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New trends in hair analysis and scientific demands on validation and technical notes

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Abstract

This review focuses on basic aspects of method development and validation of hair testing procedures. Quality assurance is a major issue in drug testing in hair resulting in new recommendations, validation procedures and inter-laboratory comparisons. Furthermore recent trends in research concerning hair analysis are discussed, namely mechanisms of drug incorporation and retention, novel analytical procedures (especially ones using liquid chromatography–mass spectrometry (LC–MS) and alternative sample preparation techniques like solid-phase microextraction (SPME)), the determination of THC-COOH in hair samples, hair testing in drug-facilitated crimes, enantioselective hair testing procedures and the importance of hair analysis in clinical trials. Hair testing in analytical toxicology is still an area in need of further research.

Keywords: Hair testing; Validation; Drugs; THC-COOH, SPME

1. Introduction

The analysis of drugs in hair samples has become very popular in recent years with possible applications in forensic and in clinical toxicology as well as in work-place drug testing procedures. Except for a few reviews or monographs [1–4], an ever increasing number of recent publications in scientific journals deals with novel analytical procedures, case reports as well as special problems like useful decontamination procedures, data about drug incorporation rates and mechanisms of incorporation and retention of a drug. The aim of the present paper is to give

- a summary of the scientific demands on, e.g. technical notes and case reports in scientific publications like in *Forensic Science International*, and
- a listing of new trends in the research concerning hair analysis.

2. Scientific demands on analytical procedures

In 2004 the Society of Hair Testing (SOHT) has published new recommendations for hair testing in forensic cases [5]. Beyond that the guidance documents recently issued by the German speaking Society of Toxicological and Forensic Chemistry (GTFCh) comprise some more details especially concerning method validation in forensic toxicological analytical methods [6] and special recommendations for hair testing procedures [7].

2.1. Development of analytical procedures

The various steps of hair analysis are listed in Table 1; each step contains several possible sources of error and such pitfalls in hair analysis were summarized in a recent excellent presentation of Pragst [8]. The extraction of drugs from hair is the most sensible step of hair testing procedures. The drugs are firmly enclosed in the hair structure and partly bound to proteins, melanin or lipids of the cell membrane complex. The extraction yield depends on the drug's structure, the state of the hair matrix, polarity of the solvent, duration and manner of extraction (e.g. grinding, use of ultrasonic bath). Analytical conditions have to be optimised [9–13] and following points have to taken into consideration during development of a complete hair testing procedure.

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Table 1
Steps of hair analysis and sources of error according to Pragst [8]

Step	Sources of error
Case of investigation	Insufficient information about case history and purpose of investigation
	Wrong choice of hair as sample material
	Wrong decision with respect to segment lengths
Sampling and storage	No unambiguous identification of the individual
	Insufficient sample amount and order of hair tuft
	Insufficient labelling, mix up with other samples
	Danger of contamination and degradation
Decontamination	Choice of wrong solvent or solvent sequence
	No analysis of the wash solution
Extraction	Inappropriate choice of extraction or digestion method
	Wrong time and temperature of extraction
	Decomposition of the drug
	High levels of impurities
Analysis	Insufficient specificity, sensitivity, accuracy
	No deuterated standards in GC/MS
	Loss of substance in clean-up
	False-positive or -negative result
Expertise	Inadequate interpretation of the concentration
	Inadequate interpretation concerning exposure time
	Case specificity and sample properties not considered

These points have to be considered in a scientific publication.

2.1.1. Decontamination

There is always the possibility that a drug in hair does not originate from consumption but has been incorporated from external sources. Therefore, prior to analysis of hair samples a decontamination strategy has to be performed and washing solutions should be stored for later analysis, if necessary. There exists no consensus about an optimal decontamination procedure and in general it has to be investigated whether a washing using different solvents is sufficient for clean-up or already leads to an untimely extraction of incorporated analytes. Additionally, in the next step of hair testing the decontamination procedure can affect the extraction efficiency of incorporated drugs and has to be considered in the interpretation of quantitative results. The detection of metabolites in a typical ratio to the parent drug can often be considered as a definite proof of ingestion; e.g. after cocaine abuse, besides cocaine the metabolites benzoylecgonine, ecgonine methylester and norcocaine and in case of simultaneous alcohol consumption also cocaethylene can be found (Fig. 1). However, the hydrolytic metabolites benzoylecgonine and ecgonine methylester may also be formed outside of the body. The only unambiguously endogenous metabolites are norcocaine and cocaethylene.

2.1.2. Hair disintegration, extraction and clean-up

The most important procedures are hair hydrolysis by sodium hydroxide with subsequent solid-phase extraction and extraction with methanol or aqueous buffer in an ultrasonic bath. The sodium hydroxide method provides high yields for alkaline-stable drugs such as cannabinoids or amphetamines, whereas under such alkaline conditions, e.g. cocaine is converted to benzoylecgonine. Methanolic extraction is universally applicable but the extracts have a high degree of contamination from hair matrix and a further clean-up is recommended in most cases. Basic drugs are best extracted by a neutral or slightly acidic aqueous buffer and subsequent clean-up. A frequent error in hair extraction is a too short extraction time, which can be controlled in a step-by-step extraction experiment, e.g. the less polar compounds heroin and 6-monoacetylmorphine (6-MAM) are extracted faster than the more polar drugs morphine or codeine (Fig. 2). Different analytical procedures can produce different quantitative results. Additionally, degradation compounds may

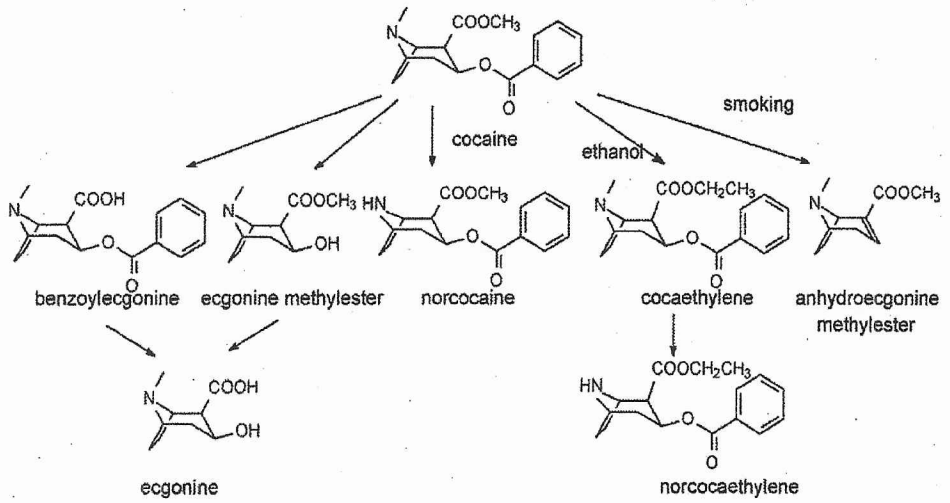

Fig. 1. The detection of metabolites in a typical ratio to the drug can often be considered as a proof of ingestion (e.g. benzoylecgonine/cocaine >0.05). After cocaine abuse, besides cocaine the metabolites benzoylecgonine, ecgonine methylester and norcocaine (and with highly sensitive methods also ecgonine) and, in case of simultaneous alcohol consumption or smoking of the drug, also cocaethylene or anhydroecgonine methylester can be found in hair samples. However, the hydrolytic metabolites benzoylecgonine and ecgonine methylester may also be formed outside of the body. The only unambiguously endogenous metabolites are norcocaine and cocaethylene.

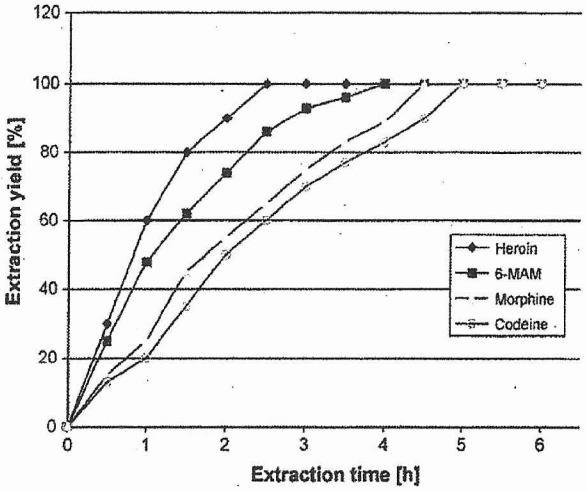

Fig. 2. Extraction of opiates from hair by methanol/ultrasonic bath at 50 8C. Step-by-step extraction of the hair sample of an opiate-associated fatality. From this experiment the optimal extraction time can be determined. Non-polar substances (e.g. heroin) are extracted faster than polar compounds (e.g. codeine). However, the extraction rate depends on the individual sample.

be produced during the sample preparation (e.g. heroin → 6-MAM → morphine). In order to asses the degree of conversion (as well as a possible influence of the internal standard), the laboratory must include adequate controls.

2.1.3. Criteria for mass spectrometric analysis

International guidelines for mass spectrometric identification and quantification of drugs have to be taken into account. Detailed and current values concerning chromatographic retention times and identification criteria, e.g. number of detected ions and peak area ratios (of at least three ions in gas chromatography–mass spectrometry (GC–MS)) along with other characteristics, are essentially comparable. In the validation of LC–MS procedures the lack of matrix effects (e.g. suppression/enhancement) throughout the application of the method have to be ensured [14]. Additionally, the possible influence of the internal standard (at low concentrations) must be assessed and documented.

2.1.4. Quality control

Quality control in hair testing is more difficult than for homogeneous body fluids, since spiked control samples cannot substitute for the actual hair of a drug user. However, for internal quality control spiked samples may be substituted for hair from drug users, if properly prepared according to special techniques [5]. For external quality control only authentic standard hair specimens should be used.

2.1.5. Interpretation of results

There is only a limited correlation between the frequency of drug use or the ingested dose and the drug concentration in hair, which is based on inter-individual differences in dosage and thus serum concentrations as well as on differing drug incorporation rates, hair pigmentation and physical state of the hair (shampooing, bleaching, dyeing, permanent wave). Therefore a statistic evaluation of the data of each laboratory with its special methodologies is proposed for the interpretation of results.

2.2. Validation of procedures

As there are several, and sometimes very comprehensive validation recommendations for various areas of bioanalytical procedures [15–17], the GTFCh has agreed on a minimum consensus with regard to the specific features in hair testing (e.g. heterogeneous matrix, stability of drugs, influence of sample preparation). In Table 2, the validation steps regarded to be essential are summarised.

Table 2
Validation parameters and minimum requirements in hair testing procedures according to the Society of Toxicological and Forensic Chemistry (GTFCh) [6,7]

Selectivity (exclusion of interference)
Six different blank samples must be analysed
Two zero samples (blank sample spiked with internal standard) must be analysed
Linearity
Calibration (matrix-based)
Five replicate analyses at four different concentrations, respectively
Approximately equidistant spacing between calibrators
Statistical evaluation
Verification of adequate linearity
Checking of the y-intercept
Use of a non-linear calibration model when necessary
Precision and accuracy
Preparation of two spiked control samples
Concentrations at the lower and upper limit of the calibration range
Two replicate analyses of each control sample on five different days
Calculation of the concentration of the control samples
Acceptance limits
99% confidence interval within ±50% of the target value (includes precision and accuracy)
Analytical limits
Calculation according to DIN 32645
Limit of detection (LOD) is defined as a 50% probability of the analyte being present or not present and is calculated according to the formula: $X_{LOD} = S_{x0} \times t_{f,a} \times \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{\bar{X}^2}{Q_x}}$
Limit of quantification (LOQ) refers to the minimum quantity which can be determined with both defined probability level and acceptable relative uncertainty and is calculated according to the formula[a]: $X_{LOQ} = k \times S_{x0} \times t_{f,a} \times \sqrt{\frac{1}{m} + \frac{1}{n} + \frac{(X_{LOQ} \times \bar{X})^2}{Q_x}} = k \times DX_{LOQ}$
Alternative estimation based on signal-to-noise ratio
Stability
Information about stability of reagents, standards and samples
Testing of stability of analytes and internal standards during sample preparation (decontamination, extraction, clean-up)

[a] S_x is the method standard deviation; m the number of measurements; n the number of calibration points; Q the sum of the deviation squares; t the quantile of t-distribution; $\bar{X}$ the mean value; k the relative uncertainty of results; a is the probability of error.

As a matter of principle a basic validation procedure and analytical strategy as described above is absolutely necessary if an analytical procedure is to be published in an international scientific journal. For selected case reports, validation of the procedure may be conducted in an abbreviated fashion, the final interpretation of (quantitative) results however has to take into account a such proceeding [17].

3. Trends in research

In the past few years publications concerning hair testing dealed with novel analytical procedures, interesting case reports and special problems like useful decontamination procedures, data about drug incorporation rates and mechanisms of incorporation and retention of a drug. In the following a short overview about new trends and interesting themes for further research is given.

3.1. Mechanisms of drug incorporation and retention

The mechanisms of drug incorporation into hair and retention of the compounds have not been clarified at all and many factors are likely to affect the concentration of drugs in hair, which complicates the interpretation of results. These factors include, but are not limited to hair colour or melanin content, physicochemical properties of drugs such as lipophilicity, melanin affinity and membrane permeability, structural factors of drugs, external contamination and cosmetic treatment of hair.

The effects of physicochemical properties of drugs on the incorporation rates (ICR) into hair were extensively investigated by Nakahara et al., who designed a model experiment with pigmented Dark Agouti rats to compare the ICRs of many kinds of drugs [18]. This model is still the basis for further research on other drugs (e.g. benzodiazepines) [19]. Another way to investigate drug incorporation is the determination of isotopically labelled drugs in hair samples after their administration under laboratory clinical conditions [20].

Several *in vivo* and *in vitro* binding studies on drug–melanin interaction have been carried out. For *in vivo* studies results from pigmented and non-pigmented hair fibres are compared and striking differences in drug concentrations were found for various substances [21–27]. For *in vitro* studies investigation focuses on the interaction of various candidate drugs with synthetic melanin subtypes [28,29], melanin during its tyrosine-mediated formation [30] and melanin granules [31] as well as on the drug uptake in HaCaT and Sk-Mel-1 cells [32,33]. These cell lines maintain the expression and synthesis of keratin and melanin and may therefore serve as a model for long term drug exposure of the cell populations present in the anagen hair root. As proposed by some groups drug concentration in hair can be normalized in reference to melanin or eumelanin content which adds significance to therapeutic compliance monitoring [22]. However, additional studies on the relationship of melanin and different drugs should be performed to further investigate the role of pigmentation on drug incorporation into hair.

Furthermore it was observed that drug concentration declined dramatically after cosmetic treatment (bleaching, dyeing and permanent waving) as well as UV exposure or exposure to water, soil or weathering [34–38]. Such effects have to be taken into account when interpreting drug abuse analyses in hair samples.

As described in Section 2.1.1 false positive hair testing results are caused by passive exposure to drugs in the environment. To distinguish between actual consumption and contamination there exist a number of approaches like decontamination using various solvents, the measurement of metabolite to parent drug concentration ratios, the use of cut-off levels setting the limits for passive endogenous drug exposure and the reproducibility of results (including segmental analysis) using a newly collected hair specimen. Baumgartner and Hill described a specific but very labour-intensive procedure to establish wash kinetics, in which the amount of drug found in the various washes was compared with the amount of drug in the washed and extracted hair specimen to distinguish between passive exposure and active ingestion [39]. However, even such a sophisticated procedure was not sufficient for a definite differentiation between active use of drugs and passive exposition [40,41]. Recently, Romano et al. demonstrated the problem that even using sophisticated decontamination procedures it was not possible to distinguish a drug-contaminated subject from an active user after a single external contamination with small amounts of drugs [42,43]. A lot of strategies were recently tested [44–50], however, there exists no consensus about an optimal decontamination procedure and it has to be considered individually whether a washing using different solvents is sufficient or already leads to an untimely extraction of incorporated analytes. It has to be taken into account that a decontamination procedure can affect the extraction efficiency of incorporated drugs and has to be considered in the interpretation of quantitative results. Furthermore, only limited data concerning the disappearance of drugs from hair after abstinence are available [51]. Further research is still necessary to substantiate the use of suitable extensive washing and application of wash criteria in combination with cut-off and metabolite criteria.

3.2. Novel analytical procedures

3.2.1. Immunological screening procedures

According to Spiehler [52] immunoassays for hair testing must satisfy three requirements: (1) they must have cross-reactivity with both the parent drug and its lipophilic metabolites actually found in hair, (2) they must not experience interference from dissolved hair matrix, and (3) they must be designed for cut-offs appropriate to the drug concentrations found in hair. Lately more and more enzyme-linked immunosorbent assay (ELISA) kits using coated well technology have been developed. These tests are less susceptible to matrix interference, target the parent drugs and lipophilic metabolites found in alternative specimens and are suitable for oral fluid and hair testing as well [53–61]. However, a validation seems to

be necessary for forensic purposes which should be of interest for further studies.

3.2.2. Alternative sample preparation techniques

Sample preparation procedures for analysis of illicit or therapeutic drugs in hair generally involve extraction or digestion of the hair matrix and subsequent clean-up by solid-phase extraction. Such multi-step-methods require a high degree of experience to obtain reproducible results. Besides such classical procedures alternative sample preparation techniques were described. Reproducibility and the extraction rate of supercritical fluid extraction (SFE) was not comparable with classical methods [62–64], but headspace solid-phase microextraction (HS-SPME) has proved to be a simple but efficient alternative (Fig. 3). All steps of the conventional liquid–liquid extraction (LLE) such as extraction, concentration, (derivatization) and transfer to the chromatograph are integrated into one step and one device, considerably simplifying the sample preparation procedure. HS-SPME uses a fused-silica fibre that is coated on the outside with an appropriate stationary phase, for example polyacrylate (PA), polydimethylsiloxane (PDMS), polydimethylsiloxane/polydivinylbenzene (PDMS/DVB) or polydivinylbenzene/polyethylenglycol (DVB/Carbowax). The analytes in the sample are directly absorbed by and concentrated on the fibre coating until the three-phase equilibrium is reached. Then the fibre can directly be injected into a GC injection port for thermal desorption. In contrast to the direct extraction from an aqueous medium (direct immersion, DI-SPME), which was described for the determination of cocainics [65,66] in hair samples, the HS technique combines great advantages like the avoidance of organic solvents, the simple technical performance, and the very low chromatographic background resulting in a long lifetime of the fibre. In order to obtain optimal conditions in the sample preparation, the conditions of hydrolysis, addition of salts (salting out effect), incubation time and temperature, agitator speed, extraction time, derivatization time and amount of derivatization reagent, desorption time and temperature as well as the depth of fiber insertion into the GC injection port have to be optimised prior to the validation of the whole procedure. Recently, an experimental design was introduced as a novel procedure to optimise SPME: by generating various surface plots describing the relationship between operating variables and predicted extraction yields optimum conditions with the highest desirability (minimal extraction time, highest yield for all components of interest) are found [67]. For example, studying three factors at five levels in a complete design would normally require 5^3 or 125 samples, whereas central composite design requires only 20 samples and still completely covers the experimental space and allows to

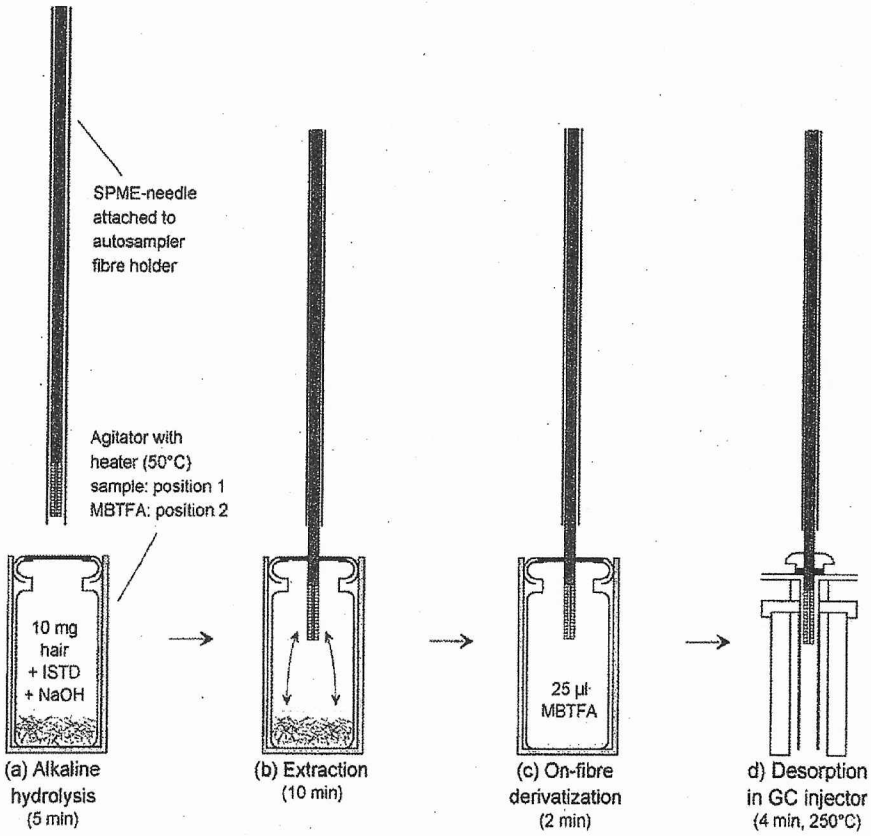

Fig. 3. Schematic description of the SPME technique for the determination of amphetamine and designer amphetamines in hair samples. The analytes in the headspace are directly absorbed by and concentrated on a fused-silica fibre with an outside coating of an appropriate stationary phase (for example PDMS). After on-fibre derivatization the fibre can directly be injected into a GC injection port for thermal desorption. For details see [72].

calculate all interactions and non-linearities. Until now, HS-SPME has been used in hair analysis for the determination of methadone and its metabolites [68–70], amphetamines/butanamines [71–74], lidocaine [75], benzodiazepines and other psychotropic drugs [76]. Using a multipurpose sampler highly reproducible and fully automated procedures were developed. A further development is the technique of automated HS solid-phase dynamic extraction (HS-SPDE) coupled with GC/MS [77,78]. In an inside needle capillary absorption trap a hollow needle with an internal coating of PDMS with charcoal is used as extraction and pre-concentration medium. Sampling is performed on the solution headspace by actively (thus "dynamic") passing the gas through the deviceusing a syringe. Analytes present in the sample are sorbed onto the deposited stationary phase. The syringe needle is placed into the injection port of a GC where rapid heating of the metal needle induces the desorption of analytes (Fig. 4). The absolute extraction recovery with SPDE was demonstrated to be 50% higher compared to a SPME fiber. A new approach is the analysis of fatty acid ethyl esters in hair specimens as possible markers of chronically elevated alcohol consumption by HS-SPME followed by GC/MS [79–81], another marker substance in hair being ethyl glucuronide [82–85]. Recently, with surfactant enhanced liquid-phase microextration (SE-LPME) an alternative concept for three-phase microextraction was introduced [86].

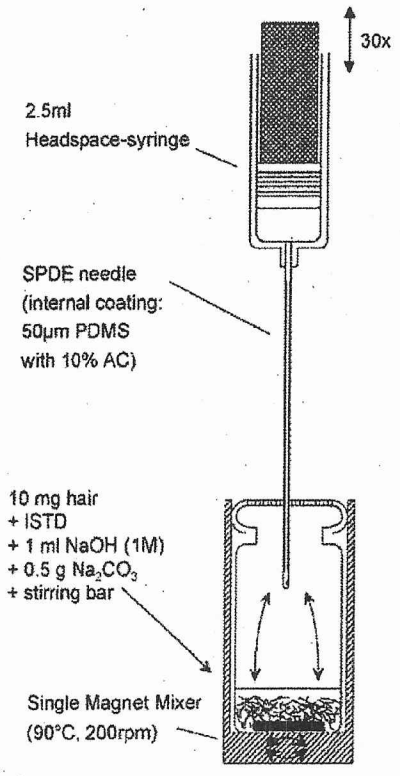

Fig. 4. Schematic description of the SPDE technique for the determination of cannabinoids and methadone in hair samples. Sampling is performed on the solution headspace (hair specimens were hydrolysed) by actively (dynamic) passing the gas through the device using a syringe. Analytes present in the sample are sorbed onto the deposited stationary phase. The syringe needle is placed into the injection port of a GC where rapid heating of the metal needle induces the desorption of analytes. For details see [77].

In summary, SPME reduces the time required for sample preparation, decreases purchase and disposal costs of solvents and can improve detection limits. The SPME technique is ideally suited for MS applications, combining a simple and efficient sample preparation with versatile and sensitive detection. By adaptation of the conditions to special properties of a particular substance an extension of SPME or SPDE to other drugs and a further decrease in detection limits are possible.

3.2.3. LC–MS procedures

The ability of HPLC to separate a large range of underivatized substances, coupled with the milder working conditions of the technique, makes it particularly valuable especially for the analysis of a wide range of more thermolabile substances like benzodiazepines and related compounds which are of interest in forensic toxicology. Recently, LC–MS/MS methods were described for the simultaneous determination of a large number of benzodiazepines and metabolites together with zolpidem and zopiclone in hair specimens [87,88]. The analysis of single benzodiazepines in hair specimens was also described in the context of drug-facilitated crimes (see 3.4). Other groups described LC–MS/MS procedures – sometimes including library search algorithms – for the analysis of a number of neuroleptics and other psychopharmaceuticals and their major metabolites in hair specimens [89–91]. LC–MS screening for drugs has improved with the development of in-source collision-induced dissociation (CID) mass spectral libraries for general unknown screening procedures. MS/MS target analysis has been applied for the subsequent identification of compounds by analysis in product ion scan mode together with MS/MS library searching. According to Weinmann [92] the development of the hybrid triple quadrupole linear ion trap technology (QTrap) offers the possibility of simultaneous detection of a drug and its identification by information-dependent acquisition (IDA) in one single run. The working-group described a multi-target screening analysis for 301 pre-selected target compounds in blood, plasma or serum extracts. Also Marquet et al. developed a general unknown screening procedure using a QTrap [93]. Others described screening procedures using ion trap MS [94], a quadrupole time-of-flight (Q-TOF) instrument switching automatically from MS to MS/MS mode when exceeding a given threshold [95] or exact mass measurement of product ions for identification of unknown compounds [96]. Such sophisticated screening procedures have to be adapted to hair testing in the future. However, in the case of LC–MS/MS based procedures, appropriate measures have to be taken to ensure the lack of matrix effects (e.g. suppression/enhancement) throughout the application of the method [14].

3.3. The determination of THC-COOH in hair samples

Hair testing for cannabinoids is a challenge in forensic toxicology. In routine laboratories analysis is mainly focused

on the determination of the psychoactive compound D^9-tetrahydrocannabinol (THC). Sometimes for plausibility control the analysis is extended to include cannabinol (CBN) and cannabidiol (CBD). Nevertheless, in hair these substances might have come from environmental exposure, and only detection of metabolites really prevents false positive results caused by a probands' passive exposure to cannabis smoke. The main metabolite of THC is 11-nor-D^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), the concentration of which is extremely low in hair due to the weak incorporation rate of this acidic metabolite into the hair matrix. Therefore, a sensitive determination of THC-COOH can only be performed in a sophisticated separate examination using GC–MS/MS [97–101], GC–MS-negative ion chemical ionisation (NCI) with high volume injection [102] or an additional clean-up before GC–MS-NCI [103].

Hair testing for THC-COOH supports the statement that developments in analytical techniques allow for new findings in the field of hair analysis. The development of novel methods resulting in an enhanced specificity and sensitivity is still necessary for future research.

3.4. Hair analysis in drug-facilitated crimes

In recent years an increase in drug-facilitated sexual assaults (DFSA) as well as in the use of drugs in robberies has been reported. Especially in cases of DFSA blood and urine analyses can be of limited use due to the frequently long delays between the actual assault and the involvement of the police. In order to enlarge the window of detection, hair analysis has proved to be suitable [104]. Drugs involved in DFSA or in robberies which can be detected by means of hair analysis are pharmaceuticals such as benzodiazepines (e.g. diazepam, lorazepam [105], bromazepam [106,107], clonazepam [107,108], flunitrazepam [109], alprazolam [110]), hypnotics (zopiclone or zolpidem [111,112]), sedatives (neuroleptics, some histamine H_1 antagonists), anesthetics (g-hydroxybutyrate or GHB [113], ketamine) as well as drugs of abuse (e.g. cannabis, ecstasy), substitutes like buprenorphine [114], or more often ethanol. Using novel and highly sophisticated analytical procedures in hair analysis resulting in enhanced sensitivity, for some pharmaceuticals even a single drug exposure was detectable.

3.5. Rare and new analytes in hair analysis

As described in Section 3.4 the developments in chromatographic procedures resulting in an enhanced sensitivity allow for the determination of an increasing number of rare drugs in hair samples, which previously eluded forensic analysis. Such seldom analysed drugs are g-hydroxy butyric acid (GHB) [115,116], kavain [117], cathinone [118], ketamine [119] as well as a lot of pharmaceutical drugs like benzodiazepines, antidepressants, neuroleptics, antiepileptics, sedatives and hypnotics, anaesthetics, cardiovascular drugs, antibiotics, chemotherapeutics and other drugs as recently summarized in an excellent chapter by Pragst [120].

3.6. Enantioselective hair testing procedures

Enantioselective determination procedures in hair specimens have mainly been described for methadone and its main metabolite [121–123] as well as for amphetamine, methamphetamine and designer amphetamines [124–128]. Together with the development of novel enantioselective procedures the enantioselective determination of special analytes in hair specimens opens up the possibility to conduct further research, e.g. distinguishing between possible sources of a drug in positive cases.

3.7. Clinical applications

Until now, most applications of hair testing have focused on forensic considerations. However, another exciting application of hair analysis are clinical investigations, because hair analysis complements blood and urine analyses by providing long-term information on an individual's drug use. Following clinical applications have to be taken into consideration.

3.7.1. Hair testing procedures during treatment programs and in risk populations

Monitoring of prescribed and non-prescribed drug use during (drug) treatment or maintenance programs provides valuable information for the diagnosis and management of patients. It has been repeatedly shown that the patients' information on their personal history of drug use is far from accurate and bioassays like urine or hair testing procedures can provide important information on an individual's drug use [129,130]. Self-report and urine test alone miss most drugs, especially when considering cocaine users in different risk populations [131–137]. In heroin maintenance programs hair analysis is a useful tool to differentiate between the ingestion of pharmaceutical heroin and an (additional) abuse of illicit heroin. In contrast to the pure pharmaceutical drug illicit heroin preparations contain codeine and acetylcodeine, the latter being discussed as a definite marker substance for the ingestion of illicit heroin preparations [138–140]. Also a possible reduction of an additional abuse of cocaine during the German heroin maintenance program in comparison to a methadone substituted group is being assessed by means of hair analysis [141].

3.7.2. Hair testing as a tool of clinical diagnosis

Urine-based toxicological screening procedures are limited to the detection of drugs over a short retrospective period. There are a lot of publications reporting on cases in which an exposure to pharmaceuticals or a not expected application of a special drug was found to be the cause of clinical (intoxication) symptoms, and in which hair testing procedures were used to produce the forensic evidence (e.g. chloroquine exposure [142] or chronic beta-blocker administration [143], amineptine abuse in a man with severe acne lesions [144] or diagnosis of toxic hepatitis after ecstasy abuse [145]). Especially in the Munchausen syndrome by proxy – an

extreme form of child abuse with a special forensic challenge – hair testing is a useful means to determine poisoning with different substances [146].

3.7.3. Hair testing as evidence of gestational drug exposure

Newborns of women exposed to drugs during pregnancy should be identified soon after birth so that appropriate intervention and follow-up can be done. In utero drug exposure can have a severe impact not only on the development of the fetus, but also on the child during later stages of life. Neonates born to drug-addicted mothers can suffer from neonatal withdrawal syndrome, which requires immediate and intensive substitution treatment. The results of hair analysis make it possible to confirm a fetal drug exposure and to reinforce the observation-based diagnosis [147,148].

3.7.4. Patient compliance

Generally, therapeutic drug monitoring (TDM) during pharmaceutical therapy is based on clinical observation, but also on urine or blood testing results. During the past few years hair analysis was used more and more frequently for such purposes to gain long-term information on an individual's drug use and patient compliance. Hair testing was established in epileptic management [120,149–151] as well as in the management of neuroleptic, antidepressant or antipsychotic medication [89,91,152], the treatment with indinavir [153–155] or in pain management with analgesic opioids [156,157]. For more details also on other pharmaceutical drugs see Sections 3.4 and 3.5 and the summary of Pragst [120]. In further studies the potential of hair as a biological specimen in therapeutic drug monitoring has to be proven, and possible correlations between hair concentration, blood levels, dose and therapeutic effects of various drugs have to be established. First results indicate a fair correlation between plasma and hair for several but not all medicines. As recently proposed, hair has potential as a biological specimen in TDM – at least as far as compliance is concerned and possibly as a longer term record of drug concentrations – and correlation between hair concentration, blood levels and clinical efficacy should be investigated for all drugs for which TDM is indicated [158].

4. Conclusions

Hair testing can complement conventional blood and urine analysis as it enlarges the window of detection and, by segmentation, permits differentiationbetween long-term therapeutic use and single exposure. Selectivity and sensitivity of MS/MS are a pre-requisite. Although there is still controversy on how to interpret hair testing results, particularly concerning drug incorporation and retention in hair, external contamination or cosmetic treatments, developments in analytical techniques today even allow the determination of low dosage compounds like THC-COOH, fentanyl, benzodiazepines or neuroleptics. The determination of rare or previously "undetectable" substances in hair specimens is a large research area. Therefore, quality assurance is a major issue of drug testing in hair resulting in new recommendations, validation procedures and inter-laboratory comparisons.

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Potential problems with the interpretation of hair analysis results

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Abstract

Due to differences in hair growth rate depending on anatomical region, age, gender, ethnicity and interindividual variability, interpretation of parent drug or/and metabolite concentrations in hair is not easy. Furthermore, as drug incorporation mechanisms into hair matrix is not yet fully understood, it is rather difficult to extrapolate details on time and dose from hair segment analysis. If incorporation sources other than from bloodstream (skin secretions and/or external/environmental contamination) are considered, interpretation becomes even more complicated. For evaluating possible passive contamination, it is essential to consider specific identification of metabolites, use of metabolite-to-parent drug ratios, assays of decontamination washes and analysis of specimens collected from other body parts. Cosmetic hair treatment, natural and artificial hair colour, differences in hair structure and specificity of analytical methodology may represent other bias sources affecting concentrations of drugs in hair. A suitable cut-off level related to the LOD will allow correct identification of drugs or metabolites in hair. Regarding the performance of different hair testing laboratories, little information is available at this time to what extent test results are comparable and their interpretation is consistent. Frequency of drug consumption and time intervals between multiple consumption or lag time between consumption and appearance in the hair has not been fully investigated and needs further research.

1. Introduction

Considerable amounts of recent publications related to alternative matrices in forensic

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Table 1
Classical versus alternative matrices

Specimens	Detection window	Use	Observations
Saliva	1 h–24 h	Concentration of parent drug	Special collection devices (Salivette)
Sweat	3 h–2 d/1 w	Only parent drug detection	Drug Wipe/Patch tests Cumulative exposure
Hair	>3d–m/y	Concentration of parent drug after chronic consumption	Easy to store at room temperature
Plasma/Serum	3 h–2 d	Concentration of parent drug allows evaluation of short term toxicity e.g. evaluation of performance (DUI)	Stored cooled/frozen
Urine	6 h–3 d	Only drug metabolites detection	Stored cooled/frozen

toxicology have appeared in the scientific literature [1–8]. Some aspects of these matrices compared to classical matrices can be summarised in Table 1. The major practical advantages of hair testing are: Larger detection windows depending on the length of hair shaft and evaluation of long term history compared to short term history (e.g. if urine is analysed).

One of the most critical issues in hair testing is the interpretation of the analytical results.

The first item to consider for interpretation is the purpose of these hair testings:

- Drug-related fatalities
- Revocation/restoration of driver's licenses
- Criminal responsibility
- Prenatal exposure
- Offences of narcotics law

2. Recommendations by the society of hair testing

To facilitate interpretation and to facilitate consensus approach the Society of Hair Testing [9] has already made some statements and recommendations about:

- *Specimen collection*
  - Hair should be collected from posterior vertex region of scalp
  - Hair should be cut as close as possible to the skin
  - Hair should be tied together
  - Approx. 200 mg hair should be collected wrapped in aluminium foil to maintain integrity and to avoid contamination and stored at room temperature.
- *Criteria for obtaining a positive result*
  - Evaluation of possible passive contamination
  - Identification of metabolites
  - Use of metabolites-to-parent drug ratios
  - Use of assay values of decontamination washes
  - Use of threshold values (cut-off values)

- *Metabolites recommended to be assayed*
  - Cocaine: Benzoylecgonine, cocaethylene
  - Heroin: 6-monoacetylmorphine, morphine
  - Cannabis: THC-COOH
  - Amphetamines: None for the moment
- *Metabolite-to-parent drug ratios*
  - Cocaine: benzoylecgonine/cocaine >0.05
  - Heroin: 6-monoacetylmorphine/morphine >1.3

3. Important parameters for interpreting hair results

3.1. Mechanisms of incorporation of drugs in hair

Before getting into details for interpretation of results, it may be useful to summarise the anatomy and physiology of hair [10–12].

Hair is a complex tissue and an annex of skin, originated in hair follicle in which the germination centre is formed by matrix cells, which are in active proliferation. In the hair shaft keratinized cells with different layers including cuticle and medulla are located. The hair surface is hydrophobic and the hair core is hygroscopic.

The hair shaft is constituted by fibrous proteins which are mostly α-keratins (85–93%), melanins (complex polymers derived from tyrosine (eumelanins and phaeomelanins), water (3–5%), lipids (1–9%) and mineral compounds (0.25–0.95%). There are some structural similarities between hair of different colour, ethnic origin and body region.

Different stages of hair growth have been described:

- Anagen stage: growing stage (4–6 years)
- Catagen stage: transition stage (few weeks)
- Telogen stage: resting stage (4–6 months)

The duration of stages growth may influence length of hair and density of a scalp. Hair growth varies between 0.7 and 3.6 cm/month [6], a mean of 1 cm/month is generally accepted.

Differences between scalp hair and hair from other anatomical regions are not yet fully investigated, as far as growth rates and anagen/telogen ratios are concerned. For example, the telogen stage of pubic hair is approximately half of its lifetime. Several exocrine glands associated with hair follicles have to be considered (merocrine glands= small sweat glands at hair scalp, apocrine glands=big sweat glands in the axially, holocrine glands=sebaceous glands).

Several models have been proposed for drug incorporation into hair [5,13–15]

1. Active or/and passive diffusion from blood into growing cells in hair follicle
2. Idem 1+diffusion from body secretions e.g. sweat and sebum during formation of hair shaft
3. Idem 2+external environmental sources after hair shaft formation

Other parameters influencing transport of drugs across biomembranes have also been shown to influence drug absorption by matrix cells in growing hair follicles: molecular size and structure of drug, microenvironment (concentration gradient/pH gradient), nature of biomembranes, blood flow, plasma protein binding, lipid solubility of the drug (log $P_{o/w}$) and ratio of ionized to non-ionized drugs (PK_a, Henderson-Hasselbalch equation) [16].

It must also be noted that several enzymes are found in hair follicles e.g. phase I and phase II drug metabolism enzymes like alcohol/aldehyde dehydrogenases, carboxylases, esterase D, cytochrome P-450 containing enzymes, glutathionreductase, glucuronosyl-transferase (UGT), glutathione-S-epoxide-transferase and sulfotransferase [16].

In summary, it may be said that the main factors influencing drug incorporation in hair are melanin affinity, lipophilicity, basicity (pK_a) of drugs.

In a study evaluating the role of melanin in drug incorporation, it appears that removal of melanin from hair does not eliminate totally hair colour bias for cocaine [17]. A study with 550 former cocaine users with 250 black, 150 brown, 100 red and 50 blond hair specimens is planned in the US [18]. As a model for the drug accumulation in melanin pigments an affinity chromatography study was made by Knorle et al. [19]. In this study melanin was immobilised on aminopropyl-silica to investigate drug affinities to melanin. The drugs tested were: chloroquine, haloperidol, zotepin, desipramine, clonazepam, flunitrazepam, trimipramine, sulpiride.

3.2. *Cut-off values and frequency of consumption*

Cut-off values are of major importance for interpreting hair results. An interesting proposal was made by Kintz [7] and is summarised in Table 2.

Precise estimation of consumption date of drug consumption is not possible. Estimation can only be made in intervals of months not in weeks, days or hours. Cut-offs and frequency of consumption have been investigated by Pépin and Gaillard [20] and are summarised in Table 3.

Table 2
Proposed cut-off values

Compound	Cut-off (ng/mg)
6-Monoacetylmorphine	0.5
Cocaine	0.5
THC-COOH	0.001
Amphetamines	0.5

Table 3
Cut-offs and levels of positivity[a]

Analyte	Cut-off	Levels of positivity		
		Low	Medium	High
6-MAM	0.5 ng/mg	<2 ng/mg	2–10 ng/mg	>10 ng/mg
Cocaine	1 ng/mg	<4 ng/mg	4–20 ng/mg	>20 ng/mg

[a] Pépin et al. [20]: These links are based on self-reported drugs use and subject to some caution.

3.3. Relationship between drug dose and hair concentration

Relationship between drug dose and hair concentration is another important parameter to consider. Several attempts to find a relationship between drug dose and hair concentration have been made by Cone et al. [13], Henderson et al. [14,15], Nakahara et al. [21], Pötsch et al. [16] and Pragst et al. [22]. The prerequisites for the studies of the relationship dose/hair concentration are maintenance of chain of custody, consideration of external contamination, proper definitions of positive results, performance with acceptable analytical methodology in qualified laboratories internal and external quality control/assurance and participation in proficiency testing with satisfactory performance.

3.4. Time course of appearance in hair

Beard hair is considered an interesting specimen since it grows at a similar rate to scalp hair and could be collected on a daily basis. Recent studies in beard hair provided interesting findings to evaluate the time course of appearance of a drug in hair after uptake of a single dose (Table 4) [23–27]. For the time being the time course of appearance in human hair is only known for a few drugs.

Moreover, animal experiments were used to investigate time course of appearance in hair. In a study with acute methamphetamine (MA) rat poisoning [28]. MA was detectable in hair at any time up to 24 h even if not detectable in plasma. There was a concentration peak at 2 h in the hair. In another study with a single dose of cocaine 5 mg/kg rabbit [29] was used. Appearance and peak of cocaine and benzoylecgonine concentration during day 1, declining rapidly on day 2 and remaining detectable up to 10 days was observed.

Table 4
Approximate time course of appearance in beard hair

Analyte	Days
Amphetamines	1
Codeine	1
Morphine	7–8
Meprobamate	4–5
Propyphenazone	3

3.5. Possible bias in hair testing

- *Racial bias*

Some studies suggested that variations exist in drug content depending on ethnic head hair types [30].

So ethnicity (caucasoid type, africoid type or mongoloid type) must be considered.

- *Bias by passive contamination*
- Another bias could be generated by environmental contamination. Hair may be easily contaminated from exterior because of its high surface to volume ratio Thus it is important to distinguish between passive contamination and active consumption [31–34]. Therefore decontamination procedures are also compulsory. Laboratories which also analyse large amounts of street drugs should be very careful and avoid contamination. There is also a need for assessing the occupational exposure of narcotics officers.

3.6. Cosmetic treatment of hair

The cosmetic history of hair must be considered to avoid pitfalls of interpretation, because there can be an alteration of all components due to colouring, bleaching, perming and UV-radiation [35–37]. These studies have shown that drug levels can be reduced but not eliminated. Moreover, opiates were more degraded after bleaching than cocaine and metabolites, In a recent study, Yegles et al. have shown that benzodiazepines are also degraded after bleaching of hair [38].

3.7. Influence of analytical techniques on interpretation

Detection techniques will also have an influence on cut-offs e.g. GC/MS versus more hyphenated technologies. Modified isolation methods may also generate results difficult to compare with existing data [39].

3.8. Quality assurance

An important issue in hair testing is quality assurance at all stages (preanalytical and analytical phases). Some attempts have already been made in this area like the NIST interlaboratory studies [40], the exercise made by the Society of Hair Testing [41] and SFTA validation studies [7]. But there is still a great need for further multi-centre proficiency testing which have to have wide availability.

4. Discussion and conclusions

Interpretation of hair analysis results is rather difficult because drug incorporation into hair depends on a multiplicity of different factors like growth rate, ethnicity, anatomical

origin, drug metabolism, type/colour, bioavailability, age, interindividual variability and gender. Nevertheless, some evaluation is possible between hair concentrations and consumed dose using terms of high, medium and low concentrations of xenobiotics in hair. Some correlation is also possible between hair segments and consumption time, if hair segments have been cut close to the root.

But there are also some important issues in hair analysis still open:

- Importance of external contamination
- Incorporation/storage in hair is highly controversial
- Need for international standardisation of analytical and interpretational criteria
- Lack of cut-off consensus for most important drugs.

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31 August 1999

Ms. Crickett Sweet
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Dear Ms. Sweet:

This letter is in response to questions posed by you to me regarding drug testing in hair. I will attempt to respond to each of the questions. A good review of the literature on drug testing in hair is contained in : Kintz, P. (Ed.) Drug Testing In Hair, Boca Raton, Florida: CRC Press, 1996. (ISBN 0-8493-8112-6). I suggest obtaining a copy of this text for a comprehensive review of the literature on this subject. The following are listed your questions and my responses based up available research literature.

1. *Can hair samples to be tested for drugs be contaminated by environmental exposure?*

Current findings suggest that cocaine and benzoylecgonine (and likely other drugs) may be readily incorporated into hair from environmental exposure and not removed by any one of several decontamination techniques. Radiotracer procedures allow a longer number of specimens to be analyzed with high precision under a variety of conditions. Several authors have proposed wash ratios as one criteria to distinguish active from passive exposure. Research on externally contaminated samples found that the suggested criteria were inadequate to identify the samples as contaminated. Several decontamination scenarios employing isopropanol, phosphates and solvents have also been subjected to research. None completely decontaminated the hair. For some drugs, metabolites in hair may distinguish active use from passive exposure.

Several variables must be considered in the analysis of hair besides the decontamination procedure. The most important is the method by which drugs are to be extracted from the hair matrix. Because no extraction solution can remove all of the drug present, dissolution of the hair matrix before extraction of the drug appears essential.

Research reviewed presents serious concern that external contamination of hair by drugs of abuse can easily occur. Any interpretation of hair analysis data should consider the prospect that the sample could have been externally contaminated. The pharmacokinetics of the incorporation of drugs into many tissues is well documented. However, substantial additional information on the

mechanisms for incorporation of drugs into hair, the decontamination of hair, and the meaning of the presence of metabolites in the hair are needed before hair analysis can be employed in forensic application.

The lack of a firm scientific basis was partially responsible for the 1990 consensus option of the Society of Forensic Toxicology (SOFT) that stated "Hair may be a useful specimen in forensic investigation when supported by other evidence of drug use." Review of the literature extends the SOFT consensus option that hair should not be used (even in preemployment testing) without corroborative evidence of drug use because the source of the drugs cannot be firmly established at the present state of knowledge of hair testing.

2. *Does there exist a potential for bias in hair testing for drugs of abuse?*

A primary concern in drug testing is that the methodology should provide an objective means for assessing drug use by an individual. Drug testing methods are biased if particular ethnic groups are predisposed to test positive more often in comparison to other groups. Presently, there is mounting evidence which suggests that bias exists in hair testing for drugs of abuse due to selective accumulation of drug by particular hair types. Evidence indicated that the binding of drug to Africoid and Mongoloid hair is substantially greater in comparison to Caucasoid hair. Hair color and cosmetic treatments all affect the binding of drugs to different hair types. Present data indicate that black hair binds more drug in comparison to brown hair and blond hair, and that bleached hair binds more drug in comparison to untreated hair.

There is currently no clear explanation for selective binding of drug by particular hair types, but differences in ultrastructure, morphology, and protein structure between hair types may be the basis for the observed differences. For instance, the binding of drugs may be greater to heavily pigmented black hair such as Mongoloid and Africoid hair in comparison to blond and brown Caucasoid hair. Structural differences could also account for the greater accumulation of drugs by brown and black hair. A recent explanation for the observed differences states that the binding of drugs to hair occurs largely in medullated sections. The degree of medullation in Mongoloid hair is greater than Caucasoid hair, and this could explain greater accumulation of drug by Mongoloid hair. For bleached hair, the binding of drugs may be greater than for untreated hair since bleached hair is highly permeable.

Although there are strong indications for selective accumulation of drugs by particular hair types, hair testing is currently applied in a variety of situations to assess drug use by an individual. The use of hair testing to make individual decisions may be unjust if selective accumulation of drugs by different hair types occurs which predisposes certain populations to test positive.

3. *Does GC/MS Provide a confirmation procedure for drug testing in hair?*

The concentration of drugs in hair are in the ng/mL range. No information is available about the minimum dose of drug intake which can be detected by hair analysis. The GC/MS procedure is state of the art. The GC/MS methods exceed by far all other chromatographic methods. Research indicates two different GC/MS methods would be best to meet the requirements of

analysis and confirmation. Studies also indicate that extraction with 0.1N HCL is as efficient to remove the target compounds from hair as enzymatic digestion that dissolved the hair.

GC/MS with either EI or CI provides accurate determinations of the target compounds. External contamination was incompletely removed by all approaches tested, making it difficult to differentiate incorporated drug from external contamination.

In preparing this letter, and its contents, 345 research and theoretical articles were reviewed by my staff and myself. Thus, the amount of time necessary to prepare this letter. If you would desire a list of all articles I will be happy to have it prepared and sent to you. If you have any questions, please call me at (616) 387-3350.

Sincerely,

C. Dennis Simpson, Ed.D.
Professor And Director