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DOCUMENT

Standard operating procedures for the detection of substances of abuse in biological matrices in drug-related violence cases

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ABSTRACT

The "Pink Code" (*Codice Rosa*) is a protocol established in several Italian healthcare centers designed to offer a multidisciplinary approach to support victims of psychological, physical, sexual, or economic violence, including cases involving coercion and deprivation of freedom, as defined by the Istanbul Convention. This Convention is considered the most comprehensive legal framework for preventing and addressing violence against women and domestic abuse, which are recognized as violations of human rights. Emergency departments serve as the initial point of contact and play a key role in identifying cases that might otherwise remain undisclosed. The national framework is outlined in the Prime Ministerial Decree (DPCM) dated November 24th, 2017, entitled National Guidelines for Emergency and Socio-Health Care and Hospital Organization for Women Victims of Violence. This decree highlights the medico-legal importance of all healthcare personnel's actions in these circumstances. In situations of recent physical violence, medical care must address both clinical treatment and legal requirements. The careful collection of biological samples and strict adherence to the chain of custody are essential to preserve evidence that can be used in legal proceedings. Nevertheless, early-stage collection and transport may face challenges such as contamination or degradation if protocols are not properly followed. To mitigate these risks, the present procedure is based on fundamental principles: ensuring the highest level of privacy protection for the victim, fostering thorough coordination among all involved staff, providing dedicated spaces for each step of care, and implementing clear protocols that guarantee the integrity and legal validity of medico-legal processes.

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Introduction

In toxicology, the terms Chemical Submission (CS) or Drug-Facilitated Crime (DFC)[1-3] are used when there is suspicion of violence associated with criminal practices, particularly sexual assault, rape, robbery, extortion, and the deliberate abuse of adults, elderly, and children under the influence of psychotropic substances.

In other words, DFCs are criminal acts committed by administering a pharmacologically active substance to an individual with the intent of altering her/his behavior, perceptions, or decision-making ability. These crimes can also target non-consenting individuals with disabilities, even if they have voluntarily consumed a disabling substance.

The significant worldwide increase in reported DFC cases has drawn the attention of state authorities, prompting the development of a network of focal points to ensure specific knowledge and monitoring of the phenomenon.

Many substances used in DFCs are potent, fast-acting central nervous system depressants, with effects that mimic alcohol intoxication or anesthesia-like states. The pharmacological effects can include relaxation, euphoria, loss of inhibition, amnesia, altered perception, difficulty maintaining balance, slurred speech, drowsiness, loss of motor function, vomiting, incontinence, loss of consciousness, and even death (Supplementary Digital Material 1: Supplementary Table I).

Drug-Facilitated Sexual Assault (DFSA) is a subset of DFC and occurs when a person (male or female) is subjected to sexual acts while being unable to understand or is unconscious following the voluntary or involuntary ingestion of alcohol or psychotropic substances causing incapability of reacting to violence.

The involved substances (Supplementary Table I) may be surreptitiously administered to the victim or designated victims (Proactive DFSA), or the perpetrator may take advantage of a victim who has voluntarily consumed the substance(s) (Opportunistic DFSA).

The use of social media, online platforms, and dating apps has significantly increased the risk of new forms of sexual assault, including "date rape," where the perpetrator, often someone known to the victim, uses psychotropic substances or specific drugs (rape drugs) to sedate the victims, render them defenseless, and prevent them from remembering the assault.[3]

Several substances (including ethanol) can be used in DFSA. Gamma-hydroxybutyric acid (GHB) and flunitrazepam (Rohypnol®) are the most common "date rape drugs." However, many other central nervous system depressants and prescription over-the-counter drugs can be also used.[4]

Currently, there is no accurate data on the true prevalence of DFCs in Italy, and no accurate estimates of the number of DFSA cases annually occurring, although reports of such assaults are increasing. Many DFSA cases are still not reported because victims are reluctant to report the incidents due to embarrassment, guilt, or perceived responsibility, because they do not clearly remember the assault.[5]

Moreover, there are no standardized national or international procedures to facilitate the detection and identification of substances that may be used in DFCs.

Aim of the document

Currently, no specific Forensic Toxicology Laboratories have been designated for the detection of psychotropic substances in biological matrices of drug-related violence victims in different Italian regions. The aim of these Standard Operating Procedures is to promote the identification of at least one regional reference laboratory to ensure uniform intervention across the national territory and to suggest a list of specific procedures to provide the judicial authority with reliable points of reference and professionally trained personnel for analytical investigations in drug-related violence cases, which may extend over several months.

The standard operating procedures

Victims access to the emergency room

The protocol for victims of violence and abuse includes the collection of biological samples at the Emergency Departments for toxicological analysis, both for clinical and forensic purposes, subject to the victim consent, regardless of the time elapsed since the reported violence.

Biological samples have to be accompanied by appropriate chain of custody documentation, especially when they may have evidence-based value and medico-legal significance.

Furthermore, it is recommended that biological samples from possible violence victims have to be collected before administration of any medications by hospital staff. All collected samples have to be properly labeled, indicating the date and time of collection, and signed by both the operator who collects the samples and the victim. Finally, the samples have to be immediately sealed and stored in a secure location.[6-11]

Chain of custody

In medico-legal analytical assessments, the certainty of the biological sample's identity and its traceability has to be

assured throughout all operating phases, from acquisition of the sample to its analysis. For this purpose, the "chain of custody procedure" has to be applied, to document, with appropriate forms, all pre-analytical phases, manipulation, sample storage, as well as analysis and final report. The personal details of the victim, along with those of the healthcare professional collecting the sample, as well as the identities of the individuals delivering and receiving the samples, have to be recorded on the appropriate form. Additionally, all subsequent manipulations of the sample have to also be documented. Each performed operation on the sample must be signed and dated by all involved personnel. All documentation has to be maintained in paper format for a minimum of one year, after which it should be stored electronically for no less than five years from the date of the report with the operator signature whenever they receive and use the sample.

Official documents published on the topic by the National Institute of Health and the Forensic Toxicologists Group[6, 7, 9-11] provide a reference for all the procedures regarding the collection and analysis of samples.

Sample collection and storage of biological samples

While DFC cases that do not involve sexual violence may require only the collection of a urine and a blood sample, cases of sexual violence (DFSA) may require the collection of multiple biological matrices, as below listed.

Urine sample

Urine collection has to be carried out according to a procedure that ensures the identity, integrity and authenticity of the sample while respecting the privacy of the individual. The urine sample should be collected in a sterile container and for medico-legal purposes and it has to be divided into two aliquots (A and B) of about 10 mL each.

The analytical pharmaco-toxicology laboratory, which performs analyses with forensic and medico-legal value, will use the A aliquot for the quali-quantitative examinations, while the aliquot B will be sealed and stored at -20 °C at the same laboratory for any possible requests for counter-analysis and in any case at the disposal of the judicial authority. Urine must be collected in a timely manner upon arrival of the victim to the emergency room and, in any case, within 48 hours of the alleged assault, as many substances may be in the process of being eliminated and therefore undetectable.[6-9]

Blood sample

Blood must be collected at the same time as urine upon the victim 's arrival in the emergency room, and no later than 48 hours after the alleged assault. In this case, as already mentioned for the urine sample, many substances could be in the process of elimination and therefore may no longer be detectable. It is essential that the blood sampling is performed using a non-alcoholic disinfectant for skin cleaning. Two 5 mL tubes (A and B) should be used, preferably containing sodium fluoride and potassium oxalate to prevent matrix degradation and coagulation.[10]

As with the urine sample, one tube (A) will be used by the laboratory for analytical testing, while the other (B) will be stored at -20°C at the analytical pharmaco-toxicology laboratory which performs analysis with forensic and medico-legal value for any requests for counter-analysis and in any case at the disposal of the judicial authority.[6, 9, 10]

Hair sample

It may be essential to collect a hair sample to assess a possible exposure of the victim to an illicit substance when an assault is reported several hours after the incident, as the latter may no longer be present in the blood. It is crucial to plan with the victim, a hair collection to be carried out within a time interval of 30 to 45 days from the event of violence. Depending on the length of the collected hair, segmental analysis can be performed on different portions of the sample, allowing for a retrospective chronological reconstruction of potential substance exposure in the weeks preceding the assault.[11-13]

The amount of hair needed for analysis is a lock of approximately the pencil thickness, divided into two aliquots (A and B), and cut as close as possible to the scalp. It is very important that properly trained personnel perform the sampling, ensuring that informed consent and chain of custody forms are completely filled.

Cosmetic treatments performed on the hair after the assault can have negative effects on the analytical determination of psychotropic drugs due to the potential degradation of the substances under investigation.

Additionally, to detect even a single administration of so-called "date rape drugs," a micro-segmental hair analysis is necessary.[14]

Micro-segmental analysis involves cutting the hair into sub-millimeter intervals (approximately 0.4 mm), with each segment analyzed independently, unlike conventional segmental analysis, which examines one-centimeter segments.

This method enables the detection of substance exposure on a daily basis (with an average daily growth rate of 0.4 mm/day), and, more importantly, it allows the identification of these substances at very low concentrations, typi-

cally in the range of picograms per milligram of hair (pg/mg).[14] However, the laboratory will be able to follow the most suitable analytical strategy according to the specific context of each case, while acknowledging that a general recommendation to use segments of at least 0.5–1 cm is likely the most appropriate.

Storage and transport of samples to the analytical pharmacotoxicology laboratory for forensic and medico-legal analysis

Samples analyzed within a few days can be stored at a temperature of +4 °C. For long-term storage (months/years), samples have to be kept at a temperature of -18 °C to -22 °C.[3, 5] The keratin matrix should be stored in a dry place at room temperature. Stored samples must be kept locked and accessible only to authorized personnel responsible for their handling.[11] All sample processing procedures, from collection to storage, transfer, and possible disposal, must strictly adhere with the procedures established for maintaining the chain of custody.[6, 7, 9-11]

Analysis of biological samples using chromatographic techniques coupled with mass spectrometry

The use of chromatographic methods coupled with mass spectrometry in its multiple methodological possibilities (*e.g.*, gas chromatography or liquid chromatography combined with triple-quadrupole mass spectrometry or high-resolution mass spectrometry) is essential to demonstrate the possible administration of "rape drugs" and to provide a result that is technically, scientifically and legally valid and reliable. These techniques, known as "hyphenated techniques," are the only ones scientifically accepted and recognized as having forensic validity.[2, 4]

Toxicological analysis of biological samples (such as blood, urine, hair and saliva) must be conducted following scientifically recognized and technically validated protocols. To ensure the reliability and reproducibility of analytical results, it is essential to apply methods validated in accordance with internationally accepted procedures.[15] The validation process used by laboratories must consider a number of parameters, each of which must meet the acceptance criteria defined by established guidelines.[15, 16] The use of an internal standard, preferably in deuterated form, is essential to ensure the accuracy of analytical results. The introduction of the internal standard prior to sample preparation allows both extraction efficiency and reproducibility of instrumental separation to be monitored.

In addition, internal standards play a central role in quantitative analysis, particularly in the development of customized calibration curves for each analyte. These curves are constructed by comparing the analytical signal, typically the chromatographic peak areas of the analyte and its internal standard, of the unknown sample with those obtained from a series of solutions of known concentration. To ensure the accuracy, reliability and forensic applicability of toxicological data, laboratories are required to regularly participate in external quality assessment programs organized by scientifically recognized regional, national or international bodies.

Interpretation of results

The interpretation of analytical results in forensic toxicology requires an integrated approach that considers both the clinical and forensic context in which the data were obtained. Results should not be evaluated in isolation but must be contextualized according to the biological matrix analyzed, the time elapsed since the event, the pharmacokinetic profile of the substance detected and the clinical condition of the individual at the time of collection.

The detection of psychoactive substances in blood is indicative of recent intake (hours) and may be associated with the psychophysical state of the individual at the time of sample collection. However, due to the rapid elimination of many of these substances, timely blood sampling is essential to maximize the evidentiary value of the analysis. Although urine is less suitable for assessing consciousness at the time of the event, it remains useful for detecting drug use several hours to several days before collection. For forensic purposes, urine samples should ideally be collected within 48 hours of the suspected incident, as the interpretive value of the analysis decreases significantly beyond this timeframe. In addition, the precise time of intake of the substance cannot be reliably determined.

Hair analysis, if performed within 30-45 days of the suspected incident, is a valuable tool for the retrospective assessment of exposure to psychoactive substances. Segmental analysis can identify specific periods of use, making it possible to reconstruct a time profile that may correspond to the incident under investigation. In cases involving potentially endogenous compounds, such as GHB, it is crucial to distinguish between physiological concentrations and levels indicative of exogenous administration. This distinction is based on comparison with reference values reported in the scientific literature,[17, 18] to avoid a misinterpretation of the result.

Conclusions

DFC, including DFSA, pose a significant forensic and public health challenge. This manuscript outlines standardized

procedures for the collection, handling, and toxicological analysis of biological samples from suspected victims, emphasizing the importance of maintaining the chain of custody and using validated analytical techniques.

The integration of these procedures into emergency clinical pathways, particularly within the framework of multidisciplinary protocols such as the "Codice Rosa," ensures both the preservation of forensic evidence and the protection of victim rights. Including multiple biological matrices, such as urine, blood, and hair, enhances the temporal window for substance detection, while advanced methods such as micro-segmental hair analysis enable retrospective assessment in delayed cases.

Implementing a national network of accredited forensic toxicology laboratories is strongly recommended to ensure uniformity, reliability, and legal admissibility of results across the country. These standardized operational guidelines represent a crucial step toward improving the medico-legal response to drug-facilitated violence and strengthening the collaboration between healthcare and judicial systems.

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Conflicts of interest
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