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ORIGINAL ARTICLE

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Ultrastructural alterations and environmental exposure influence the opiate concentrations in hair of drug addicts

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Abstract Hair samples were taken at autopsy from the head of 1 male and 1 female subject both known as drug abusers. Some of the strands were bleached by in-vitro cosmetic treatment.The bleached hair as well as the original hair samples were partly exposed to water or soil prior to further investigations and drug monitoring. The exposure times were 4 weeks or 6 months for water and 6 months for soil. The hair fibers were examined by transmission electron microscope (TEM) and by scanning electron microscope (SEM) investigations. The electron microscope studies confirmed that all experimental conditions had produced morphological alterations in the hair fibers. After exposure to water or to soil for 6 months as well as after storage of the clipped bleached hair in tap water at room temperature for 4 weeks, drug monitoring of formerly positive hair samples gave negative results. After storage of natural hair in soil or in water for 4 weeks the opiate levels had dramatically decreased. The samples were screened by fluorescence polarization immunoassay after enzymatic digestion. The results were confirmed by GC/MS.

Key words Hair · Opiates · Drug monitoring · Ultrastructure · Environmental conditions · Hair damage · Cosmetic treatment

Introduction

In the last few years a growing number of studies on testing human hair for drugs of abuse have been published [27]. By increasing distance from the scalp of the hair segment under analysis, decreasing drug levels in the hair specimen were frequently observed. Nakahara et al. [20] and other investigators [27] found that the drug levels in the sections towards the root were higher than in the sections nearest the tip. Nakahara assumed that some drugs may leave the hair within a period of 6 months or gradually decompose in the hair shaft.

Up to now little attention has been focussed on the ultrastructure of the keratinized hair shaft and on the physical and chemical properties of human hair with reference to the analytical results. This paper describes observations on the correlation of the morphology of the hair fibers with the drug level of the sample as well as on the influence of environmental exposure on the results of drug monitoring and their interpretation.

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Materials and methods

Hair samples were collected at autopsy from the posterior vertex from 2 documented abusers, both Caucasians. The colors of the hair specimens were dark brown and light brown for the female and the male respectively. The hair samples were divided into strands with a diameter of about 5 mm. The length of the hair fibers was 10–22 cm. All experiments were conducted in triplicate.

Morphological investigations

Light microscopy (Zeiss, Oberkochem, Germany): the hair samples were examined for the type of medullation from the root to the tip. Cross sections from the root side as well as from the tip side were investigated for pigmentation and medullation. The shape, the diameter and the cross-sectional area of the hair fibers were determined. Special attention was paid to findings caused by cosmetic treatment such as coloration or bleaching.

Cosmetic treatment applied *in vitro*

1. Bleaching was performed by immersing the hair strands in an aqueous hydrogen peroxide/sodium persulfate solution (6% or 10%, 1:1, v/v), adjusted to pH 10 with 25% NH_4OH, for 45 minutes at 35°C and subsequently by thoroughly rinsing with tap water.

2. Commercially available bleaching treatment (Poly Blond Ultra, Henkel, Düsseldorf, Germany) was applied to the hair strands according to the manufacturer's instruction.

Environmental conditions

1. Exposure to water: each strand was immersed separately in a jar filled with 250 mL of tap water. The hair samples were removed after 4 weeks or after 6 months, air dried and investigated by SEM, TEM and drug analysis.

2. Exposure to soil: each hair strand was put into a tissue embedding cassette (Medizinische Diagnostik Methoden GmbH, Giessen, Germany) and placed in soil 10 cm under the surface in a garden and recovered after 6 months. The hair samples were washed under running tap water to remove visible particles, then air dried before further investigations.

Scanning electron microscopy (SEM)

Hair fibers from the original hair strands as well as from the hair strands that had been exposed to the cosmetic treatment and to the different experimental environmental conditions were investigated at the root side and at the tip side. Prior to examination the samples were stored in a desiccator for 4 days, sprayed with a layer of gold and viewed in a Zeiss 926 scanning electron microscope at 15 KV (Zeiss, Oberkochem, Germany).

Transmission electron microscopy (TEM)

Hair fiber segments from the root side and from the tip side were fixed for 4 hours at 4°C in a 5% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4), and in a 1% osmium tetroxide solution in the same buffer, dehydrated in alcohol solutions of graded concentrations and propylene oxide, and embedded in Epon 812 (Serva, Heidelberg, Germany). Ultrathin sections were cut with diamond knives on a Reichert Ultramicrotome OM U2 (Reichert-Jung, Heidelberg, Germany). Longitudinal and transverse sections of the hair fibers, stained with a 1% uranyl acetate solution as well as unstained, were viewed in a Philips 301 transmission electron microscope (Philips, Eindhoven, Netherlands).

Drug analysis

The hair fibers were briefly rinsed with ether and air dried. Snippets from hair segments (100 mg) taken 6 cm above the scalp were digested in 3.0 mL 0.1 M Tris buffer (pH 8.3) containing 2 mg pronase E (Merck, Darmstadt, Germany) and 20 mg 1.4-dithioerythritol (Merck, Darmstadt, Germany) overnight at 40°C. After centrifugation the supernatant was removed to a clean vial. Drug screening was performed by fluorescence polarization immunoassay (Abbott, Wiesbaden, Germany) on an Abbott ADx according to the manufacturer's instruction. ADx calibration was established by drug-free and spiked hair samples [14].

All samples were confirmed by gas chromatography coupled with mass spectrometry (GC/MS) after solid phase extraction by means of Bond Elut Certify (Analytichem International, Frankfurt, Germany). Deuterated opiate substances were added as internal standard (Sigma, München, Germany). Derivatization was performed with 100 µL of a mixture of BSTFA, N,O Bis (trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Fluka, Buchs, Switzerland), and trifluoroacetic acid (9:1 v/v) for 20 min at 70°C. The reaction solution was evaporated under nitrogen, and the residue was dissolved in 30 µl ethyl acetate. Aliquots of 2 µl were injected into a GC/MS system consisting of a Perkin Elmer gas chromatograph 8600 (Perkin Elmer, Überlingen, Germany) with an ion trap detector (800), operated in the EI mode with 70 eV. The flow of carrier gas (helium) through the column (Permaphase DMS capillary column, 25 m × 0.32 mm, 0.15 µm film thickness) was 2 mL/min. The injection temperature was 280°C. The gas chromatograph was temperature-programmed from 100°C (2 min hold) to 280°C at 30°C/min (11 min hold). For each substance the standard deviation from the blank value ($\bar{x}_B$) was determined, the limit of detection (LOD) and linearity (LOL) were determined ($n = 6$) and calibration was done by spiking hair of drug-free subjects according to Möller et al. [18]. For morphine the LOD was 0.1 ng/mg hair; the LOL was 0.19 ng/mg hair.

Results and discussion

While almost all reports on hair analysis for drugs of abuse address the importance of exogenous drug contamination and washing procedures [1, 2, 11], only a few investigators discussed the stability of drugs in hair [13, 19, 20] or a possible linkage with pigmentation [21, 31]. Up to now little attention was paid to the effects due to hair degradation, the environmental exposure of the hair fibers to the climatic elements or to other so-called hazards of urban existence and the alterations caused by cosmetic treatments on drug findings.

Morphological investigations of the hair fibers prior to analysis showed that the degree of hair degradation can be observed by electron microscope studies. Intact ultrastructure and hair surface was found in the proximal hair segments. The distal segments from 6–8 cm above the scalp in the starting material showed slight amounts of hair damage. This damage can be frequently observed and it

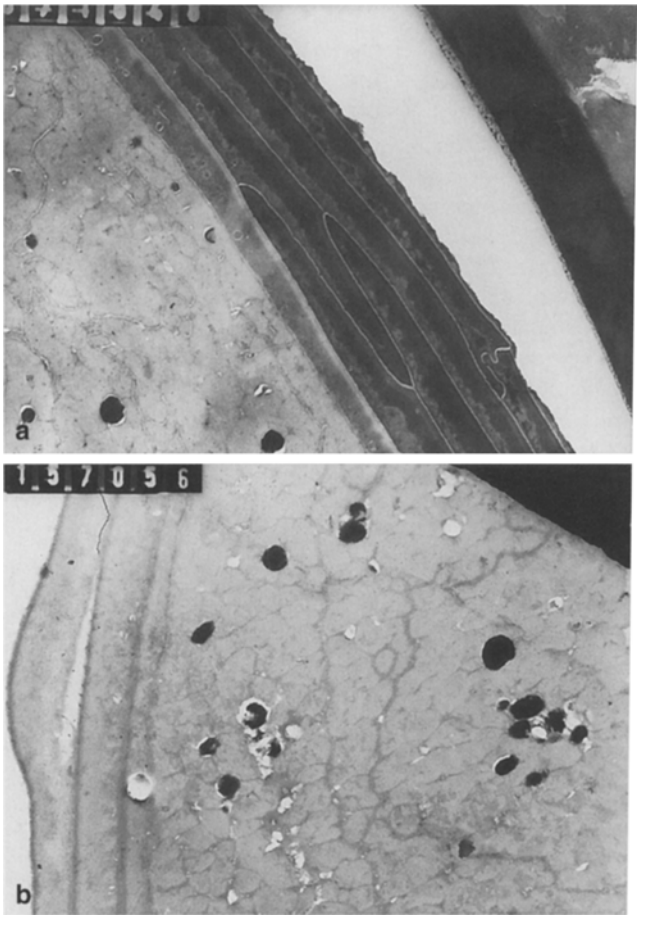

Fig. 1 Ultrastructure of human hair. TEM. (**a**) Intact structure of natural human hair fiber prior to bleaching. Magnification ×14 200. (**b**) Ultrastructure of slightly bleached hair. Magnification ×18 200

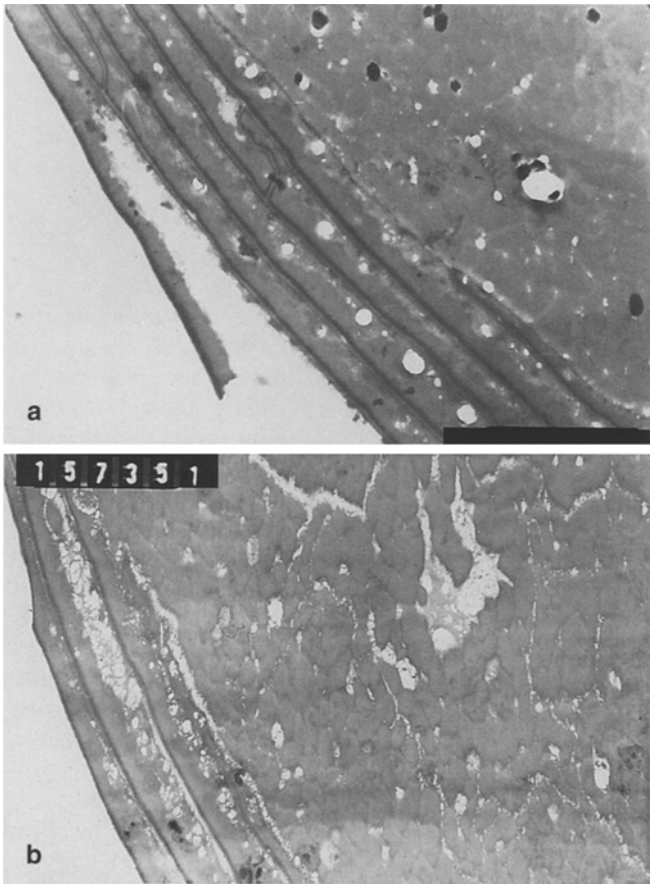

Fig. 2 Ultrastructural findings on human hair after exposure to water for 6 months. (**a**) Natural hair fiber (Fig. 1a). Magnification ×14 800. (**b**) Slightly bleached hair (Fig. 1b). Magnification ×18 200

Table 1 Results of drug monitoring for opiates on hair samples (n = 2) collected at autopsy prior to and after storage in water at room temperature for 4 weeks and 6 months and in soil for 6 months. n.d: not detected. GC/MS pos*: below limit of linearity

Hair samples	Subject 1 female	Subject 2 male	Subject 1 female	Subject 2 male
100 mg hair sample	A: natural hair fibers ng/mg hair		B: in vitro bleached hair fibers ng/mg hair	
Hair fibers prior to exposure to water				
ADx	pos	pos	neg	pos
GC/MS: Dihydro-codeine-TMS	11.3	3.7	n.d.	pos*
Morphine-TMS	pos*	pos*	n.d.	n.d.
MAM-TMS	1.5	1.0	n.d.	n.d.
Codeine-TMS	n.d.	n.d.	n.d.	n.d.
Hair fibers after exposure to water for 4 weeks				
ADx	pos	pos	neg	neg
GC/MS: Dihydro-codeine-TMS	0.2	0.3	n.d.	n.d
Morphine-TMS	n.d.	n.d.	n.d.	n.d.
MAM-TMS	n.d.	n.d.	n.d	n.d.
Codeine-TMS	n.d.	n.d.	n.d.	n.d.
Hair fibers after exposure to water for 6 months				
ADx	neg.	neg.	neg.	neg.
GC/MS: Dihydro-codeine-TMS	n.d.	n.d.	n.d.	n.d.
Morphine-TMS	n.d.	n.d.	n.d.	n.d.
MAM-TMS	n.d.	n.d.	n.d.	n.d.
Codeine-TMS	n.d.	n.d.	n.d.	n.d.
Hair fibers after exposure to soil for 6 months				
ADx	pos	pos	neg.	neg.
GC/MS: Dihydro-codeine-TMS	1.2	0.8	n.d.	n.d.
Morphine-TMS	n.d.	n.d.	n.d.	n.d.
MAM-TMS	pos*	n.d.	n.d.	n.d.
Codeine-TMS	n.d.	n.d.	n.d.	n.d.

obviously results from a combination of stress due to the climatic elements and cosmetic treatment.

It is well known that the hair shaft is gradually damaged by ageing. Hair is continuously subjected to a number of natural factors due to weather, atmospheric pollution and to repetitive or poorly applied cosmetic treatments such as hair washing, combing, towelling, brushing, perming, bleaching and permanent coloring [5, 16, 22, 28].

In natural human hair above the scalp the flat cuticle cells are regular and aligned close together to the fiber. Weathering effects can be seen more often as the distance from the scalp increases. The cuticle cells are irregular, partly broken, detached and lifted from the fiber surface. Sometimes they have become completely lost and the cortex cells are exposed. Split ends are also frequently observed. The bleaching of hair not only results in a light colored sample, but also has an enormous effect on the hair structure. During the bleaching reaction melanin granules are progressively dissolved. Severe bleaching results in a complete breakdown of the melanin granules [32] thus producing holes in the fiber (Fig. 1).

Electron microscope investigation confirmed that the experimental environmental conditions had produced alterations in the ultrastructure of the hair fibers. Briefly, the degree of degradation induced seems to be strongly influenced by the starting material as well as to depend on the exposure conditions and the exposure time. Where alterations in the ultrastructure of the starting material were already present, the additional damage caused by the experimental environmental conditions was more severe (Fig. 2).

Exposure to water often resulted in the lifting and/or loss of cuticle cells, high porosity in the endocuticle and in the partial disappearance of the intercellular cement which also means cell membrane complex degradation. Exposure to soil showed the greatest effect when a medulla was present. Alterations due to microorganisms could be observed on the hair surface in one of the samples [23, 25].

For drug screening tests on hair samples an enzymatic digestion was preferred because less personnel is needed and additional alterations such as hydrolysis of the drug molecule by various chemical agents can be avoided. For

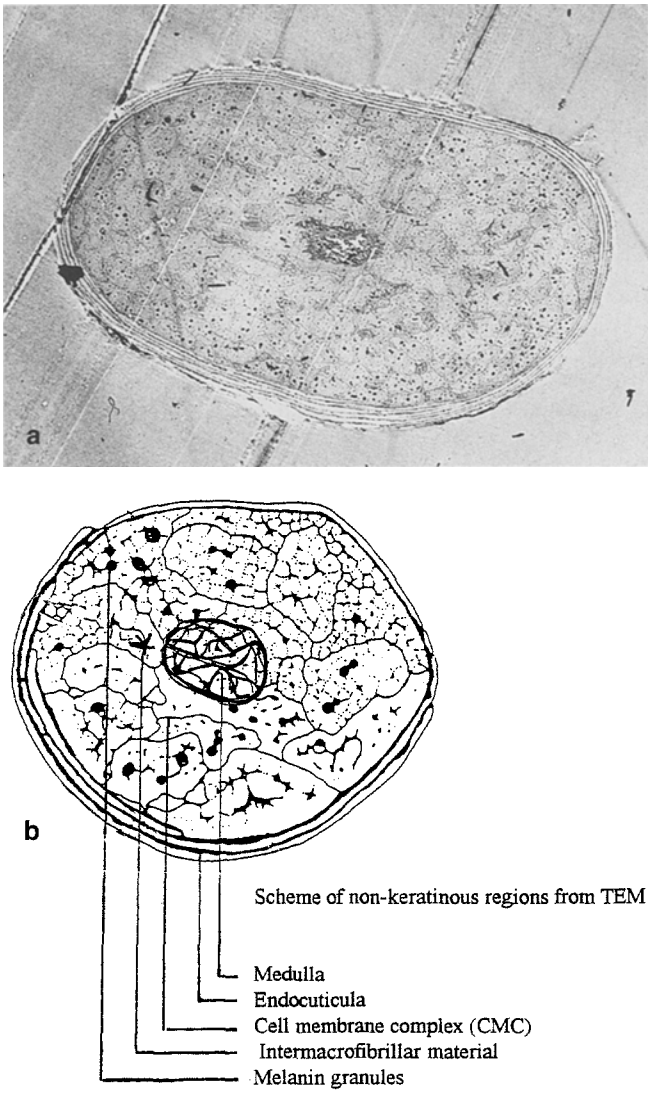

Fig. 3 (**a**)Transverse section of medullated human hair fiber. TEM. Magnification ×1225. (**b**) Scheme of non-keratinous regions from TEM: endocuticula, cell membrane complex (CMC), intermacrofibrillar material, melanin granules and medulla. Modification according to Kaplin et al. [10b]

screening purposes a fluorescence polarization immunoassay can be used on such samples, while the results obtained by immunoassays based on light absorption are strongly influenced by color and turbidity [14]. Our results demonstrated that neither the hair biomatrix nor artificial hair color substances altered the capability of ADx for opiate screening in human hair and confirmed the observations of Kintz and Mangin [14], who suggested that ADx by comparison with GC/MS can be used as a rapid screening test. Negative and positive results were confirmed by GC/MS.

In the starting material high concentrations of dihydrocodeine were found. In addition the concentration of 6-monoacetylmorphine (MAM) exceeded the concentration of morphine by several times indicating abuse of heroin [18]. The most interesting finding was that ultrastructural changes seemed to show a certain correlation with the results of drug analysis [24]. In vitro applied cosmetic treatment such as bleaching on clipped drug-positive hair samples and whenever water molecules were present for a certain exposure time, resulted in reduced drug levels. After storage of the clipped drug-positive hair samples in water at room temperature for 4 weeks even formerly drug positive hair fibers of intact ultrastructure gave negative results for MAM and morphine (Table 1).

Our findings support the data and the opinion of other investigators [12] that the results of drug monitoring of scalp hair may be biased towards false negatives rather than false positives. The experimental observations presented as well as the negative results obtained from occasionally performed drug monitoring on hair of drowned persons known as drug abusers and recent reports of decreased drug finding rates after different storage conditions of clipped hair samples [17, Sachs H. pers. comm.] point to the same direction. The results obtained after prolonged exposure to water molecules indicate that inaccessible domains in the keratinized hair shaft, where the drug substances may be located [1] and which have never been demonstrated, do not exist. There is strong evidence from electron microscope studies, from data of other investigators [6, 7, 11] and at least from the chemical and physical behavior of human hair [3, 4, 26, 29], that the drug substances in keratinized hair shaft material may be mainly linked to the areas of the non-keratinous proteins. Non-keratinous regions from TEM-findings are: endocuticula, cell membrane complex, intermacrofibrillar material, melanin granules and medulla (Fig. 3). The cell membrane complex (CMC) forms one of the main components of the non-keratinous regions in human hair with relatively few cysteine bridges and is always present even in non-medullated hair fibers. It is a typical product of keratinization processes and is formed from the 2 plasma membranes of adjacent living cells [7, 10a, 15, 30]. Feughelman [8, 9] has suggested a 2-phase model consisting of water-impermeable microfibrils oriented parallel with the fiber axis which are embedded in a water-penetrable matrix. This 2-phase model helps to explain many of the chemical and physical properties of the keratin fibers. The so-called CMC forms the only continuous phase in the hair and allows diffusion-controlled reactions to occur even within the intact ultrastructure of the hair fiber [26]. Diffusion is facilitated when ultrastructural degradation is present. Reviewing the literature under this aspect, many of the published data on hair analysis are found to be in accordance with our hypothesis, that the drug substances in the keratinized hair shaft may be mainly linked to the non-keratin protein components. Further studies on morphological parameters of the hair fibers as well as experiments that demonstrate a correlation of the results of hair analysis with the physical and chemical behavior of human hair are already underway and will be published soon. The experiments and the results presented in this paper already indicate that the interpretation of the analytical results obtained from hair samples according to the quantity of drug uptake must be done with caution.

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