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Sample preparation

Determination of drugs of abuse in biological samples by accelerated solvent extraction and LC-MS/MS

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Introduction

Consumption of drugs of abuse is a scourge of the modern world. Abuse, drug addiction, and their consequences are major current problems of European society because of the significant repercussions to individuals, families, and social and economic levels. The term Drugs of Abuse (DoA) can be applied to a wide variety of natural and synthetic compounds prevalent in human culture and consumption. Analytical confirmation of DoA requires a high level of data confidence and an extremely low margin of error. The variety of biological specimens that are sent to the forensic department for these types of analysis include complex matrices (blood, urines, bile, hair, etc.) and require, therefore, very careful preparation of the samples that can influence the outcome and the reproducibility of the analysis. For several years solid-phase extraction (SPE) has been a common technique for the extraction of molecules of forensic interest and is today used as a standard procedure in the preparation of samples in many laboratories around the world. It allows efficient purification and concentration of samples before a liquid or gas chromatographic analysis. In some cases, it can extract several compounds, thus permitting the detection of substances that are often difficult to extract and would not be detectable with standard procedures. However, as a manual method this technique is time-consuming, operator-dependent, and subject to possible human mistakes that could invalidate the results.

Among the available extraction techniques, accelerated solvent extraction (ASE) is characterized by shorter extraction times and reduced solvent consumption. Accelerated solvent extraction (a.k.a. pressurized fluid extraction) uses high temperatures combined with high pressure. A high temperature allows a higher rate of extraction due to a reduction of the viscosity and surface tension and increases the solubility and diffusion rate into the sample. At the same time, high pressure prevents the solvents from reaching their boiling point and promotes penetration into the sample. Many studies have validated the ASE extraction method in the animal[1-8] and botanical[9-19] fields. However, there is a paucity of studies regarding the validation of an ASE extraction of human biological samples. Thus far studies are limited to the meconium[20,21], the bone matrix[22,23] and blood.[24,25]

In this report, we investigate the feasibility of high-throughput measurements of 36 compounds in forensic toxicology by reducing time-consuming sample preparation steps and employing HPLC-MS/MS analyses. The target analytes belong to the group of psychoactive drugs (14), antagonists (3), medications (16), and anesthetics (3) (Table 1).

Table 1. Target molecules

Main category	Cluster number	Main molecules/family	
Psychoactive drugs	1	Morphine	
		6-MAM	
		Codeine	
		Ketamine	
	2	Amphetamine	
		Methamphetamine	
		MDA	
		MDMA	
		MDEA	
	3	Cocaine	
		BZE	
	4	Methadone	
		EDDP	
	5	LSD	
Antagonist substances	6	Naloxone	
		Naltrexone	
		Flumazenil	
Medications	7	Benzodiazepines	Diazepam
			Flurazepam
			Bromazepam
			Delorazepam
			Midazolam
	8	Barbiturates	Phenobarbital
			Thiopental
	9	Neuroleptics/ Antipsychotic	Carbamazepine
			Citalopram
			Sertraline
			Chlorpromazine
			Promazine
			Haloperidol
			Clozapine
			Olanzapine
			Quetiapine
Anesthetics	10	Fentanyl	
		Remifentanil	
		Propofol	

Experimental

Biological Samples

Samples were taken from 10 human cadavers during the post mortem examination at the Bureau of Legal Medicine of the University of Milan. A sample of urine and blood was taken from each cadaver, for a total of 20 samples. Every sample was split into two parts, one for the traditional SPE analysis and one for the pressurized fluid extraction. All the samples were collected using sterilized syringes, placed in sealed vials, and stored at -20 °C until analysis to prevent decomposition. To avoid an overload of the SPE cartridge, the target molecules were divided into main categories (psychoactive drugs, antagonists, medications, and anesthetics) and in 10 additional clusters, as shown in Table 1. Two samples of blood and two samples of urine, originating from the same cadaver, were spiked with the molecules of a single cluster and extracted: one sample of blood and one sample of urine with the classical SPE extraction, another sample of blood and urine with pressurized fluid extraction.

Equipment

A standard 12-port vacuum manifold and 1 mL/130 mg Thermo Scientific™ HyperSep™ Verify CX Cartridges were used for the SPE analysis. The pressurized fluid extractions were carried out with a Thermo Scientific™ Dionex™ ASE 350™ Accelerated Solvent Extractor (Figure 1 – left) equipped with 10 mL stainless steel extraction cells. The extracts were collected in 60 mL vials and evaporated to dryness under reduced pressure. The samples were analyzed with a Thermo Scientific™ TSQ Fortis™ Triple-Quadrupole Mass Spectrometer (Figure 1 – right).

Chemicals and reagents

Morphine, 6-MAM, codeine, ketamine, amphetamine, methamphetamine, methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), Cocaine, benzoylecgonine (BZE), methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), lysergic acid diethylamide (LSD), naloxone, naltrexone, flumazenil, diazepam, flurazepam, bromazepam, delorazepam, midazolam, phenobarbital, thiopental, carbamazepine, citalopram, sertraline, chlorpromazine, promazine, haloperidol, clozapine, olanzapine, quetiapine, fentanyl, remifentanil, propofol and the internal standard proadifen (SKF-525A) were purchased from Sigma-Aldrich. Methanol, hydrochloric acid, and chloroform were purchased from Sigma-Aldrich. Acetone, ethyl acetate, dichloromethane, isopropanol, and n-hexane were purchased from VWR. Buffer solution pH 6.88 was purchased from PanReac AppliChem ITW Reagents. Diatomaceous Earth and ASE glass fiber filters were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Experimental procedure

Every biological sample was split into two parts, one for the traditional SPE analysis and one for pressurized fluid extraction (Figure 2).

Figure 1. Dionex ASE 350 accelerated solvent extractor (left) and TSQ Fortis triple-quadrupole mass spectrometer (right)

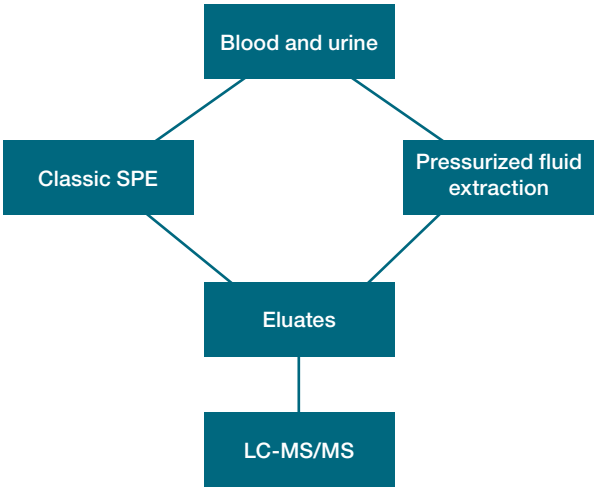

Figure 2. Experimental procedure

The whole blood was stabilized with sodium fluoride and potassium oxalate. Investigations of matrix effects and extraction efficiency were based on negative authentic samples (pre-screened for all kinds of licit and illicit drugs received by the laboratory, either from autopsy cases or from living persons). The whole blood was stored at -20 °C until use.

A stock solution containing a mixture of all the LC standards at a concentration of 1000 mg/L (ppm) was prepared in methanol. From this stock, three working solutions were prepared in water, at concentrations of 100 ppm, 10 ppm, and 1 ppm, respectively. The stock solution was stored at -20 °C. Working solutions were stored at -80 °C. A solution of SKF-525A at a concentration of 0.1 ppm was prepared in methanol, stored at -80 °C, and used as an Internal Standard (IS). Calibrators were made by spiking 0.500 mL of whole blood and urine with different concentrations of working standard solutions to yield final concentrations of 50, 100, 250, 500, 1000, and 2000 µg/L (ppb).

Solid-phase extraction

According to the standard procedure, 100 ppb of Internal Standard SKF 525-A (proadifen hydrochloride) was added to 0.5 mL of every sample. The samples were successively diluted to 5 mL using a pH 6.88 phosphate buffering solution and spiked according to the cut-off conditions of the standards.[26] The resulting solutions were briefly vortexed, centrifuged for 10 min at 3500 rpm, and loaded on the Verify CX cartridges, which were previously conditioned with 2 mL of methanol and 2 mL of pH 6.88 phosphate buffer. A first elution was made with 2 mL of pH 6.88 phosphate buffer, followed by 1.5 mL of 0.01 M hydrochloric acid solution and finally by 0.3 mL of methanol. The cartridges were successively dried under vacuum for 30 min. The final elution was made with a 1:1 mixture of chloroform and acetone, to yield an acid/neutral extract. Basic molecules were extracted with 1 mL of a 2% solution of ammonia in ethyl acetate, followed by 1 mL of a 2% solution of ammonia in dichloromethane-isopropanol pH 8:2. The extracts were concentrated to dryness in a vacuum rotary evaporator, reconstituted in 100 µL of methanol, and submitted to analysis by a TSQ Fortis triple-quadrupole mass spectrometer.

Pressurized fluid extraction

A cellulose filter was placed in the bottom of a 10 mL extraction cell. 0.5 mL of sample was added with 100 ppm of Internal Standard SKF 525-A in a centrifuge tube vial. Positive controls were prepared by spiking blank samples of urine and blood in equal concentrations as in the classical SPE extraction. After a short vortex, the samples were quantitatively transferred to the extraction cell filled with ASE Prep DE and extracted according to the conditions reported in Table 2. The extraction of one sample required 12 min. and 30 mL of solvent.

Table 2. Conditions for the pressurized fluid extraction

ASE 350 system parameters	
Extraction solvent	*n*-hexane: acetone (4:1)
Temperature	80 °C
Pressure	1500 psi
Static time	5 min
Static cycles	1
Rinse volume	60%
Purge time	100 sec
Total extraction time	12 min/sample
Total volume	30 mL/sample

The extracts were concentrated to dryness in a vacuum rotary evaporator, reconstituted in 100 µL of methanol, and submitted to analysis by a TSQ Fortis triple-quadrupole mass spectrometer.

LC-MS conditions

The liquid chromatography/mass spectrometry was performed using a TSQ Fortis triple-quadrupole mass spectrometer coupled to an HPLC system constituted by a Surveyor MS Quaternary Pump with Degasser, Surveyor AS Auto-Sampler, oven with Rheodyne valve and a 20 µL loop*. The instrument conditions are given in Table 3.

Mass spectrometry was performed using a TSQ Fortis triple-quadrupole mass spectrometer equipped with a heated electrospray ionization source (HESI). The instrument conditions are given in Table 4.

*The Thermo Scientific™ Vanquish™ Flex UHPLC System is the recommended instrument for this application. Click here to learn more.

Table 3. Conditions for the liquid chromatography

LC system parameters			
Column		Thermo Scientific™ HyperSil Gold™ C18 Column, 50 mm x 2.1 mm, 1.9 µm	
Column temperature		35 °C	
Mobile Phase A		20 mM ammonium formate and 0.1% formic acid	
Mobile Phase B		Methanol	
Flow rate		0.4 mL/min	
Injection volume		0.5 µL	
Mobile phase gradient	Time	A%	B%
	0	10	90
	1	10	90
	4	95	5
	7	95	5
	9	10	90
	15	10	90

Table 4. Conditions for the TSQ Fortis triple-quadrupole mass spectrometer

TSQ Fortis triple-quadrupole MS parameters	
Parameter	**Value**
Positive ion spray voltage	3,500 V
Sheath gas	45 Arb
Aux gas	20 Arb
Sweep gas	10 Arb
Capillary temperature	330 °C
Vaporizer temperature	280 °C
Q1 resolution	0.4 FWHM
Q3 resolution	0.7 FWHM
CID gas	1.5 m Torr

Full-scan acquisition was combined with a DIA (Data-Independent Acquisition) protocol providing MS/MS spectrum for confirmation response according to the inclusion list. The resolution power of the FS was set at 70.000 FWHM. The mass range was set to 50-650. Automatic gain control (AGC) was set at 1 x 10-6 and maximum injection time was set at 200 ms. The DIA segment operated with a positive mode at 35.000 FWHM and the AGC target was set at 5x10-4 with a maximum injection time of 100 ms. The quadrupole filtered precursor ions with an isolation range of 2 m/z. Fragmentation of the precursors was optimized with normalized collision energy in 3 steps (NCE) (10-40-60 eV).

Method validation

Evaluation of method performance including selectivity, carry-over, the limit of detection (LOD), lower limit of quantification (LLOQ), calibration curves, accuracy, precision, extraction recovery, and stability was performed according to the Scientific Working Group for Forensic Toxicology.[26]

Selectivity

The selectivity of the method could be influenced by the presence of interfering molecules in the blank samples. For this reason, all the biological samples collected were previously analyzed to prove the selectivity of the matrices, thus excluding the presence of any drugs that could interfere with standard molecules spiked in the specimens. Therefore, the samples can be considered “clean”. Moreover, to check for any interferences between the substances of each cluster, the evaluation of multiple mass transitions and the ratio of their relative intensities were performed to discriminate the target molecules from any interference.

Carry-over

The carry-over effect was investigated and minimized by injecting a blank sample of blood or urine after each calibration point with the highest concentration and after each spiked sample: the response was observed at the retention time of the investigated molecules. In each blank sample injected the carry-over effect was < 20% of the LLOQ of the previous spiked sample and < 5% for the Internal Standard (IS).

Limit of Detection (LOD) and Lower Limit of Quantification (LLOQ)

The LOD was evaluated as the lowest concentration that gives a reproducible instrument response with a signal-to-noise ratio (S/N) ≥ 3. The LLOQ, considered as the lowest concentration that gives a reproducible instrument response with a coefficient variation (CV%) < 10% and an S/N ratio ≥ 10, was obtained by adding scalar quantities of standard molecules to six matrices obtained from six different individuals. The LLOQ of each molecule was used as the lowest point for the calibration curves of the standards.

Calibration curves

Quantification was performed using the external calibration method. Calibration curves were prepared for each substance, using six matrices obtained from six different individuals, with six calibration points assessed twice per compound: a variation lower than 9% between the calibration points was maintained throughout the preparation of each calibration curve in each calibration point. The LLOQ of each molecule was used as the lowest point of the calibration curve. The calibration curves were injected at the beginning and end of the sequence, by the guidelines. The concentration range of the calibration points per substance was selected based on forensic interest concentrations and on the expected concentration of each substance in the sample examined.

Accuracy (bias %)

The accuracy was analyzed by measuring five replicates in three different analytical sessions using three samples with forensically relevant concentrations to cover the range of the calibration method. The bias % was below the 15% guidance at all the concentration levels[26]. Measurement of the accuracy at the concentration equal to the LLOQ yielded a bias% lower than the 20% guidance.[26]

Precision

The precision was evaluated starting from the sampling until the analyses of the specimens and investigated with a pool of samples (blood and urine) of different individuals spiked with low, medium, or high concentrations of the drugs under investigation. The coefficient of variation was evaluated in less than 30 days with 10 distinct measurements per level of concentration (low, medium, high) in the same or different analytical session. The precision was calculated lower than the 15% of the %CV, with the concentration at the LLOQ lower than 20%.

Recovery

Recovery tests, performed six times during the development of the method, were carried out using the method of standard addition (recovery of the extraction): the biological matrices, spiked with the standard solutions, were analyzed following the guidelines.[26] The recovery was determined as the amount of extracted standard compared with the concentration of standard solutions with whom the matrices were spiked. Recoveries were assessed by three different operators twice a day for three days to evaluate intra-day and inter-day repeatability. The values were in the accepted range between 60% and 140%, as reported in Table 4 and with an error lower the 10%.

Matrix effect

The matrix effect (ME) is connected to the elution of some components of the matrices that may influence the charge state inside the ion source. During the study, six samples of blood and six of urine were collected from different individuals to evaluate the ME. The blank matrices are extracted and later they are spiked with a known concentration of analytes. The area of analytes obtained from each sample is compared with the areas of substances obtained from a solution with the same concentration of the spiked samples. From all the substances, together with the IS, a low, medium, and high concentration of analytes are analyzed. In conclusion, the normalized matrix effect was calculated within the ± 15% criteria, with %CV, per matrix, below the 10%.[26]

Stability tests

Stability tests were performed to guarantee the correct administration of all the analytes. All the parameters and conditions respected the normal procedure times of the laboratory. The stability was evaluated on three independent samples of two different concentrations of the analytes under investigation (two levels of the calibration curves). In all the analytes the median of the two concentrations was below 15%, calculated from the concentration determined at time zero in respect with the median concentration of the level concentrations prepared with new standards. The blood matrices were stored in test tubes with anticoagulants and the urine matrices were stored in test tubes. The analytes were evaluated in the matrices individually and then evaluated in clusters of substances (as analyzed during the study). The matrices, spiked with the analytes, were kept at ambient temperature for 24 h under direct light. To evaluate the long-term stability, the analytes were kept at ambient temperature for 15 days. In the end, the stability test was performed on the samples after the extraction; the samples were maintained at 4 °C (the temperature of the autosampler of laboratory's HPLC system) for 72 h.

Results and discussion

In this work, two different extraction techniques, classical SPE and pressurized fluid extraction were compared. The analytical results clearly show the efficiency of the ASE extraction when compared to the more common SPE extraction. Recovery tests show that while most of the molecules of interest can be equally extracted with both methods with satisfactory results (psychoactive drugs and medications have similar average extractions via SPE and ASE methods), benzodiazepines are more efficiently extracted with the ASE technique (diazepam has a 92.7% of recovery with ASE compared to an 85.22% of recovery with SPE extraction in the blood). On the other hand, some psychoactive substances have a greater affinity for SPE extraction (LSD, as an example, has an average recovery of 88.21% with SPE in urine compared to a 77.59% of recovery with ASE with a urine sample). Recoveries were better in urine compared to blood samples. The recovery of every substance extracted with the two different methodologies is summarized in Table 5. Recovery tests were assessed three times for each substance for both extractive methods. Recovery was determined as the amount of extracted standard compared with the number of standard solutions with whom the matrices were spiked. Calibration curves for the validation of the study were prepared for each substance with 3 calibration points assessed twice for every compound: a variation lower than 10% was maintained throughout the preparation. Moreover, the background of the samples extracted by pressurized fluid extraction was lower

compared to the classical SPE procedure. This turns into a better qualitative resolution of the chromatographic peaks and, in general, into more reliable and higher quality results. Stability tests of the standards were not assessed because data on the stabilities of our analytes are already present in the literature.

ASE extraction has the advantage of solid robustness as variation due to operator-dependent steps is mostly eliminated due to the almost complete automatization of the procedure. Chromatographic profiles indicate that ASE-generated extracts are nearly identical in composition to those generated by conventional techniques.

Table 5. Average recoveries in both matrices with SPE and pressurized fluid extraction

Molecules	SPE (Average % rec., n = 6)		ASE (Average % rec., n = 6)	
	Blood	Urine	Blood	Urine
Morphine	83.08	86.01	88.02	88.99
6-MAM	84.10	88.03	85.98	89.11
Codeine	85.00	88.20	85.10	90.01
Ketamine	88.67	90.11	89.67	92.00
Amphetamine	89.99	90.08	91.65	94.97
Methamphetamine	79.01	82.12	80.03	81.04
MDA	88.60	89.75	91.01	92.33
MDMA	79.99	81.89	80.77	84.02
MDEA	89.81	91.59	91.21	93.18
Cocaine	88.98	90.87	86.93	89.99
BZE	81.01	86.02	80.20	81.58
Methadone	92.02	92.41	91.33	91.81
EDDP	90.59	92.40	88.70	89.10
LSD	87.10	88.21	84.45	77.59
Naloxone	91.35	92.93	90.13	91.02
Naltrexone	95.72	96.01	90.06	91.31
Flumazenil	89.00	89.19	91.01	91.07
Diazepam	85.22	87.89	92.70	94.88
Flurazepam	92.89	95.97	95.93	96.80
Bromazepam	93.71	94.98	92.22	95.89
Delorazepam	92.01	92.11	96.09	96.30
Midazolam	93.42	93.99	94.34	94.98
Phenobarbital	74.42	76.11	74.31	76.80
Thiopental	78.38	69.20	84.99	85.1
Carbamazepine	85.09	85.89	86.01	88.77
Citalopram	84.59	85.91	94.52	94.14
Sertraline	91.54	92.52	89.81	92.13
Chlorpromazine	87.53	89.62	97.61	99.81
Promazine	90.27	90.80	92.48	93.01
Haloperidol	92.78	94.33	91.80	96.12
Clozapine	86.81	87.08	89.07	91.22
Olanzapine	91.08	91.21	89.60	92.00
Quetiapine	84.69	84.99	84.90	78.11
Fentanyl	83.89	84.10	88.95	89.59
Remifentanil	90.99	93.19	78.19	85.20
Propofol	78.78	79.23	78.14	80.71

Conclusion

This report shows that the ASE extraction is an efficient alternative method of extraction to the classical SPE technique for the purification of biological matrices with a toxicological/forensic purpose. The authors suggest the use of the ASE technique for standard toxicological-forensic extractions because this extractive method reduces solvent consumption, improves extractive processes, reduces the time required for multiple extractions, greatly decreases operator bias, and increases sample throughput. The versatility of this extraction procedure allows method customization for peculiar molecules or samples of different nature. Specifically, pressure, temperature, and time of extraction, as well as solvent mixtures, can be modified to target specific molecules and increase the process efficiency.

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