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Toxicological analysis of some drugs of abuse in biological samples

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Abstract

Consumption of drugs of abuse is a scourge of modern world. Abuse, drug addiction and their consequences are one of the major current problems of European society because of the significant repercussions in individual, family, social and economic level. In this context, toxicological analysis of the drugs of abuse in biological samples is a useful tool for: diagnosis of drug addiction, checking an auto-response, mandatory screening in some treatment programs, identification of a substance in the case of an overdose, determining compliance of the treatment.

The present paper aims to address the needs of healthcare professionals involved in drugs addiction treatment through systematic presentation of information regarding their toxicological analysis. Basically, it is a tool that help you to select the suitable biological sample and the right collecting time, as well as the proper analysis technique, depending on the purpose of analysis, pharmacokinetic characteristics of the drugs of abuse, available equipment and staff expertise.

Introduction

Consumption of drugs of abuse is a scourge of modern world, regardless the fact that we are talking about high-risk drugs or about the off label use of certain authorized medicinal products. It is a large-scale, multifactorial, dynamic phenomenon which affects all the age groups, but predominantly the one between 14 and 35 years old. Abuse and drug addiction, as well as their consequences are one of the major problems in the current European society (1- 3). This is due to their significant repercussions in individual, family and social level (crime, social marginalization, and death due to overdose or by suicide) as well as in the economic level: dependence treatment costs but also the costs of the therapy for viral and bacterial infections associated with the intravenous consumption (AIDS, HVC, or reappearance of TBC) (4, 5). Given the major risks associated with the drugs of abuse, their analysis in biological samples is a useful tool for:

- Initial diagnosis of drug addiction
- Checking an auto-response, a declaration
- Mandatory *screening* in some treatment programs
- *Screening* as a method of tracking drug effects over time
- Identification of the substance in case of an overdose
- Determination of treatment compliance

Discussion

Toxicological analysis represents the whole analytical processes through which the presence of a toxic substance in an analysed sample is determined. It includes the physicochemical methods for the isolation, identification and quantification of toxic substances in the air, water, soil, food, delict objects and organic products for the prevention or diagnosis of intoxications (6- 9).

Drugs of abuse are those substances which, as a result of pleasant effects they produce, are used for other purposes than the ones they are intended to. For example, the therapeutic effect in the

benzodiazepines case or the industrial use in volatile solvents case. Drugs of abuse are those substances whose possession, transport or storage is restricted by law, due to potential harmful effect on the consumer and include materials manufactured under license, as well as illicit products manufactured in clandestine laboratories or natural products (10- 14).

The methodology of toxicological analyses of drugs of abuse is developed based on:

- Type of sample used
- Scope of analysis
- Pharmacokinetic features and biotransformation of the illicit substance
- Available equipment and reagents
- Staff expertise
- Costs.

Biological samples. Depending on the purpose of the analysis, the substances of abuse may be determined from different biological samples.

Blood/plasma: first choice for the quantitative determination of drugs; therapeutic levels in the blood are low but, when they are consumed abusively, the concentrations may be 2-3 times higher.

Urine: first choice for screening of drugs of abuse. It is available in sufficient quantity and substances or metabolites are present in relatively high concentrations.

Hair: it is used for the determination of the history of an abuse substance consumption. Detection is possible at10-14 days to 90 days after ingestion.

Saliva: it is used for the screening of drugs of abuse consumed within the last 24 hours.

Meconium: reveals maternal history of drugs of abuse consumption in the last 20 weeks of pregnancy and allow the choice of therapy for mother and new-born.

Breast milk: it is used for the determination of the exposure extent of the infant to drugs of abuse.

For example, in table no. 1 are presented the chromatographic techniques used for the analysis of methadone cited in the literature, grouped according to biological samples in which the determination is carried out (15- 16).

Table 1. *Chromatographic analysis of methadone according to the biological samples*

BIOLOGICAL SAMPLE	GC-MS	HPLC	LC-MS
Plasma		Schmidt N. et al.,1992 Foster D. et al, 2001 Foster D. et al, 2003 Hallinan R. et al, 2006	Liang H.R. et al., 2004 Whittington D. et al., 2004 Etter M.L. et al., 2005 Lehotay D.C. et al., 2005 Rook E.J. et al., 2005 Quintela O. et al., 2006 Jenkins R. et al., 2006 Shakleya D.M. et al, 2007
Urine	Larson M. et al, 2009 Moore C. et al., 2001	Cheng Y.-F. et al., 1999	Dams R. et al, 2003 Shakleya D.M. et al, 2010
Breast milk			Choo R.E. et al, 2007 Jansson L.M. et al, 2007
Meconium			Choo R. E. et al, 2005
Umbilical cord	Nikolaou P.D. et al., 2008		de Castro A. et al., 2009
Placenta		Nanovskaya T.N. et al., 2004	
Saliva			Concheiro M. et al, 2010 Rodriguez Rosasa M.E. et al., 2003 Ortelli D. et al., 2000
Hair	Moeller M.R. et al., 1993 Lucas A.C.S. et al., 2000 Girod C. et al., 2001		Kintz P. et al., 2009
Sweating	Brunet B.R. et al., 2008		

The detection time of abuse substances is varying in different biological samples. For example, drugs of abuse are detected in saliva within minutes after consumption and in urine only after 4-8 hours (17- 19).

Biological samples matrix is very complex and contains other endogenous or exogenous substances in addition to substances of interest. This is the reason that, in most cases, is necessary to use specific isolation procedures (20- 24).

Table 2. *The detection time of certain substances of abuse in saliva and urine*

Abuse substance	**Saliva**	**Urine**
Marijuana	12-24 hours	Days/wk. Depending on the frequency of use
Opioid	12-24 hours	2-4 days
Amphetamine	24-48 hours	1-2 days
Benzodiazepine	24-48 hours	1 week
Cocaine	12-24 hours	2-3 days

Procedures for extraction of drugs of abuse from biological samples:

The *liquid - liquid extraction* (LLS): the method used for emergency analysis and for unknown analysis when substances with physico-chemical properties must be extracted. This process facilitates the extraction of a drug from aqueous solutions in organic solvents and involves a relatively high consumption of solvents and multiple operations of extraction and separation.

Solid phase extraction (SPE): the aim of this method is the extraction, purification, and, sometimes, the concentration of non-volatile or semi-volatile substances for analysis. It involves passing aqueous solution through a column with silica based desiccant, active carbon and resins. It is a more expensive process and often less sensitive (25- 29).

Testing of substances of abuse extracted from biological samples

The tests for substances of abuse shall be sub-divided into two types of analytical procedures:

- *Screening tests*: are quick, simple and requires a minimum previous processing of the sample. Examples: immunoassays, Thin Layer Chromatography (TLC).
- *Confirmatory tests*: are performant, sensitive, selective methods that reduce the number of false-positive / false-negative results. Examples: Gas Chromatography-Mass Spectrometry (GCMS), High performance liquid chromatography (HPLC), Liquid chromatography-Mass Spectrometry (LCMS).

1. Screening methods for the determination of drugs of abuse: thin layer chromatography, immunoassay

For the toxicological screening of drugs of abuse simple, quick and inexpensive analytical methods are required. Screening methods plays an important role in the forensic medicine laboratories, both in the analysis of incriminated objects as well as in the analysis of biological samples. Due to the large diversity of samples it is practically impossible to use extraction methods and sophisticated and time-consuming instrumental techniques for analysis of all samples. Therefore, it is absolutely necessary to use simple screening tests to restrict further research area (30- 33).

The conditions that have to be met by a method of analysis to be used as screening test:

- easy to performed
- quick
- not require a complicated and unaffordable equipment
- require few usual reagents
- not require highly qualified personnel
- inexpensive
- able to be performed also outside a lab
- require a minimum processing of the samples.

The interpretation of screening tests results is a complex process, which requires an overview on limitations raised by the analysis method, by pharmacokinetic and biotransformation characteristics of the incriminated substance, but also by psychological, physiological and pathological pattern of the patient (including history of drug dependence) (34). A negative result does not necessarily indicate the absence of the substance, that can be present but at a level below the detection limit of the method. A true-positive result from a screening test will not indicate the dose, the time or the route of administration, and it doesn't make the difference between an occasional or a chronic administration. That's why, it is recommended to use more specific and performant analytical methods for confirming the screening test results (35- 38).

Screening tests are used in several purposes: forensic (analysis of incriminated samples), clinical or medical care (admission in substitution treatment, compliance of treatment, testing abstinence during therapy), occupational medicine, doping tests. The most commonly used screening tests at the present time are *thin-layer chromatography (TLC) and immunoassays.*

Thin-layer chromatography (TLC) is a wide spread technique used for the separation and identification of substances. It is used to analyse bulk active substances, pharmaceutical products, but also illicit substances or biological samples. Conventional TLC is a fast and low-cost method for qualitative analysis. Requires a minimum and readily available equipment, and experimental techniques are easily acquired. These determinations are not expensive, and can be carried out in laboratories with limited facilities (39- 42).

The Committee of Systematic Toxicological Examination of the International Association of Forensic Toxicologists (TIAFT), recommends 10 separation systems to identify medicinal substances and drugs of abuse, depending on their acid-base character. The correspondence between the different psycho-active substances and TIAFT recommended systems is shown in table number 5.

Table 5. *TLC methods for the analysis of psycho-active substances according to TIAFT*

Drug of abuse	**TA**	**TB**	**TC**	**TE**	**TL**	**TAE**	**TAF**	**TAJ**	**TAK**	**TAL**
5-methyltryptamine	56	-	-	-	-	-	-	-	-	-
amphetamine	43	20	9	43	18	12	75	-	-	-
benzphetamine	73	67	70	87	70	60	-	-	-	-
benzoylecgonine	21	0	1	-	-	-	-	-	-	-
cannabidiol	94	-	-	95	-	-	-	88	76	97
cannabinol	94	-	-	95	-	-	-	90	77	97
cocaine	65	45	47	77	54	35	30	13	0	2
Δ^9- THC	11	-	-	31	-	-	-	0	1	31
diamorphine	47	15	38	49	4	26	33	25	5	64
dimethyltryptamine	46	15	10	63	11	14	56	2	3	41

dimethyltryptamine	40	9	9	50	6	14	39	-	-	-
DOM	51	15	17	41	16	9	76	-	-	-
ketamine	63	37	63	79	64	68	72	47	4	43
lysergic acid	58	0	0	0	0	70	16	48	7	79
mescaline	20	3	10	24	12	6	63	2	9	51
methamphetamine	31	28	13	42	5	9	63	0	3	45
methadone	48	59	20	77	27	16	60	8	0	45
morphine	37	0	9	20	1	18	23	0	0	15
psilocin	39	5	9	47	9	14	48	-	-	-
psilocybin	5	0	-	0	0	80	1	-	-	-

Immunoassays are commonly used as screening tests for testing drugs of abuse. Often, they are not making any discrimination between the related compounds, so the results obtained are likely to be cross-reactive. For this reason, such methods are followed by confirmation using performant separation technique such as GC-MS for qualitative analysis and HPLC for quantitative analysis.

Used on a larger scale, the immunoassays methods are based on the antigen - antibody reaction. The quality of antibody is critical for the sensitivity, precision and accuracy of the determination. In order to generate a measurable signal, the immunoassay technique uses a specific antibody for the identified compound or class of compounds and a labelled form of the same compound or the antibody. The labelling may be done with a radioisotope in case of radioimmunoassay (RIA), an active enzyme in case of enzyme-linked immunosorbent assay (ELISA) or a fluorescent compound in case of fluorescence immunoassay (FIAS) (43- 46). Polarization Immunoassay (FPIA) use fluorescein attached to one compound (antigen) as marker. When it is bound to antibody, fluorescein molecular rotation slows down and leads to changes in the polarization of fluorescent emission. The polarization p is inversely proportional to the concentration of the unbound compound. The main advantage is the exceptional stability of the FPIA reagents, which enable the tracing of the calibration curve valid for longer time and the automation of the determination (47).

In the immunoassay methods, the biological sample requires a minimum previous preparation (e.g. simple centrifugation in the case of urine). After the initial immunoassay test of the screening program, usually we proceed to identify the particular compound involved using performant separation methods from complex matrices as biological samples (48- 50).

2. Toxicological examination of the drugs of abuse - confirmatory methods

In order to eliminate false positive or false negative screening tests, toxicological analysis is continued with confirmatory tests.

Confirmatory tests:

- Are effective methods, sensitive, selective, accurate, reproducible;
- Are performant column chromatographic methods: GC-MS, HPLC, LC-MS;
- Requires a laborious sample preparation stage;
- Requires expensive equipment and highly qualified personnel;
- Are analysed with higher costs.

Chromatography is a method of separating components of a mixture on the basis of their different distribution between two phases, one of which is stationary - generally fixed on a support (glass or aluminium plate, paper sheet, steel column, etc.) and other, mobile, which moves in relation to the fixed phase. This conducted to a different migration of the components leading to their separation. The mobile phase is gas in GC-MS methods and liquid in HPLC and LC-MS methods. The chromatography is used both for qualitative and quantitative determination of the chemical substances. The identification is based on the time required for the migration of the substance into the separation system. The assay is based on the proportionality of the amount to the peak area (51).

Confirmatory Tests - gas chromatography coupled with mass spectrometry (GC-MS)

The GC-MS is the gold standard for a reliable identification of the drugs of abuse in all kinds of samples. It combines the advantages of gas chromatography with those of mass-spectrometry. Mass

spectrometry is an analytical technique used to identify organic substances, based on pattern recognition's of fragments resulting from the ionization.

Table 6. *GC-MS methods for heroin and its metabolites analysis in biological samples*

References	**United Nations International Drug Control Programme**	**Goldberger B. A. et al.**	**De Giovanni N. et al.**	**Wang W.L. et al.**
Detected compounds	Drugs of abuse	Heroin, 6-acetyl morphine	Heroin, cocaine and metabolites	Cocaine, morphine, codeine, heroin and metabolites
Column	Diffrent capillary colum 12 m x 0,2 mm d.i.	Rtx-5, 15m x 25 mm d.i.	HP-1, 12 m x 0,2 mm d.i.	HP-1, 12 m x 0,2 mm d.i., film de 0,33 μm
Carrier gas	He	He ultrapur		He
Flow rate	1,9 mL/min	1,2 mL/min		1 mL/min
Injection type		splitless		splitless
Injector temperature	250°C	250 °C	250 °C	250 °C
Oven temperature	150 – 300 °C, with gradient of 12 °C/min	1 min. to 150 °C, followed by increasing to 200 °C with a gradient of 12.5 °C / min, maintained at 200 °C for 15 s, increasing to 290 °C with 30 °C/min, held for 4 min	120 °C for 1 min, increasing to 220 °C with a gradient of 20 °C / min, then to 260 °C with 5 °C / min and finally to 280 ° C with a 20 ° C / min, held for 2 min	70 ° for 1 min, increasing to 220 °C with a gradient of 35 °C / min, constant at 220 °C 0.25 min, increasing to 250 °C at 10 °C / min, held at 250 ° C for 3 min
Derivatization	SIM or TFA	-	BSTFA -1%TMCS	-
Observation	guide of recommended methods for analysing substances of abuse	Heroin: LOD 10 μg/L; range 1,0 – 250 μg/L, r 0,995 6-acetyl morphine: LOD 1,0 μg/L, range 1,0 – 500 μg/L, r >0,995	LOD 50 ng/mL; range 50-500 ng/mL	Hair: range 0,1 – 10,0 ng/mg Urine, saliva, plasma: range 1 – 100 ng/mL
Extraction	Liquid-liquid extraction or SPE with C18 cartridge	SPE with ZSDAU020 cartridge	SPE with C18 cartridge	SPE
Biological samples	Blood, saliva and urine	Blood, plasma, saliva and urine	Urine	Blood, saliva, urine and hair

The mass spectrum recorded is compared with libraries of mass spectrum. In table number 6 are listed a few GC-MS methods for heroin and its metabolites analysis in biological samples.

Confirmatory Tests – High Performance Liquid Chromatography (HPLC)

HPLC method is the first choise for the quantitative determination of drugs of abuse in all kinds of samples. Quantitative determination is based on proportionality between peak area and amount of the analyte in the sample. In Table number 7 several HPLC methods for analysis of the heroin and its metabolites in biological samples are presented (52).

Table 7. *HPLC methods for analysis of the heroin and its metabolites in biological samples*

	United Nations International Drug Control Programme	**United Nations International Drug Control Programme**	**Katagi M. et al.**	**Bourquin D. et al.**	**Low A.S. et al.**	**Umans J. G. et al.**
Detected compounds			Heroin and metabolites (monoacetylmorphinae, diacetylmorphine)	Heroin, morphine, codeine and their metabolites	Heroin, 6-monoacetyl-morphine, codeine, pholcodine, dihydrocodeine, morphine	Heroin, 6-monoacetyl-morphine, morphine
Detection	UV, λ=218nm	Electrochemical	Mass spectrometry	DAD, λ=210 nm	UV, λ=280 nm	UV, λ=218 nm
Column	LiChrosorb 60, 5 µm, 30cm x 4 mm d.i.	ODS, 5µm, 25 cm x 4,6 mm d.i.	Capcell Pak SCX 1,5 mm d.i. x 150 mm, 5 µm	C18, 125 x 2 mm d.i., 3 µm	Hypersil 3µm, 200 x 2 mm d.i.	LiChrosorb Si 60 5µm, 30 x 4 mm d.i.,
Faza mobilă	acetonitril e: ammonia: methanol: acetic glacial acid soluţia B	acetonitrile - 0.2M sodium perchlorate buffer / 0.005 M sodium citrate (1: 9, v / v)	10 mM ammonium acetate (pH 6.0) - acetonitrile (30:70, v / v)	o-phosphoric acid, dicyclohexylamine, acetonitrile, water,	dichloromethane, pentane, methanol, diethylamine	acetonitrile, ethanol concentrated ammonia, methanol, glacial acetic acid
Elution	Izocratic	Izocratic	Izocratic	Gradient	Izocratic	Izocratic
Flow rate	1,3 mL/min	1,9 mL/min		0.2 mL/min	0,4 mL/min	80 mL/ora (1.33 mL/min)
Extracţie	LLE or SPE with C-18 cartridge	LLE or SPE with C-18 cartridge	SPE	ex SPE with C-18 cartridge	SPE with C-18 cartridge	LLE
Biological samples	Blood, saliva and urine	Blood, saliva and urine	Urine	PLasma	Urine	Blood

Confirmation Tests - liquid chromatography coupled with mass spectrometry (LC-MS)

Liquid chromatography coupled with mass spectrometry is a modern hyphenated technique which combines the advantages of HPLC with those of mass-spectrometry. The mass spectrum recorded is

compared with libraries of mass spectrum. It is used for both qualitative and quantitative determination, having as advantages the selectivity and the increased sensitivity (53).

In table number 8 a few LC-MS methods for analysis of the methadone and its metabolites in biological samples are listed (54).

Table 8. *LC-MS methods for methadone and its metabolites determination in biological samples*

References	Vlase L. et al.	Dams R. et al.	Danielson T.J. et al.	Widschwendter C. G. et al.	Kelly T. et al.	Rosas M.E. et al.
Detected compounds	Methadone	Opioids, cocaine and metabolites	Methadone and her metabolites (EDDP, EMDP)	Methadone and quetiapine	Enantiomers of the methadone and of EDDP, EMDP	Enantiomers of the methadone and of EDDP
Detection	MRM (310 → 265)	SRM	MRM	MRM (m/z 310 → 265; 384 → 253)	MRM	SIM (m/z 310, 278, 313, 281)
Ionization	ESI	API	?	API	API	ESI
Column	Zorbax SB-C18 (100 x 3.0 mm, 3.5 µm I.D.)	Synergi Polar RP (150 x 2 mm, 4 µm)	?	Waters Acquity, C18 (50 x 2,1 mm, 1,7 µm)	AGP alpha-glicopro-tein	AGP alpha-glicopro-tein
Internal standard	-	Deuterated isotopes of the analysed compounds	Deuterated isotopes of the analysed compounds	D3-methadone	-	D3-methadone and D3-EDDP
Mobile phase	Acetonitrile: 0.2% formic acid 45:55 (v/v)	Mixture of 10 mM ammonium formate or 0.001% formic acid and acetonitrilein various proportions		Mixture of acetonitrile and 5 mM formic acid	20 mM acetic acid: isopropanol 93: 7 (v/v)	Acetonitrile: ammonium acetate buffer 18:82 (v/v)
Elution	isochratic	gradient		gradient	isochratic	isochratic
Flow rate	1 mL/min	300 µL/min		0,25 mL/min	0,9 mL/min	0,9 mL/min
Column temperature	45ºC	25ºC	-	-	-	25ºC
Extraction	De-proteinized plasma with methanol Urine diluted with water	Without	?	With acetonitrile and centrifugation	?	?
Biological samples	Plasma Urine	Urine	Blood Liver	Urine	Hair	Saliva

Conclusions

The methodology of a toxicological analysis of the substances of abuse shall be developed on the basis of: test sample type, analysis purpose, pharmacokinetic and biotransformation particularities of substance, equipment and reagents available, stuff expertise, cost.

Immunoassays offers a flexible approach of the analyses of drugs of abuse from different biological samples and represents a convenient method and a quick screening test for a large number of samples, with different matrixes. A true-positive result of an initial screening test, will not indicate on its own the dose, the time or the route of administration and it will not make the difference between an occasional administration or a chronic one.

Screening tests require subsequently performing confirmation tests for removing false positiveâ/ false negative results. Confirmation tests are modern chromatographic techniques (GC-MS, HPLC, LC-MS), high-performance, sensitive, selective, accurate. Confirmation tests have as disadvantages: time-consuming step for the processing of the samples, expensive equipment, highly qualified staff and high cost.

The identification and the assay of drugs of abuse and their metabolites in biological samples provides to the specialists (doctors, authorities in the field of health, representatives of law) an objective tool for the diagnostic of abuse or for the monitoring of addictions treatment. Interpretation of the results is a complex process and requires an overview of: the analysis method, pharmacokinetic and biotransformation particularities of the substance, clinical pattern of the patient (including history of drug dependence).

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