof of deliberate cocaine consumption. Following analysis of 90 hair samples from cocaine users for cocaine, benzoylecgonine, norcocaine, cocaethylene, and tropococaine by GC-MS, cocaine usage was stratified as: light (0.5–3 ng/mg hair), moderate (3.1–10 ng/mg) and heavy (10.1–40 ng/mg). Cocaine concentrations ranged from 1.63–39.29 (mean 9.49) and benzoylecgonine ranged from 0.19–5.77 (mean 1.40). The benzoylecgonine:cocaine ratio was $\geq$5% (6.4–26.1%) in all samples. Norcocaine was present in 60% of samples (range: 0.22–3.14 ng/mg) and was strongly predictive only of heavy cocaine use (sensitivity 100% for cocaine concentrations $>$9.6 ng/mg). Cocaethylene was detected in 20 samples from moderate/heavy users (range 0.22–1.98, mean 0.73 ng/mg). However, both norcocaine and cocaethylene may occur in "street" cocaine if processing has involved the use of ethanol and potassium permanganate, hence hydroxycocaine and/or hydroxybenzoylecgonine are assumed to be more specific markers of cocaine exposure [90].

Regarding the Society of Hair Testing consensus for the use of alcohol markers in hair for assessment of both abstinence and chronic excessive alcohol consumption [91], the direct determination of ethanol itself in hair is not possible due to its volatility and its potential absorption from external sources. Instead, the minor ethanol metabolites ethyl glucuronide, ethyl sulfate, and/or fatty acid ethyl esters can be measured in hair as *indirect* markers of alcohol consumption. The fatty acid ethyl esters measured were ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate. For the interpretation of results prior to 2016 the Society of Hair Testing recommended that the sum of the concentrations of these four esters should be used when testing for alcohol use, but the 2016 consensus guidelines now suggest that only ethyl palmitate should be used. The fact that legal judgments will have been made based on the earlier, superseded guidelines

Table 2. Alcohol use and ethyl glucuronide concentrations in hair, 2010–2016.

Subjects (ethanol dose)	Hair length (cm)	Ethyl glucuronide (pg/mg) Range	Mean	Remark	Reference
23 Abstainers	2	<2	–	Prospective study	Kronstrand et al. [95]
7 M (32 g/day)		<2–11 (b)			
14 F (16 g/day)		<2–3			
43 Teetotalers (children)	3	0–10 (a)	0.6	Daily self-report	Kharbouche et al. [96]
44 Low risk ≤20/30 g/day		0–32	4.9		
38 At risk >20/30 g/day		1–1190 (b)	88.3		
19 Teetotalers (children)	1 or 3	0–37 (a)	1/19 > 30 (a)	Self-reported	Lees et al. [97]
51 Low risk 1–20 units per week		0–510	5/51 > 30		
11 Increasing risk 21–50 units per week		0–698	5/11 > 30 (b)		
19 High risk >50 units per week		0–504	11/19 > 30 (b)		
317 Abstinent	3	0–493 (a)	2	Self-reported drinking & parents in child protection cases	Suesse et al. [98]
65 Low-moderate		0–90	3		
672 Moderate		0–1340	14.5		
322 Excessive		0–1420	66		

(a) Instances in which self-reported abstainers exceed the Society of Hair Testing defined ethyl glucuronide cut-off of 7 pg/mg hair; (b) chronic users of more than 30 g ethanol/day with ethyl glucuronide values below the Society of Hair Testing 30 pg/mg cut-off.

inevitably raises questions as to the reliability of the decisions made.

Measurement of markers to identify long-term alcohol consumption is generally used to corroborate claims of alcohol abstinence. Remarkably, it is mentioned that occasional drinking events cannot always be excluded. The concentration of ethyl glucuronide and fatty acid ethyl esters in hair can be influenced by cosmetic treatments and thermal hair straightening tools. Therefore, the type of cosmetic hair treatment should be documented during sampling and considered during interpretation. For instance, bleaching, perming, and/or dying hair may lead to lower concentrations of ethyl glucuronide or even false negative results, and may also influence concentrations of fatty acid ethyl esters. Moreover, the use of ethanol-containing hair care products such as hair sprays or lotions may lead to falsely elevated concentrations of fatty acid ethyl esters. The complex topic of hair analysis for markers of alcohol use has been summarized by Pragst [92].

The Society of Hair Testing consensus states that for abstinence assessment over a pre-defined time period, which includes: prerequisite for regaining a suspended driving licence, child custody cases, clinical contexts, forensic cases, etc., ethyl glucuronide should be the first choice. For ethyl glucuronide, a concentration ≥7 pg ethyl glucuronide/mg hair in the 0-3 up to 0–6 cm proximal scalp hair segment *strongly suggests* repeated alcohol consumption. However, an extensive meta-analysis shows that the proposed 7 pg/mg "cut-off" is only useful for indicating active alcohol use, and not for proving abstinence [93]. An ethyl glucuronide result ≥7 pg/mg cannot be overruled by a negative fatty acid ethyl esters result. The analysis of fatty acid ethyl esters alone is not recommended to assess ethanol abstinence, but may be used in cases of suspected false negative ethyl glucuronide results, utilizing a fatty acid ethyl esters cutoff concentration of 0.2 ng/mg hair for a 0–3 cm proximal scalp hair segment or 0.4 ng/mg for a 0–6 cm proximal scalp hair segment. A positive fatty acid ethyl ester result combined with an ethyl glucuronide result below 7 pg/mg does not clearly disprove abstinence, but indicates the need for further monitoring.

Often samples are sent for hair analysis for alcohol markers in the belief that the interpretation is straightforward. The Society of Hair Testing consensus may say that "It is not advisable to use the results of hair testing for alcohol markers in isolation; all relevant factors surrounding a case must be considered when providing expert interpretation and opinion", but those who use the results may lack the competence to understand what is really meant by this statement.

To assess chronic excessive alcohol consumption the Society of Hair Testing consensus states that ethyl glucuronide and fatty acid ethyl esters can be used either alone, or in combination. It has been reported that the lowest false negative and false positive rates were obtained using the results of hair ethyl glucuronide and fatty acid ethyl esters together. But even here the markers showed relatively poor correlation [94]. It is stated that an ethyl glucuronide concentration of ≥30 pg/mg hair in the 0–3 up to 0–6 cm proximal scalp hair segment strongly suggests chronic excessive alcohol consumption. A cut-off concentration of 0.5 ng/mg hair for the sum of the four fatty acid ethyl esters in scalp hair is considered strongly suggestive of chronic excessive alcohol consumption when measured in the 0–3 cm proximal segment. But, many reports from 2010-2016 document instances in which admittedly self-reported abstainers and even teetotalers exceed the Society of Hair Testing SoHT defined ethyl glucuronide cut-off of 7 pg/mg hair [Table 2(a)]. On the other hand, chronic users of more than 30 g ethanol/day are reported with ethyl glucuronide values below the 30 pg/mg cut-off [Table 2(b)].

Conclusions

As more of the pitfalls and problems associated with the interpretation of hair analysis results have become apparent, those marketing the testing have embarked on efforts to assure clients of the reliability of the claims made on the

basis of the results. However, doubts continue to surface, especially since definitive evidence of the flawed assumptions made for the so-called "decontamination" procedures has called into question much of the literature on this topic. Qualitative analysis, however, still has a place – the simple fact of unequivocal detection of a compound in any biological sample from an individual requires understanding of how it may have got there [99], although here the difficult question of limit of detection/sensitivity/accurate measurement remains. To attempt to assess the mechanism of exposure, and the extent and duration of exposure, requires far more information than can be provided by an analysis of head hair alone.

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