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Penetration and Identification of Drug Use and Abuse in Hair by Various Advanced Analytical Techniques

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ABSTRACT

Now a days, besides blood, urine, saliva & hair is being recognized as an alternative & fundamental biological specimen for drug testing in the field of forensic analysis & clinical chemistry. Hair testing was used in legal cases, historical researches. This review on methodologically & practically used to the application of hair as a biological indication of drug use & abuse. The mechanism of drug incorporation into the hair were commented. This article include sample (hair) collection, preparation & extraction methods as well as the advanced analytical techniques such as immunoassay, HPLC-CE, GC-LC & GC-MS/MS. The outcome of hair analysis had been review for this categories: drugs of abuse(Opiates, Cocaines. Amphetamine, Cannabinoids) & Benzodizapines, prescribed drugs etc. finally the specific purpose of hair testing was discussed along with their interpretation.

Keywords: hair, drugs of abuse, prescribed drugs, advanced analytical techniques.

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INTRODUCTION

Human hair has a significant potential in forensic anthropology and has been extensively used in forensic investigations. Human hair has also been successfully used to assess drug and substance abuse as drugs of abuse cannot be often detected in body fluids[1]. Using very sophisticated analytical techniques such as immunoassay, GC-LC, GC-MS/MS, HPLC-CE investigators are now extensively using human hair to solve cases of poisoning and drug and substance abuse. Hairs may be defined as slender filamentous outgrowths of the skin and are primarily composed of keratin. It differs from one animal species to another in the basis of length, color, shape, root appearance and morphological characteristics. There is also a considerable deal of variability in the types of hairs that are found on the body of a particular animal. In humans, hairs are distributed on the head, pubic region, arms, legs, and other body areas[1].

Hair appears to be a fairly uniform structure, is very complex and its biology is only partially understood[2]. Hair has two separate domains the hair shafts (external domain) which are cylindrical structures made up of tightly compacted cells that grow from the follicles (internal domain) which are small sac-like organs in the skin (figure 1). Hair follicles are embedded in the epidermal epithelium of the skin and are associated with the sebaceous glands. In the axillary and pubic areas hair follicles are also associated with the apocrine gland. Both sebaceous and apocrine glands empty their ducts into the follicle. The eccrine sweat glands are located near the follicles but do not empty their ducts into them. The innermost zone of the follicle, called bulb, is the site of biosynthesis of hair cells and it is in contact with capillaries. The cells in the bulb divide every 23 to 72 hours, faster than every other cell type in the body. Directly above the bulb is located the keratinogenous zone where the hair undergoes hardening and solidification. The final zone is the permanent hair shaft. The composition of human hair (depending on its moisture

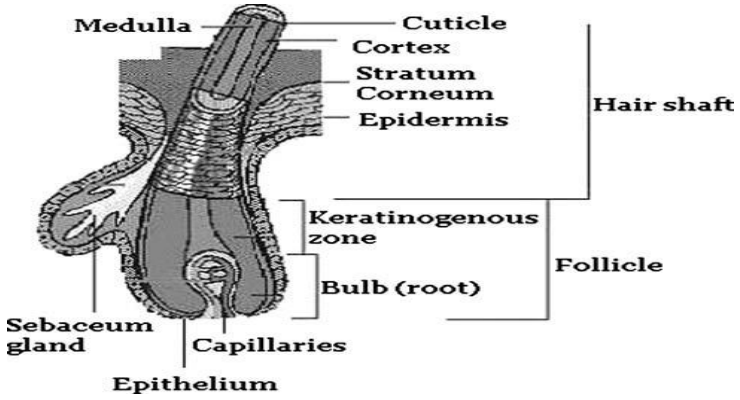

Figure 1: Simplified diagram of the hair structure, the associated sebaceous gland and the surrounding tissues.

content) is 65–95% protein, 15–35% water and 1–9% lipids. Mineral hair content varies from 0.25 to 0.95% (dry weight basis). The lipids found in hair are derived from sebum and the secretions of apocrine glands. They consist of free fatty acids, mono- di- and triglycerides, wax esters, hydrocarbons and alcohols. Hair proteins are rich in the amino acids glycine, threonine, aspartic and glutamic acid, lysine and cysteine[1].

Each hair has the same basic structure. There are three layers: cuticle, cortex and medulla (figure 2) CUTICLE is a outer layer of colourless cells, which forms a protective surface to the hair. It consists of elongated, overlapping (like roof shingles; if you rub a hair from base to tip it feels smooth, but if you rub it from tip to base it feels rough) individual cuticle cells, each having 0.5 to 1.0 μm thickness and about 45 μm length[1]. It regulates the chemicals entering and damaging the hair, and protects the hair from excessive heat, light and drying. CORTEX, the middle and largest layer, consisting of long spiral chains of cells like springs. is composed of long keratinized cortical cells, which form long fibers, about 100 μm in length[1].These are composed of small bundles of macro fibrils which in turn are formed from even smaller bundles of proto-fibrils - all long, spiralling, ladder like chains. Between the cortex cells, very small spaces are located called fusi. Fusi are filled initially with fluid but as the hair grows and dries out the fluid is replaced by air. In cortical cells are also found pigment granules containing mainly melanin. Melanin is synthesized in specialized cells, the melanocytes, located in the hair bulb. The amount, the density and the type of melanin in melanocytes determines the exact color of hair[1] whereas the 5–10 layers of shingle-like cells of the non-pigmented cuticle are responsible for the high chemical and physical resistance properties and the glossiness or shine of the hair.

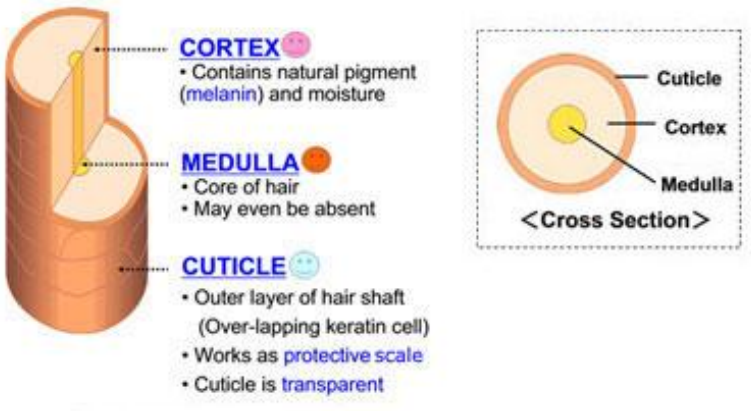

Figure 2 : Simple diagram of three layers of the hair

MEDULLA, the central layer of the hair, it consists of medullar cells. In human hair, medulla comprises a small percentage of the hair mass continuous along the central axis or discontinuous or it is completely absent. In general, the size of medulla increases as the hair fiber's diameter

increases. Individual human hair shaft ranges in diameter from 15 to 120 μm depending upon the hair type and the body region the follicle is located. It serves no useful purpose, and is not always present.

Hair formation and growth:

Hair formation begins in the third month of fetal life. Each hair grows through a follicle and is made up of epidermal cells that grow under the dermis. Initially the epidermis thickens and cells begin to grow down into the dermis. This down growth forms a cap over some of the connective tissue to create papillae whose cells multiply to form the hair. As these cells are pushed up the central canal of the hair shaft and away from their source of nourishment, they become impregnated with keratin. The morphogenesis of most hairs follow a cyclic pattern of cell proliferation and differentiation initiated in mid to late embryonic development and repeated throughout life[3].As hairs undergo a cyclical growth, intermediate and resting phases, the visible morphological characteristics under the microscope are sufficient to determine the phases of growth of the hair..

Stages of Growth & the life cycle of hair is composed of the anagen (active growth), catagen (transition) and telogen (resting) stages. The individual length of hairs depends on stage duration and growth rate. The average values for the above stages are 4–8 years, a few weeks, and 4–6 months, respectively[4]. ANAGEN, the active growing stage of the hair, a period of activity of the papilla and germinal matrix. This stage may last from a few months to several years. It is at this stage of formation at the base of the follicle that the hair's thickness, shape and texture is determined. Hair colour is formed in the early part of anagen[5]. During this phase the hair grows about 1 cm every 28 days. Scalp hair stays in this phase for 2–6 years. The hair on the arms, legs, eyelashes and eyebrows have an anagen phase of about 30–45 days. The average rate of hair growth it is usually stated to be 0.44 mm per day (range 0.38–0.48) for men and 0.45 mm per day (range 0.40–0.55) for women in the vertex region of the scalp[6]. The hair growth rate depends on the anatomical location, race, gender and age[1].CATAGEN, a period when the hair stops growing but cellular activity continues at the papilla and the hair shaft becomes fully keratinized. The catagen phase is the short transitional phase that enters the hair, following the anagen phase. The follicle becomes considerably shorter. This phase lasts for about 2–3 weeks. The hair bulb gradually separates from the papilla and moves further up the follicle[1].TELOGEN, the final stage, when there is no further growth or activity at the papilla. The follicle begins to shrink, and completely separates from the papilla area. This resting stage does not last long: towards the end of the telogen stage, cells begin to activate in preparation for the new anagen stage of regrowth.

The new anagen period involves the hair follicle beginning to grow down again. Vigorous papilla activity generates a new hair at the germinal matrix. At the same time the old hair is slowly making its way up and out of the follicle. Often the old and new hair can be seen at the same time in the follicle[5] The resting phase lasts for about 10 weeks for scalp hair while for the rest body surface hair lasts for about 2–6 years. On a healthy head, 80–90% of the hair follicles are in the anagen phase, 2% in the catagen phase and 10–18% in the telogen phase[1,7] .

Hair Structure:

Hair is a fibrous outgrowth from the skin of mammals which grows from papillae embedded in the bases of follicles situated in the dermis or true skin[4]. Hairs are fine strands of tissue which appear above the skin surface. They cover most of the body, with the exception of the eyelids, the palms of the hands and the soles of the feet. There are three different types of hair. LANUGO HAIR is the hair type produced in the very first cycle of hair growth when a hair follicle enters shortly after it develops in the embryo. These hairs are fine and soft, and they grow all over the baby's body and its main function is to retain body heat. They all grow at the same rate and so are of the same length. Around the eighth month of development this hair is usually shed and often a second generation of lanugo hairs then starts growing and lasts until the first three or four months of extra uterine life are completed. VELLUS HAIR is short, fine, "peach fuzz" unpigmented hair. It is very soft, much softer than lanugo and grows in most places on the human body in both sexes. It is usually less than two centimeters long and the follicles are not connected to sebaceous glands. Vellus hair is also present among pre-adolescents. TERMINAL HAIR is developed hair, which is generally longer, coarser, thicker, and darker than vellus hair. The phases of growth in terminal hair are more apparent than in vellus hair and the former generally has a longer anagen phase. Terminal hair contains a large hair follicle and sometimes, a medulla. During puberty many hair follicles in the pubic region, armpits, legs, chest and face, the latter two in case of males, transform from vellus hair to terminal hair under the influence of hormones. Terminal hairs can be further subdivided into different types depending on their nature and/or position of growth on the body. The different types of terminal hair types include hair of the eyebrows, eyelashes, head hair, beard hair, pubic hair and perianal hair. Longer, coarser hair, found on the head, on the faces of men, in ears and eyebrows, on the arms, legs and chest, and in the pubic region[8].

Mechanisms of Drug Incorporation into Hair:

The precise mechanisms by which drugs incorporate into hair remain unclear and need further investigation. Three models for incorporation have been proposed: drugs can enter the hair

through (1) passive diffusion from the bloodstream into the growing hair cells at the base of the follicle and then during subsequent keratogenesis they become tightly bound in the interior of the hair shaft[1], (2) diffusion from sweat and other secretions bathing the growing or mature hair fiber, or (3) external drug deposition from vapours or powders that diffuse into the mature hair fibre[4,9,10]. Indeed, a combination of these three routes is probably most realistic, and a full picture of the incorporation of drugs into hair shafts is likely to involve all three to greater or lesser extents. The incorporation is dependent on the drug concentration in blood, which depends on the ingested drug dose. Since hair is assumed to be growing at a constant rate, this model forms the scientific base for determining the time-course of drug use by performing segmental hair analysis. This means that the position the drugs are found along the hair shaft can be correlated with the time the drugs were present in the bloodstream[1].

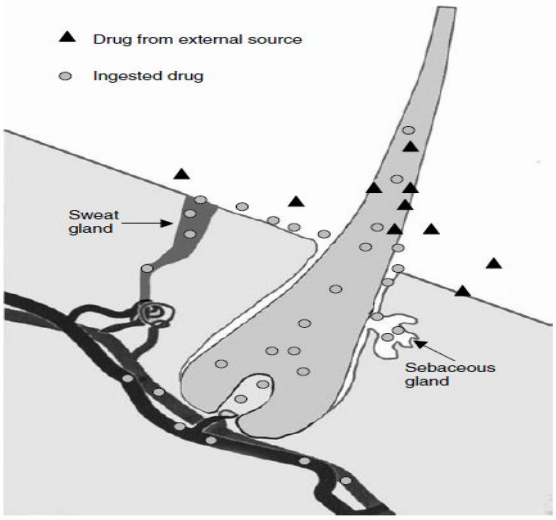

Figure. 3.: Three models of drug incorporation. Ingested drugs can enter the hair from the bloodstream feeding the dermal papilla as well as by sweat and sebum bathing the mature hair fibre. External drugs from vapours or powders may also incorporate into the mature hair fibre[9].

A more complex multi-compartment model is now acceptable in order to explain how drugs get into hair. In this model, drugs are suggested to be incorporated into hair via: (i) the blood circulation during formation; (ii) sweat and sebum after formation; and (iii) the external environment after hair formation and after the hair has emerged from the skin. Substances may also be transferred from multiple body compartments that surround the hairfollicle as well[1,11](figure 3).

External contamination of hair is divided into two categories: deposition of drugs on the surface of the hair and passive inhalation. External deposition of substances on the keratinized mature

hair fiber from air, water and cosmetic hair treatments has been suggested as a source for some of the trace elements present in hair, as well as, the reason for the difficulty in establishing cut off concentrations for trace elements in hair. Deposition from air could also be a potential route of entry into hair of substances that are smoked, such as amphetamine, cocaine, heroin, marijuana and nicotine[11,12,13]. The interpretation of false positive hair tests resulting from external contamination still remains a subject of debate. On the other hand, passive inhalation of substances that are smoked would result in the incorporation of the substances with the same mechanisms as the active use of substances[1].

Another possible mechanism of drug entrance into hair is the intradermal transfer of very lipid-soluble drugs like tetrahydrocannbinol (THC) to hair[14] and THC and especially the main metabolite THC-COOH have a very low incorporation rate into hair and THC is not highly bound to melanin, resulting in much lower concentrations in hair compared with other drugs. Additionally, THC is present in cannabis smoke and also can be incorporated into the hair only by contamination and possible explanation for the unusual elimination of cocaine and its metabolites in hair[15]. Another possible mechanism is the drug binding to melanin-related sites in skin, which would result in inter-individual differences in drug uptake into hair, reflecting the differences in melanin levels[11].

Three additional key chemical and physical factors, namely the melanin content of hair and the lipophilicity and the basicity of the drug itself, also influence drug incorporation[4,16]. The pH of melanocytes is between 3 and 5 and significant melanin affinity for basic drugs has been demonstrated in several experimental studies both with animals [17,18] and humans [19-22] or *in vitro* [23-25]. It was confirmed that drug concentration in pigmented hair was much higher than in blond or grey hair after the same dosage [21, 22,28]. The second important factor is the polarity of a drug or its metabolite. It has been many times documented that more polar metabolites benzoylecgonine, morphine or amphetamine enter the hair in a lesser extent than their more lipophilic precursors cocaine or 6-monoacetylmorphine or methamphetamine[26,27,29]. The third important factor is acidity of basicity of a drug substance. The matrix of hair is more acidic than blood pH 7.4, therefore the resulting pH gradient is more convenient for transfer of bases than for neutral molecules or acids[28].

However, the mechanism of drug incorporation into hair is very complex and still not clear. Another mechanism proposed is the binding of drugs with sulphydryl containing amino acids present in hair. There is an abundance of amino acids such as cysteine in hair, which forms crosslinking S-S bonds between polypeptides to stabilize the protein fibre network. Drugs

diffusing into hair cells could also be bound in this way[30].In conclusion, the incorporation of drugs and other chemicals into hair is proposed to occur from multiple sites, via multiple mechanisms, and at various periods during the hair growth cycle. The multi-compartment model seems to be more possible for explaining drug incorporation into hair[1].

Hair collection and preparation:

The vertex of the head has been preferred as a site for hair collection for most investigators. The vertex hair has less variability in the hair growth rate as compared to hair from other areas of the head, the number of hairs in the growing phase is more constant and the hair is less subjected to age and sex related influences. Vertex hair appears to be the most uniform in growth and the most consistent in the growth phase[1]. An average rate of head hair grows is 1 cm each month[31]. Hair collection protocols recommend clean hair that has not been dyed, permed, bleached or strightened for three months and that only the newest hair grown is sampled. For regular analysis of hair analysis, the sample collection is done using polyethylene gloves with precautionary measures to avoid contamination, which include environment (air, water, dust, oil, dirt) and also may be modified by cosmetic treatments and structure deformation of hair[32].

The amount of hair sample collection for the analysis should be about the diameter of a pencil or 100-200 mg and a sample cut from the posterior vertex region of the head, close to the scalp as possible in order to obtain the most suitable sample for the more resent drug use[1]. It is important to collect sufficient hair in order to carry out routine tests and also used for a repeat analysis or confirmation test by a second laboratory[33]. All hair samples were kept in air tight pre-washed dried plastic bag and stored in a dry place at ambient temperature in aluminium foil and envelope. Place the root ends of the hair sample at the edge of the Collection Foil where it is marked[34].

Hair collection offer some advantages over other assays; the collection of hair specimens could performed under close supervision with less embarrassing and intrusive than observed urine collection and hair does not require refrigeration and can be stored at room temperature and do not need to be directly analyzed after collection. Hair analysis allows a cumulative reflection of long-term abuse. Furthermore, the window of detection of drug abuse in hair tests is considerably wider than that of blood or urine assays and is only limited by the length of the hair[15]. Moreover, a second sample could be collected and matched to the first one by macroscopic or/and microscopic examination, or DNA/RNA procedures[35,36]. Hair is an external to the body sample and is subjected to external contamination. Any substance that might be introduced into the body by inhalation (smoking, or gas/vapor inhalation) must be considered as a possible source of

producing positive results in hair analysis through passive absorption by hair shaft. In the same manner any substance, liquid or solid, which is handled by an individual could be transferred to his hair. Thus, prior to analysis sufficient decontamination of hair is needed in order to avoid false positives due to passive environmental exposure[37]. Each stage of sample preparation carrier a risk of analyte loss or sample contamination, special measures to prevent these unwanted problem. Contamination of hair can occures from air, water, dust, perspiration, dyes, shampoos and other hair preparation; however, washing techniques can alleviate these problems (analytical methodology). Popular hair cosmetic treatments, such as bleaching or permanent waving, were found to affect the stability of incorporated drugs. The results obtained showed that the concentrations of all the drugs detected decreased in bleached hair in comparison with untreated hair. After cosmetic treatments, drug concentrations decline dramatically by 50–80% from their original concentration[4]. On the other hand, long-term effects of weather (sunshine, rain, wind) may also cause damage to the hair shaft, with subsequent impacts on detectable drug concentrations[38]. It is necessary to remove the surface contamination with minimum leaching effect when preparation of hair samples for analysis. The effect of leaching out of some elements from the inner structure of the hair are evident with some washing reagents. Hence, a washing procedure with minimum leaching effect and capability of removing loosely bound exogenous metals is desirable[32]. For decontamination of hair samples, different washing procedure are divided into the following general categories:

(1) washing with methanol, ethanol, acetone etc.[39,40,41]

(2) washing with sodium dodecyl sulfate solution or other detergents[42,43,44]

(3) washing with dichloromethane[45,46] and

(4) combined procedures using organic solvents and repetitive washings with phosphate buffers[47,48] . The washing procedure involving the use of triton X-100 adopted as a standard procedure for the present study.

The incorporation of drugs into hair was variable depending on the hair type and different colored hair. The thick black hair was most absorbent and thus it was the most resistant to decontamination, whereas thin brown hair was the less absorbent[49]. An effective decontamination procedure should include a short (e.g., 15 min) wash in an organic solvent (isopropanol) to remove water insoluble substances and a minimum of three 30-minute washes in aqueous medium (usually phosphate buffer) to allow swelling of the hair and diffusion of contaminating drugs into the wash solution. If the wash criterion fails then five additional 1-hour-washes should be applied[1,50].

Extraction Procedures:

After the extending washing of the hair samples and after a plateau has been reached in the drug concentration of the washing solvents, any drugs remaining in the hair define the drug fraction in the inaccessible domain of hair. These drugs are recovered from the hair matrix by extraction/digestion procedures and represent the drugs that were incorporated from the interior.

Hair digestion procedures normally expected to produce a clear, homogenous, particulate free liquid medium. However, use of some less effective methods can externally contaminate the hair sample and/or burn off internal elements containing within the hair matrix.

Standard Solutions: Stock solutions for standard curves were prepared for each drug at 1 mg/ml in methanol and stored at 4 °C. From these solutions, two sets of working solutions were prepared, one for plasma and one for hair analysis. For hair calibrations, the control hair samples (10 mg) were mixed with the appropriate working solution to achieve final concentrations 0.1-50 ng/mg hair.

Hair extraction procedures for drugs are divided into three main categories:

1. alkaline digestion[51,52]
2. acidic methanolic extraction [52,53,54]
3. Incubation with proteinase K9[42,43,55 ,56]
4. Basic Methanolic Extraction

Alkaline digestion (1 M NaOH Digestion)[51]:

Hair samples (10 mg) digested with 1.5 ml of 1 M NaOH overnight at 40 °C with agitation. Once cooled, the digested hair samples were filtered off and rinsed twice with 0.75 ml of 1 M NaOH. After the pH of the filtrate was adjusted to pH 5-7 with 6 M HCl, this solution was extracted with dichloromethane/isopropanol (5 : 1) by vortexing it for 1 min[57].

Digestion with alkali should be applied when alkaline stable compounds, such as Morphine, Amphetamines and Cannabinoids have to be analyzed[1].Alkaline digestions can result in hydrolysis of compounds such as Cocaine, Heroin/6-MACM and other ester compounds in hair[51].

Acidic Methanolic Extraction [MeOH/acetone/TFA (25 : 25 : 1)] [53]:

Hair samples (10 mg) containing 100 ng of the two ISs were extracted with 1.5 ml of MeOH/acetone/ TFA (25:25:1) for 1 h under sonication and stored at room temperature overnight. The hair was filtered off and washed twice with 0.75 ml of MeOH/acetone (1 : 1). The filtrate was evaporated to dryness under a nitrogen stream and the residue reconstituted in 1 ml

of pH 6.0 phosphate buffer. This solution was extracted with dichloromethane/isopropanol (5 : 1) by vortexing it for 1 min[57].

Incubation with proteinase K9:

The use of enzymes for hair analysis aims at the destruction of the hair structure and thus to the release of the incorporated drugs to the digestion buffer. For this purpose several enzymes like *β*-glucoronidase/arylsulfatase (glusulase), proteinase K, protease E, protease VIII and biopurase have been used.

Hair samples (10 mg) 100 ng of MPPH and PB-d5 (two ISs) were digested overnight in a solution containing Proteinase K (1 mg) in pH 7.5 phosphate buffer (1.5 ml) at 40 °C with agitation. Once cooled, the digested hair samples were filtered off and rinsed twice with 0.75 ml of phosphate buffer. After the pH of the filtrate was adjusted to pH 5—7 with 1 M HCl, this solution was extracted twice with 1 ml of dichloromethane/isopropanol (5 : 1) by vortexing it for 1 min[57].

All procedures are not suitable for extracting all classes of drugs, the different digestion procedures recover from hair different concentrations of drugs. Disadvantage of the enzymatic digestion of hair has been considered the fact that the resulting digest could denature, under certain conditions, the antibodies used for preliminary detection of drugs by immunoassays. However, in most cases, it is not the method of choice since it is rather expensive[1].

Basic Methanolic Extraction [MeOH/acetone/NH4OH (25%) (10 : 10 : 1)]10):

Hair samples (10 mg) with 100 ng of the two ISs were extracted with 1.5 ml of MeOH/acetone/ NH4OH (25%) (10 : 10 : 1) for one hour under sonication and stored at room temperature overnight. The hair was filtered off and washed twice with 0.75 ml of MeOH/acetone (1 : 1). The filtrate was evaporated to dryness under a nitrogen stream, and the residue reconstituted in 1 ml of pH 6.0 phosphate buffer. This solution was extracted with dichloromethane/isopropanol (5 : 1) by vortexing it for 1 min[57].

Hair analysis techniques:

The procedures for the detection of drugs in hair specimen are either the same or slightly modified than the procedures used for the detection of drugs from urine, blood or other biological fluid specimen. The hair analysis methods are Immunoassay technique , gas chromatography, liquid chromatography, radioimmunoassay, high-performance liquid chromatography, Enzyme Multiplied Immunoassay Technique, Fluorescence Polarization Immuno Assay, Thin Layer Chromatography, capillary electrophoresis, GC-MS and LC-MS/MS, Capillary gas chromatography–mass spectrometry. The use of infrared microscopy has

been also reported in hair analysis of drugs of abuse[58]. Analysis of drugs in hair has a minimum of analytical requirements, which are: sensitivity in the range of picograms per milligram of hair; specificity for parent drugs and lipophilic metabolites; and absence of matrix effects with hair digests[1].

Gas Chromatography / Mass Spectrometry (GC/MS):

For the hair analysis, GC/MS method is most frequently used techniques and that is superior than other method in specificity, selectivity and sensitivity. For this reason GC/MS tests should always be used to confirm positive test results. The GC/MS method is applicable for gathering both quantitative and qualitative results. The sensitivity levels are generally measured in nanograms or pictograms, depending on the particular drug that is being tested. GC/MS testing has a higher degree of specificity than TLC methods, and has between 100 and 1,000 times more sensitivity. The quantitation of drugs in hair is performed by selected ion monitoring (SIM) due to the low amounts of drug present while the deuterated target drugs are the usually used internal standards. These features enable the development of specific and sensitive procedures for a large variety of drugs or metabolites with sufficient accuracy at very low concentrations[16]. GC/MS has been used for the hair analysis of Opiates, Cocaine and related drugs, Amphetamines, Cannabinoids, other abused substances, Benzodiazepines, therapeutical drugs, pesticides and other environmental pollutants, as well as doping substances.

Three µl of the sample were injected into the GC/MS system operating in selected ion monitoring mode (SIM). The source and interface temperatures were 200 and 280°C respectively. The detection was performed with the following ions: Codeine *m/z* = 355 and 282, 6-MAM *m/z* = 383 and 327, Morphine *m/z* = 397 and 341, Cocaine *m/z* = 303 and 182, Methadone *m/z* = 294 and 72, MDMA *m/z* = 114 and 162, MDE *m/z* = 162 and 72, Nalorphine *m/z* = 423 and 367.

The GC/MS method involves combining a hair specimen with a specific organic solvent. The drug is then concentrated by evaporating the organic solvent before the GC/MS process begins. This process involves two distinct steps. First, GC separates the sample into its constituent parts than MS provides the exact molecular identification of the compounds. In the gas chromatography phase, a gas (generally helium) is forced through a silica column that has a polymer layer made of cross linked silicone. The vaporized drugs interact with the polymer causing them to separate. The time it takes each one to arrive at the column's end is referred to as the "retention time" (RT).

After the sample has been separated into its various compounds using gas chromatography, the

substances are then identified as the leave the gas chromatograph using mass spectrometry. This process involves firing an electron beam at each of the components. This breaks the components into different fragments and forces them to move through a magnetic field. The molecule that makes up a drug will always split into identical fragments. This is referred to as the drug's mass spectrum. Each drug has its own unique mass spectrum, similar to a person's fingerprint. A computer program is then used to compare and analyze the mass spectrum. This program can identify the parent compound, as well as the probable fragments.

GC/MS is a very specific drug detection method, however these tests are very expensive in terms of both the equipment required and the expert analysis that is needed in order to understand the results. This procedure is the most costly, averaging approximately $200 per sample to test. GC/MS involves using state-of-the-art technology which is very accurate. This level of accuracy means that they are frequently used in forensic, clinical, pharmaceutical and industrial laboratories.

The second part of the process involves inserting the silica column into the mass spectrometer. The mass spectrometer is made up of a vacuum chamber and quadropoles which surround the column of the gas chromatograph. As the drugs leave the column they are ionized by electrons and then forced to enter the quadropoles. The fragments are separated into different quadropoles according to their molecular weight and charge. The fragments are then converted into electrical pulses using an ion detector and the relevant information is feed into a special computer system. The mass spectra results are then produced to represent the original molecule [59].

Immunoassays:

Immunoassays are bioanalytical methods in which the quantitation of the analyte depends on the reaction of an antigen (analyte) and an antibody. Immunoassays have been widely used in many important areas of pharmaceutical analysis such as diagnosis of diseases, therapeutic drug monitoring, clinical pharmacokinetic and bioequivalence studies in drug discovery and pharmaceutical industries. The importance and widespread of immunoassay methods in pharmaceutical analysis are attributed to their inherent specificity, high-throughput, and high sensitivity for the analysis of wide range of analytes in biological samples. The detection system in immunoassays depends on readily detectable labels (e.g. radioisotopes or enzymes) coupled to one of the immune analytical reagents (i.e. analyte or antibody). The use of these labels in immunoassays results in assay methods with extremely high sensitivity and low limits of detection[60,61]. There are two types of immunoassays, homogenous and heterogeneous[63] depending on whether or not extra procedures are used to separate the complex of antibody

bound from the free drug derivative in the reaction mixture. These methods can be performed in either competitive or non-competitive designs. The choice from these designs is based on nature of the analyte, labelling chemistry available and the analytical parameter required from the assay (e.g. sensitivity, dynamic range, and precision)[61]. The heterogeneous immunological methods require washing or centrifugation to remove the unbound labels. On the other hand the homogeneous assays are based on the modulation of the label property by the immunoreaction[64].

Immuno assay methods applied in pharmaceutical analysis:

There are three types of immunoassays that are in common use. These are: 1) radioimmunoassay (RIA), 2) fluorescence polarization immunoassay (FPIA), 3) Enzyme linked immunosorbent assay (ELISA)[65,59].

Radioimmunoassay (RIA):

RIA has been applied for the detection in hair of Opiates[66], Cocaine/Benzoylecgonine [67], Phencyclidine[68] and Methadone[69].

Fluorescence polarization immunoassay (FPIA):

Fluorescence polarization immunoassay (FPIA) with Abbott TDx has been reported for the detection of Morphine in hair[70].

Enzyme linked immunosorbent assay (ELISA):

ELISA has been used for the detection of Buprenorphine in hair specimen [71]. With immunoassays has been also detected Fentanyl[72].

High-performance liquid chromatography:

High-performance liquid chromatography is similar to GC, except a liquid carries the sample through the chromotographic columns and the columns are not placed in a heated compartment. High-performance liquid chromatography is both sensitive and specific, and it is simpler and faster than GC. Gas chromatography and HPLC are reliable methods for screening, and they allow for simultaneous determination of a wide variety of different compounds. HPLC-UV and -FL methods, a derivatization procedure is required to increase sensitivity[73].

Analysis of drugs in hair by HPLC has not found a wide application. It has been reported the detection in human hair, of Haloperidol with HPLC-UV [74] of Phenytoin and Carbamazepine[75] Thiopental and Ketamine[76]. By HPLC-UV analysis have been also detected in hair Clonazepam, Flunitrazepam, Midazolam, Diazepam and Oxazepam[77,78] .

Capillary electrophoresis:

Capillary electrophoresis has been applied for the determination in hair sample of Heroin, Cocaine[79], Morphine and 3,4-Methylenedioxy Methamphetamine[80], 1,4-Benzodiazepines and

metabolites [78] and Methaqualone[81]. Capillary electrophoresis was accomplished using a 100mM phosphate running buffer pH 2.5 with an applied potential of 10kV at 20°C. Detection was by monitoring UV absorption at 200nm or between 190 and 400nm. Dried hair extracts were reconstituted in 0.1mM formic acid, the injection end of the capillary was then dipped in water for 5 seconds, a plug of 0.1mM phosphoric acid was loaded by applying 0.5 psi for 10 seconds, and the sample was electrokinetically injected at 10kV for 10 seconds. Using this technique, the limit of detection (S:N = 5:1) for MDMA was 2ng/ml[82].

Detection of specific classes of substances by hair analysis:

Hair Analysis for methamphetamines and Amphetamines:

It has been performed the detection of MA and AP in a single hair by HPLC-chemiluminescence (HPLC-ChemLu)1,[83]. A very sensitive and simple HPLC-FL detection method for the determination of AP related compounds such as MDMA (4,5-Methylenedioxymethamphetamine), MDA (4,5-Methylenedioxyamphetamine), AP (Amphetamine), MP (Methamphetamine)[73.] For the determination of MPs was developed with the detection limits 74.6 pg/mg and 51.4 pg/mg for MP and AP at S/N = 3, respectively. The method was applied to segmental analysis of some abuser's hair samples. After each segment was cut into 0.4 - 0.6 cm, the pieces were washed with 0.1% sodium dodecyle sulphate and ethanol, dried in dish dryer, and extracted with HCL-methanol (1:20, v/v). The quantitative results for 6 hair samples from abusers clarified the illegal use of MP for several months [84,85].

Hair Analysis for Opiates:

Hair analysis for opiates offers major advantages over urinalysis, including the reliable presence of the heroin metabolite 6-monacetylmorphine (MAM) in the hair of heroin users[41]. Hair analysis started in 1979 when Baumgartner and colleagues[66] succeeded to detect Opiates in the hair of Heroin abusers by RIA and to estimate their Opiate abuse histories by sectional analysis. In 1980, Klug detected Morphine in hair in the range of 0.1–10 ng/mg and hewas the first to fulfill the forensic toxicology requirements for hair analysis, because he confirmed RIA results by a chromatographic method (TLC with fluorescence detection)[86] . In 1986, Marigo et al. detected Morphine in the alkaline digested hair of Heroin addicts using HPLC with fluorimetric detection[87]. The use of GC-MS for the detection of opiates in hair started in 1991 with the identification of Heroin and 6-MACM[1,41].

Heroin use has to be differentiated from Codeine or Morphine use by the presence of 6-MAM in hair, with a recommended limit of quantification of 0.2 ng/mg hair using chromatographic techniques[10].Usually, the concentrations of basic drugs and metabolites were much higher in

pigmented hair than in non-pigmented hair[88,89]. 6-MAM was determined at a concentration of 0.44- 4.8 ng/mg, whereas Morphine was at 4.8-35.5 ng/mg. Overnight acid hydrolysis using hcl 0.1M was responsible for the difference, with that an unstable compound 6-MAM changed into Morphine[4].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed for the determination of Opiates in biological samples according to the emerging problem in drugs abuse. Opiates such as Heroin, 6-acetylmorphine, Morphine, Codeine, Acetylcodeine, Hydrocodone and Hydromorphone were extracted with 2ml chloroform: isopropanol (9:1) from human blood, urine, oral fluid and hair. The sample was then separated on an Allure propyl PFP column, with a mobile phase of acetonitrile-20mM ammonium acetate (7:3, v/v). Detection was implemented with MRM mode by an API4000 triple quadrupole tandem mass spectrometer[90].

In 1994, Edder et al. demonstrated the quantitative extraction of Opiates in hair[91]. Their optimum conditions for the SFE of Opiates in hair were: P = 25 Mpa; T = 40 ; flow rate = 0.7 mL/min; t = 30 min; eluent phase = CO2/MeOH/TEA/H2O (85:6:6:3 by vol.)[92].

The treatment of hair with 10% HCl for one hour at 100◦C has been considered to give quantitative extraction of Morphine from hair. It was also found that the major components in hair of Heroin addicts are 6-MACM together with Morphine[93,94]. It was shown that the extraction with methanol/trifluoroacetic acid was the best for extracting 6-MACM and Morphine with the minimum hydrolysis and the maximum recovery of 6-MACM[54]. The incorporation of Codeine and Morphine metabolites into hair samples and the recovery of opiates (6-MACM, Acetylcodeine, Morphine) during extraction has been also evaluated[95] . Moreover, combined extractions of Cocaine, Opiates and their metabolites from human head hair have been achieved in cases of polydrug poisonings. Hair analysis for Opiates is performed, in general, by using validated GC-MS methods, which are also used for the analysis of other specimen[56,94].

Hair Analysis for Cannabinoids:

To establish and validate a screening procedure for simultaneous identification and quantification of the Cannabinoids (Δ9-tetrahydrocannabinol(THC), Cannabidiol (CBD) and Cannabinol (CBN)) and the metabolite 11-nor- Δ 9-tetrahydrocannabinol-carboxylic acid (THC-COOH), in human hair, by GC/MS. The analytical procedures described in the literature for the analysis of cannabinoids in human hair by GC/MS are mainly targeted for the simultaneous detection of THC, CBD and CBN or THC and THC-COOH separately or simultaneously[96].

In head and pubic hair have been detected, with GC-MS, derivatized THC and THCA in the range 0.26–2.17 ng/mg and 0.07–0.33ng/mL respectively. By GC-NCI-MS the range of

derivatized THCA has been demonstrated to be 0.02–0.39 ng/mg[97]. The cutoff for the hair total Cannabinoid equivalents by immune detection was reported to be 0.06 ng/mg, while the LOD for THC and CBN by GC-MS was 0.04 ng/mg. In the same study was also reported the determination of THCA by GC-MS/MS at a LOQ of 0.1pg/mg hair[98] .

The determination of THCA in hair demands a more laborious examination of hair like GC-tandem MS[98], GC-MS-NCI[99] or additional cleaning steps prior to analysis[98]. Therefore, the exclusive detection of THC, CBN, and CBD, which is usually performed, will never raise the uncertainty for some cases.

In hair samples THC-COOH is present in the fg to pg/mg range, which requires special analytical procedures, such as GC/MS/MS techniques[100,101] or GC/MS in negative ion chemical ionization mode (GC/MSNCI)[15, 99,102,103].

Interpretation of hair analysis results:

Despite the fact that hair is considered an important-if not the most important-non-conventional biological sample and despite the bulk literature that exists on hair analysis the interpretation of hair analysis results, in many cases. Contamination of hair can occurs from air, water, dust, perspiration, dyes, shampoos and other hair preparation however, washing techniques can alleviate these problems. Substances deposited in hair from the environment are loosely bound to either the surface of the hair or to the hair matrix, and thus can be removed by appropriate decontamination procedures. It is necessary to remove the surface contamination with minimum leaching effect when preparation of hair samples for analysis. Decontamination procedures for hair are compulsory[44,48,104]. The second criterion that has been suggested for the discrimination of passive exposure from active use is the “metabolite criterion”. The application of this criterion consists of both the determination of the metabolites present in hair, after washing and digestion of the hair matrix and the determination of the metabolite to parent drug ratio. It has been shown that labile molecules, like Heroin and Cocaine, could be hydrolyzed in hair fibers and Cocaine could be hydrolyzed to benzoylecgonine during normal hygiene procedures with basic detergents[54]. Other sources of possible bias in interpreting hair analysis results have been considered the hair color, the hair type and the various cosmetic hair treatments. The consideration regarding hair color has been that darkly pigmented hair could bind larger quantity of drugs than lightly pigmented hair. Since the natural color component of hair is melanin several studies have dealt with the effect of the melanin content of hair on drug incorporation into hair. It is generally accepted that melanin binds, to some degree, a variety of drugs. However, it has been shown, by using non-pigmented hair, that drugs would bind in hair even in the absence of

melanin[74]. The studies that support the effect of melanin on drug incorporation into hair are based exclusively on the results of in vitro experiments with human hair soaked in Cocaine or Opiates (mostly Codeine) solutions[19] or Amphetamines[105]. Cosmetic hair treatments can alter hair texture and all hair components due to coloring, bleaching, perming and UV-radiation and the ingredients of hair cosmetic products are capable of reacting with drug molecules [106]. It has been reported loss of up to50%of Methamphetamine incorporated in hair after five months[107] and of deuterated Cocaine after six months[11]. proper interpretation of hair analysis results is the purpose of the hair testing. Hair analysis has been applied in forensic investigations (past drug use/abuse, acute drug poisoning, rape cases etc), in legal cases (criminal responsibility, addiction assessment, adoption and protective cases etc.), in historical research, in doping control, in risk assessment of prenatal exposure or chronic exposure, as well as, in other scientific or legal cases[108]. More specifically it has been applied to compliance studies of patients who received Haloperidol[109], Carbamazepine and Phenytoin[75] and various Tricyclic antidepressants[110]. In the field of prenatal exposure assessment, hair analysis has been performed in many cases in order to confirm the birth of "Cocaine babies"[40] and "Methamphetamine babies"[6]. Hair of newborns has been considered as a biological indicator of intrauterine exposure to many substances, such as Cocaine[111], Methylephedrine, Dihydrocodeine, Caffeine, Chloropheniramine; Cocaine, Opiates and Cannabinoids[6] and Nicotine[112].

CONCLUSION:

In conclusion, a positive result in hair analysis for drugs of abuse (the most studied field), after a sufficient decontamination process, should constitute specific identification of the metabolites and the parent compounds, by using the cutoff levels , and application of the metabolite and washing criteria, as well as, analysis of other body specimens for comparison. There is no doubt that hair analysis can provide valuable and valid information on previous drug use in the fields of forensics sciences and clinical toxicology. However, the confirmation of chronic environmental exposure to substances, by performing hair analysis, represents a challenge for the toxicologist and careful evaluation of the restrictions in the analysis of every class of substances is further required.

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