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Different Extraction Procedures for Multiple Drugs Abuse in Human Hair: Case Study

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ABSTRACT

In recent years, analysis of hair matrix has gained increasing importance in the determination of substances of abuse in many forensic fields. Hair analysis for drugs abuse can be expand the toxicological examination of conventional materials and thus contribute with additional important information to the complex evaluation of a certain case. Hair is a unique material for the retrospective investigation of chronic drug consumption, intentional or unintentional chronic drug toxicity in criminal cases. When compared with other biological samples, hair provides a large window for drug detection. Drugs get deposited in hair through blood circulation by various mechanisms, after its administration. The drug in hair is much stable and can be detected after a longer period of time as compared with other biological samples, such as urine, blood, and others. The main advantage of analyzing hair is its capacity to provide information over a long period of time before the sample was obtained, and also the fact that it accumulates the main drug and its metabolites. The present study discusses the analysis of drugs abuse in hair sample for the postmortem case, although the technique is common in many countries but it considers as new technique in Kuwait, and the forensic toxicology laboratory is just added this kind of analysis in Lab protocol, since the hair samples give a great and clean results of drugs and narcotics. Also, in this study different extraction procedures used to perform forensic toxicological analysis in hair as well as a sample preparation. The hair results of the postmortem case were supported by additional analysis of biological samples of both urine and blood that gives information of drugs have been taken. Immunoassay and high-performance liquid chromatography is an effective means for identifying drugs in various samples analyzed to give full survey in the present study case. As a result, hair analysis become part of forensic laboratory procedures, mostly due to better extraction methods allowing current instrumental analysis to detect lower amounts of certain drugs in hair, especially in the absence of other samples suitable for analysis.

Keywords
Kuwait, Hair analysis, Drugs abuse, Postmortem, Extraction.

Introduction
The abuse of drugs is a major social problem in all countries. To increase the ability and extent of drug testing, the development of new methods for the analysis of drugs in a single sample is required. This would be of benefit not only clinical research, but also for forensic analysis.

Urine sample usually used as a traditional sample to investigate about drug use, but urine testing only provides a short-term historical record of drug exposure. This disadvantage of urine drug testing calls for the development of alternative biological specimens that can be monitored for illicit drug use [1,2].

Human hair testing for the drugs of abuse offers the possibility of revealing an individual's recent historical record of drug exposure beginning at sampling time and dating back over a period of months to years. Since scalp hair grows at a relatively constant rate of 1 cm/month, it has been suggested that drug concentration along the hair shaft reflects degree of drug exposure and the distance from the hair segment being tested to the root is a measure of time elapsed since exposure. Furthermore, hair contains a relatively

high parent drug to metabolite ratio, which means that it is easy to identify the parent drug and the metabolites [3].

Mechanism of Formation of Hair and Hair Growth

Normal hair grows in a series of three stages: The anagen (growing stage), the catagen (transition stage), and the telogen (resting stage). During the anagen stage, the papilla promotes the hair growth, in the zone of cell and protein synthesis. It then moves up to the zone of differentiation, where the melanocytes produce the hair pigment, which is incorporated into each fiber inside the cortical and medullary cells by means of a phagocytosis-mediated mechanism. The intermediate filament proteins, consisting of helical di-sulphur bond dimers of acidic and basic-neutral filaments (kertatins), are the first proteins synthesized in the differentiating cortical cells. These dimers, at a later stage, pair to form tetramers that combine, becoming protofilaments. The cuticle is built higher up in the follicle and consists of cysteine rich proteins. The resulting cell complex, temporarily tied by desmosomes, tight and gap junctions is finally fixed by the cell membrane complex. Cells are in active proliferation in the root, whereas in the hair shaft any residual metabolism is lost. During the catagen stage, the metabolic activity of the root slows down and the base of the bulb migrates upwards in the skin through the epidermal surface. In the telogen phase, growth stops, the follicle atrophies and finally the hair is lost Figure 1C. The length and the rate of growth of hairs depend on the duration of the stages described. The duration of the stages varies from person to person, between the scalp hair and the hair of other body regions and even between different areas of the scalp of same person [4,5].

Mechanisms of Drug Incorporation into Hair

The simplest model proposed for drug incorporation into hair is the simple passive transfer. In this model chemicals move by passive diffusion from the bloodstream into the growing hair cells at the base of follicle and then during subsequent keratogenesis they become tightly bound in the interior of the hair shaft. 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. Therefore, segmental analysis can provide a "calendar" of drug use for an individual.

Several studies have shown a positive correlation between dose and amount of the drug in the hair. However, in other studies, has been stated that the distribution of substances along the hair shaft does not always correlate well with the time of exposure. 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 hair follicle as well. 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. However, more has to be known about the mechanisms and factors influencing this procedure, in order a precise correlation to be extracted, regarding concentration and location of drug in hair and the drug use history of individual Figure 1B [5,6].

Materials and Methods

Sample Collection

Sample collection should be performed by a responsible authority not necessarily a physician, respecting ethical and legal principles. If possible, hair should be collected from the posterior vertex of the scalp, and cut as close to the skin as possible. A sufficient amount of the sample should be collected to allow further analysis in needed. At least 100-200 mg sample is recommended. During collection, hair specimen orientation must be marked so that the root or tip end could be clearly identified. Analysis of hair involves a series of steps generally, starting with documenting weight, length, color and potential chemical treatment of hair and the anatomical part of

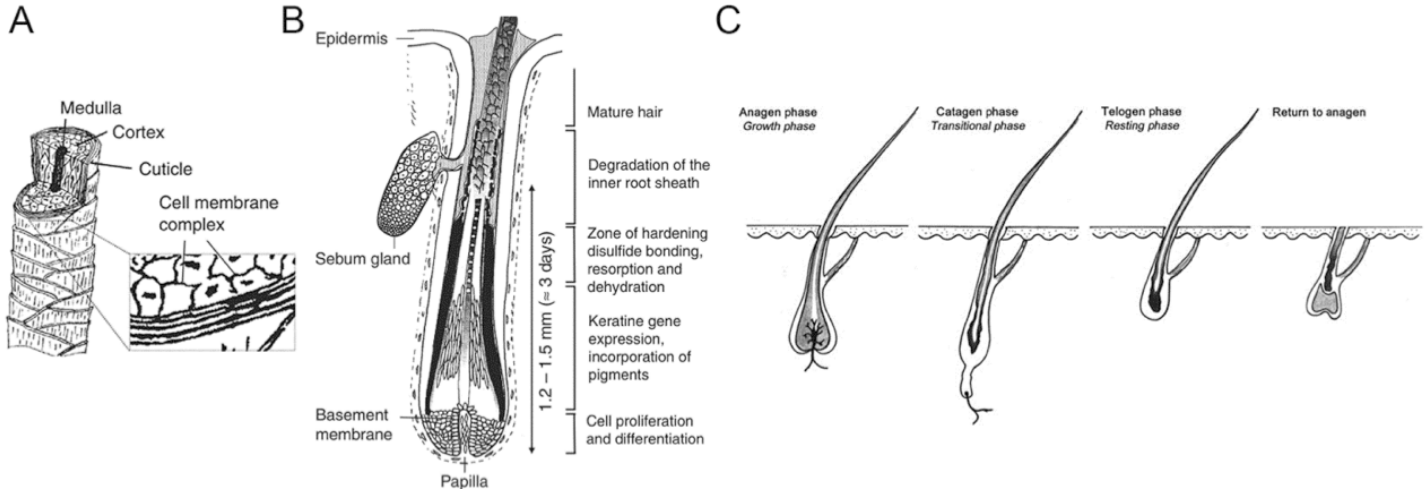

Figure 1: A) Structure and constituents of the human hair shaft. B) Formation of hair follicle from matrix cells on the basement membrane to the mature hair shaft. Drug incorporation from blood should occur in a 1.2-1.5 mm zone before completion of keratinization. C) Different phases of the hair growth cycle.

the body where the sample was collected. The collection kit should include: Chain of custody form, Foil and collection envelope, Security seal, Evidence bag, Transportation envelope, and all instructions for the collection of a hair sample should be recorded. Hair samples must be stored in a dry, dark environment at room temperature, away from direct sunlight. Hair samples should not be stored in the refrigerator or freezer, since swelling may occur and drug may lose.

Post-mortem hair samples can provide important information relating to the drug use history of the deceased. The post-mortem hair sample should be collected at the beginning of the autopsy to avoid external contamination with post-mortem body fluids or tissues leaked during the dissection. Also, if information is required relating to recent drug use, the collection of a pulled hair sample with roots is recommended in addition to the collection of a cut hair sample [7].

Decontamination and Washing

Washing the hair sample before the analytical process must be carried out to reduce external contamination that may present from the environment and passively deposited onto hair shaft surface that can cause confusion with actual drug use. As well as to reduce possible analytical interferences and improve the extraction recovery by the removal of external residues on the hair surface, such as hair care products, sweat, sebum and dust [8].

Since the achievement of remove all of external impurities from the hair matrix is practically impossible, the analyst should find the most favorable compromise by tuning the wash/rinse procedures. Non-protic washing solvents such as (dichloromethane, acetonitrile) do not cause hair to swell and consequently are believed to remove external contamination without loss of compounds incorporated in the hair matrix. In contrast, protic washing solvents as (water, methanol) cause the hair matrix to swell and therefore may also remove part of the existence drugs.

Segmentation, Grinding, and Digestion Procedures

For the segmentation hair is cut from the root into consecutive sections. The length of the segments depends on the chosen chronology for the analysis to be undertaken, using an estimated hair growth rate of 1 cm per month. After segmentation, hair should be grinded to increase the surface area during the extraction procedure. Pulverization is paramount since particle size distribution plays a decisive role in drug recovery. This means that it affects the method recovery and its reproducibility [9,10].

After segmentation and grinding, the analytes bound within the hair matrix are released. This is achieved by an extraction or digestion step. Analytes are released by enzymatic or basic digestion/extraction or by simple extraction with aqueous acid, buffer solution, or organic solvent. The three categories of hair digestion/extraction as follows:

Alkaline Digestion: Digestion with alkali should be applied when alkaline stable drugs, such as morphine, amphetamines have to be analyzed. In general, it involves incubation of the hair sample in 0.1-2.5 M NaOH, at 37°C overnight. Adjustment at pH=9 follows and the procedure continues with solid phase extraction (SPE) or liquid/liquid extraction (LLE).

Acidic Digestion: Acidic extraction of drugs from hair is usually carried out with 0.1-0.5 M HCl or 0.005 M H_2SO_4 at room temperature or at 37°C overnight. After neutralization of the solution, extraction follows.

Enzymatic Digestion: Enzymes can be used to promote the extraction of chemical substance from hair. The use of enzymes for hair analysis aims at the destruction of the hair structure and thus to released of the incorporated drugs to the digestion buffer. For this purpose, enzyme like β-glucoronidase is used. It should be underlining that different digestion procedures recover from hair different concentrations of drugs and moreover, not all procedures are suitable for extraction all classes of drugs. As the solvent extraction efficiency depends on the physical properties of the hair (whether it is thin or thick, porous or not, the type and quantity of melanin present). Differences due to variable melanin content of the hair are possible in cases where a major portion of the analyte is sequestered in the melanin granules. Thus, the choice for extracting and measuring these residual drugs from the hair matrix, should be the enzymatic digestion of the hair specimen [5].

Various liquid-liquid extraction (LLE) or solid phase extraction (SPE) procedures proposed for the separation of drugs from hair matrix are investigated.

Immunoassay Hair Screening

Screening for a range of drugs in hair is achieved through immunological methodologies. Immunoassays commonly used for rapid screening of drugs in biological techniques fluids are also available for hair. Laboratories that use immunological methods should ensure that screening assays have sufficient sensitivity to detect low drug levels in hair and are targeted to reflect the drug profile. Also, hair matrix should not interfere with the immunoassay, and all presumptive positive immunoassay screening tests must be confirmed using a more specific test for the target analyte like mass spectrometry.

Randox screening evidence investigator biochip array technology was used to perform simultaneous detection of multiple analytes from a single sample. The core of the technology is the randox biochip; a solid-state device with array of discrete testing regions containing immobilized antibodies specific to different drugs of abuse compound classes. Randox DoA hair investigator kit combines a mixture of classic and common drugs of abuse on one test. Allowing for simultaneous, multi-analyte detection. In an immunoassay, cutoff concentrations can be selected at the assay's optimal sensitivity, selectivity, and efficiency reducing the number of false positive and false negative specimens. When hair analysis is utilized, lower cut-offs should be employed [11-13]. In general, concentrations in the low picogram/ nano per milligram range are shown with its approximate equivalent cut-off in nanogram per milliliter in Table 1.

Table 1: Cut-off level of drugs abuse in screening hair test.

Analyte	Cut Off (ng/ml)	Equivalent Cut-Off (ng/mg)	Equivalent Cut-Off (pg/mg)
Amphetamine	2	0.2	300
Methamphetamine	7.5	0.2	300
Cocaine	5	0.5	500
Benzodiazepines	0.5	0.1	200
Opiate	2	0.2	300
THC-metabolites	0.5	0.1	1

LC/MS/MS Confirmation Screening

A Q-Exactive^{TM} Hybrid Quadrupole-Orbitrap^{TM} Mass Spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used to confirm the results generated from the screening test of the biological urine and blood samples. The Q Exactive system provides very good analytical performance in terms of reproducibility, linearity, and signal-to-noise, and addresses an extremely wide range of masses.

The mass spectrometer is a Benchtop LC/MS/MS system that combines quadruple precursor ion selection with High-Resolution, Accurate-Mass (HRAM) Orbitrap detection. Samples (5 µL) were injected in a 2.6 mm Accucore^{TM} Phenyl-Hexyl column (100 mm × 2.1 mm) and the LC column was heated to 40°C. Analytes were resolved at 0.5 mL/min using a mobile phase consisting of two solvents. The Mobile phases for LC-Screening are (Phase A: H_2O, $[NH_4]$+[HCOO]- 2 mM, 0.1% HCOOH), for 1 L mobile phase A use 1 L of water and add 126 mg of ammonium formate and 1 mL of formic acid. (Phase B: $[NH_4]$+[HCOO]- 2 mM, MeOH/ACN 50:50, 0.1% HCOOH, 1% H_2O), for 1 L of mobile phase B use 495 mL of methanol, 495 mL of acetonitrile, 10 mL of water, and add 126 mg of ammonium formate and 1 mL of formic acid.

The Q Exactive mass spectrometer was equipped with a Heated Electrospray Ionization Source (HESI-II) and was operated in the positive ionization mode. Parameters were optimized according to the methodologies from previous publications with some modifications [20-22], including a sheath gas flow rate of 53, an auxiliary gas flow rate of 14, and a sweep gas flow rate of 3 (manufacturer units). The spray voltage was set at 3kV, the capillary temperature was set to 269°C, the auxiliary gas heater temperature was set to 438°C, and the S-lens RF level was set to 55. The scan parameters were set as follows: Full MS scan with a resolution of 70,000, Automatic Gain Control (AGC) target 1e6, maximum Injection Time (IT) 100 ms, scan range 100 to 1000 m/z, and centroid spectrum data type.

Chemicals, Standards, and Instrumentation

All LC/MS Grade chemicals were purchased from Fisher scientific (Thermo Fisher), while dichloromethane, and ethyl acetate were purchased from Sigma Aldrich. Other chemicals are available in toxicology laboratory. Narcotics standards were purchased from lipomed (Arlesheim, Switzerland) and Chiron AS (Trondheim, Norway). Most of them received as powder of 10 mg sealed vials, and few received as liquid of 1 mg/1 mL of calibrated solution in methanol (ampoules). Crest Power Sonic Benchtop Ultrasonic was also used. Most tabletop ultrasonic baths have thin little disc transducers, while crest new power sonic line have real industrial stack transducers, ensuring uniform cleaning throughout the tank. This is achieved through a special frequency that sweeps by a margin of 3khz, removing “hotspots” and dead areas in the tank. All Crest tabletop units come with choice of 0-30 minute mechanical timer, or 0-60 digital timer, and all heaters in heated units are thermostatically controlled.

Agilent Vac.Elut. 12 and 20 position Manifold SPE unit was used in sample extraction. The corrosion resistance VacElut cartridge manifolds allow simultaneous processing of multiple bond elut. Cartridges. The clear glass base allows observation of the entire sample collection process.

Liquid-Liquid Hair Extraction

Liquid-liquid Extraction (LLE) is a widely accepted sample preparation method that uses different solubility of analytes in two immiscible or partially immiscible liquids. The advantages of LLE are the simplicity, good reproducibility and general acceptance for standard methods. The major drawbacks are the labor intensity, the large volume of solvent used and tendency for emulsion formation. LLE has been used as a sample preparation procedure for hair analysis of many narcotics and abuse drug substances. LLE is performed after hair washing and segmentation and a primary extraction procedure is needed to release the analytes from hair to a liquid phase. This extract is then submitted to an LLE extraction using several solvent mixtures according to the compound under analysis.

Solid Phase Hair Extraction

Solid-Phase Extraction (SPE) is simultaneously a separation and pre-concentration technique that uses a selective sorbent in a column. The sorbent selectivity is achieved using the appropriate material for a given analyte. The extraction procedure starts by loading the sample into the column. If the sorbent is chosen carefully, the analyte is adsorbed in the column. The next step is removing the analyte from the column, applying an appropriate solvent which elutes the analyte to the liquid phase. Solid phased extraction is used to separate different analytes from hair, such as drugs of abuse. The procedure requires a primary extraction to release the analytes for the liquid phase which is then applied to the SPE column.

Case History

A complete postmortem examination was performed for a middle age female that have been thrown dead in hospital parking area, she was known as an addict to different kinds of drugs and narcotics. A full survey was done for various biological samples as urine, blood, and hair.

Systematic toxicological analysis was performed, starting with a screening of urine, blood, and hair extract by means of immunoassay (Randox technology), as a routine analysis for the following substances: Benzodiazepines, barbiturates, tramadol, cannabinoids, amphetamine, methamphetamine, cocaine, opiates, tricyclic antidepressants, and synthetic cannabinoids. The positive

results were confirmed by High-Resolution Accurate-Mass (HRAM) Orbitrap detection. The results of biological samples were positive for many different kinds of drugs which was cause health hazards in many vital organs, as a long-term drugs user toxicity is more likely to have fatal outcome with chronic use.

Results and Discussion

Full investigation and data recovery were carried out under authority of the general department of criminal evidence in Kuwait (Forensic Toxicology Lab) including performance of all toxicology tests, in addition to Pathology Lab in Forensic Medicine Department that is deal with the autopsy and samples provided. The primary screening results were analyzed for urine, blood and hair samples by Randox technology, which is further confirmed by LC/MS/MS system. Table 2 and 3 summarized the physical characterization of the hair and observational screening and conformation biological data of postmortem case, respectively.

Table 2: Physical characterization of postmortem case.

Hair Color	Hair Length	Hair Weight
Dark Brown	8 cm	150 mg

The hair was collected from postmortem case by pathology lab in forensic medicine department, where the hair was taken from posterior vertex of the scalp. The sample was placed in aluminum foil from root and saved in envelope in dark dry place. As received in toxicology laboratory all information were checked, and all physical information was documented as in table 2.

Dichloromethane, and acetonitrile were used twice for washing to remove all external impurities from hair matrix, then the hair placed in filter paper and let it to dry naturally. The hair then should be grinded to tiny parts to increase the surface area during the extraction or pre-extraction procedure.

Different hair extraction procedures were carried out either direct, LLE or SPE. The summary of the extraction procedures is shown in Table 4.

Table 3: Screening and Confirmation Results of Postmortem Case.

Samples	Screening Results (ng/mL)	Confirmation Results
Urine	AMPH: +>500 BENZ: +>200 MAMP: +>175 OPIAT: +>1000 PGB: +>5000 ACE: +87.05	Amphetamine Diazepam, Oxazepam Methamphetamine 6-MAM, M-3-G, Codeine, Morphine Pregabalin Paracetamol
Blood	AMPH: +48.5 BENZ:+ 8.5 MAMP:+>175 OPIAT:+215 PGB:+930	Amphetamine Not detected Methamphetamine 6-MAM/6-MAC, Morphine, M-3-G, Codeine Pregabalin
Hair	ABPIN:+20.5 MAMP:+>175 OPIAT: +>1000 TRM:+>50 ACE:+29.8	AB-PINACA, UR-144 Pentanoic acid Methamphetamine 6-MAM, M-3-G, Codeine, Morphine Tramadol Paracetamol

All biological samples were positive to amphetamine and methamphetamine, which is mainly comes from addiction of "crystal meth" which is common street drug in Kuwait and it comes from nearby countries. Also, the samples were positive to opium where unchanged morphine and its metabolites occurred clearly in confirmation test. Other drugs were found either in one or two sample, and they were analyzed and investigated by LC/MS/MS.

The fatality of combination of multiple drugs and narcotics is a point of concern, especially in the presence of opium and methamphetamine. Previous studies shown a serious health problem that can be associated with drugs combination such as liver failure, cardiac arrhythmias, heart attacks, strokes and seizures. The toxicity of combination drugs is more likely to be fatal in chronic use [14,15].

Table 4: Different Extraction Procedures for Multiple Drugs in Hair.

Drug Name	Extraction Procedure
Opiates	In screw capped glass vial, 100 mg powdered hair sample was added to 1 M NaOH at 60°C 2h in ultrasonic, the pH adjusted to alkali digestion at 7.4. C_{18} column was pretreated with 2 ml methanol, 2 ml deionized water. Sample applied and washed by 0.5 ml distilled water and 0.5 ml methanol. Elution with 1.5 ml of dichloromethane/ isopropanol/ 25% NH_4OH (80:20:2). Evaporate to dryness and reconstitute with 500 µl methanol.
Amphetamine	Adding 1% HCl to 100-200 mg powdered hair and 2 ml of methanol in glass vial. Sonification at 45 °C overnight. The clear supernatant can be directly injected to LC/MS. Other method can be used instead of methanol is 1M NaOH with 2 ml acetonitrile.
Methamphetamine	Same as amphetamine extraction
Pregabalin	Alkaline digestion used to extract pregabalin from hair fragments. 1 M NaOH at 45°C 2h in ultrasonic, after separation of liquid mixture 2 ml of dichloromethane/ methanol was added and extracted. Allow to dryness and reconstitute.
Tramadol	The powdered hair was placed in screw capped glass vial then 2 ml of 0.5 M HCl was added followed by overnight incubation at 50°C. The sample was allowed to cool to room temperature, and the liquid phase was removed following centrifugation at (3000 rpm for 10 min). The sample solution was adjusted to pH=9 using potassium borate buffer before SPE extraction. C_{18} column was pretreated with 2 ml methanol, 2 ml deionized water and 2 ml of borate buffer. The sample applied to the column. After washing column with 2 ml 1% acetic acid followed with 2 ml acetonitrile, tramadol was eluted with 2 ml of n-butyl chloride. Eulation allow to dryness and reconstituted with 500 µl of methanol.
Benzodiazepines	To 100-200 mg of fine powder hair sample add 0.5 ml methanol and 0.5 ml of phosphate buffer pH= 8.5. Sonification of sample in 37°C 5h. Allow sample to cooling in room temperature, transfer the supernatant after centrifugation to LLE extraction funnel and add (90:10) of dichloromethane/ diethyl ether. Evaporate the extract to dryness under N_2 gas. Reconstitute with 500 µl of methanol/acetonitrile (1:1).
Synthetic Cannabinoids	Hair fragments were incubated overnight at 45°C in 2 ml of 0.1 m NaOH. The incubation mixture then manually extracted in sealed glass tube for 10 min with 5 ml of mixture of hexane/ethyl acetate (9:1). After centrifugation at 3500 rpm for 15 min, the organic layer was collected and dried under stream of nitrogen and reconstitute with 500 µl methanol.

Different extraction procedures can be applied to various drugs of abuse to be extracted from hair matrix where deposited drug is much stable and can be detected after a longer period of time as compared with other biological samples such as urine and blood, where pharmacokinetics of the amount of drug used and the concentration of the drug in hair remains controversial.

As summary for this postmortem case report and data analysis we can find that hair analysis gives actual support for other biological sample, thus the forensic toxicology laboratory in Kuwait needs a category to add this protocol of hair test as regular procedures.

Conclusion

The analysis of hair in forensic toxicology poses several issues, mostly that hair testing provides a 90 days drug use window, which makes hair testing an ideal solution for a number of drug testing programs. Moreover, this type of analysis may also provide information in cases of advanced decomposition of a corpse in the absence of other samples suitable for analysis. Drug hair analysis is becoming part of forensic laboratory procedures, contributing to criminal investigation. This is mostly due to novel and better extraction methods which reduce sample preparation time and increase extraction yield, allowing current instrumental analysis to detect lower amounts of certain drugs in hair.

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