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Exploring Cerebrospinal Fluid: Validation of a New Method for Quantification of 39 Drugs of Abuse by LC-MS/MS

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Abstract: The use of cerebrospinal fluid (CSF) in post-mortem (PM) toxicological analysis is an under-addressed topic, likely due to the technical complexity of the collection of a proper sample. However, it is a matrix of significant interest since it has similar chemical and physical properties to the blood and it is less exposed to risks like PM redistribution and diffusion due to its anatomical location. This study aimed to validate a sensitive analytical method for the quantification of drugs of abuse and their metabolites (i.e., cocaine, ketamine, amphetamine, MDPV, 6-monoacetylmorphine, morphine, codeine, and methadone) through liquid chromatography–tandem mass spectrometry (LC-MS/MS). CSF was collected through ventricular puncture, and 200 µL was deproteinated with acetonitrile (600 µL). Quantification was carried out, acquiring two MRM transitions for each compound in positive ionization mode. Chromatographic separation was achieved with a C18 column. Limits of quantification ranged from 0.05 to 5 ng/mL. Bias and precision were always within the acceptance criteria. Ion enhancement and suppression effects were observed depending on the substance. The method validated here was applied to a real case, proving to be suitable for PM analysis. CSF and blood were positive for methadone (460 vs. 280 ng/mL), cocaine (125 vs. 69 ng/mL), benzoylecgonine (4640 vs. 3160 ng/mL), and lorazepam (19 vs. 25 ng/mL). In the future, this will be useful for the evaluation of CSF as a valuable alternative matrix in PM investigations.

Keywords: cerebrospinal fluid; drugs of abuse; postmortem analysis; LC-MS/MS

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1. Introduction

The cerebrospinal fluid (CSF) is a blood plasma filtrate surrounding the brain and the spinal cord. The total volume is about 100–160 mL and its production rate is estimated at 25–40 mL/h, with a turnover time of 3–6 h. It is predominantly, but not exclusively, secreted by the choroid plexuses. CSF provides mechanical protection, plays a role in the elimination of waste products and toxins, and is a possible administration route for all the drugs that are not able to cross the blood–brain barrier (BBB). CSF is separated from the vascular system by the blood–CSF barrier (BCB), which is morphologically different from the BBB. However, both barriers are permeable to small molecules including psychotropic substances [1–4]. CSF analysis, besides its clinical purposes, is also of interest for PM toxicological analysis. PM drug analysis is crucial in identifying the potential cause and manner of death. However, PM modifications, such as postmortem redistribution (PMR), can lead to data misinterpretation [5]. Thus, in recent years, forensic toxicologists have been focused on the study of alternative matrices for PM investigations [6–9]. Because of its composition and anatomical position, CSF is less susceptible to PMR, so it is a potentially useful sample for drug measurement and interpretation [10]. CSF's utility for medical diagnosis has been widely proven by several studies. In recent years, many studies have been focused on CSF

in PM investigations, demonstrating its high value and suitability for the identification of drug consumption. This pilot study aimed to validate a new analytical method for the detection of 39 drugs of abuse (BDZ/Antidepressants, opioids, amphetamines, and NPS) in CSF by liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS). The new procedure was very simple and fast. Indeed, sample preparation for LC-MS/MS analysis consumes less time and resources than other instrumental techniques, such as gas chromatography–mass spectrometry (GC-MS). Moreover, MS/MS provides high specificity and sensitivity, useful for multiclass analysis. The method validated here could be employed to assess the forensic validity, in PM analysis, of CSF compared to blood or other conventional matrices.

2. Materials and Methods

2.1. Chemicals and Reagents

Acetonitrile (CH_3CN) for protein precipitation (PP) and methanol (CH_3OH) were purchased from Panreac Quimica S.L.U. (Castellar del Vallès, Spain), while ACN and water for LC-MS/MS were acquired from Biosolve Chimie SARL (Dieuze, France). Furthermore, 7-aminoclonazepam, 7-aminoflunitrazepam, 7-aminonitrazepam, α-hydroxyflunitrazepam, alprazolam, bromazepam, brotizolam, clonazepam, delorazepam, chlordiazepoxide, diazepam, flunitrazepam, flurazepam, halazepam (internal standard, IS), lorazepam, lormetazepam, midazolam, nordiazepam, oxazepam, pinazepam, prazepam, temazepam, triazolam, α-hydroxyalprazolam, and α-hydroxymidazolam were purchased from Lipomed Inc. (Cambridge, MA, USA). Moreover, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), methamphetamine, ketamine, and norketamine were obtained from Chemical Research 2000 s.r.l. (Rome, Italy). Cocaine, benzoylecgonine, codeine, dextromethorphan (DXM), methylenedioxypyrovalerone (MDPV), methadone, morphine, oxycodone, and tramadol were purchased from LGC standards (Milan, Italy). All standards were diluted to the appropriate concentration with CH_3OH.

2.2. CSF Collection

Blank CSF was collected from corpses with a known post-mortem interval of 3 days without brain traumas or known neurological/metabolic diseases and without external/internal signs of advanced putrefaction. Sampling was performed at the Section of Forensic Medical Sciences of the University of Florence during a full forensic autopsy. The recent consumption of the drugs of interest was excluded through traditional toxicologic testing. CSF was obtained through ventricular puncture by an expert forensic pathologist as previously described in the scientific literature, considering only samples whose limpidity excluded blood contamination [11].

2.3. Sample Treatments

A PP was achieved by adding 600 µL of cold CH_3CN (0° C) and 30 µL of IS (0.05 ng/µL) to CSF (200 µL). The mixture was vortexed and centrifugated (2500× *g*, 5 min), and the liquid phase was then dried under a nitrogen stream at 40 °C and reconstituted with 100 µL of water.

2.4. LC-MS/MS

The analysis was conducted using an HPLC Agilent 1290 Infinity system (Agilent Technologies, Palo Alto, CA, USA) interfaced with an Agilent 6460 Triple Quad MS (Agilent Technologies), equipped with an electrospray ion source (ESI) operating in positive mode. The ESI configuration was a gas temperature of 325 °C, a gas flow rate of 10 L/min, a nebulizer of 20 psi, and a capillary of 4000 V. Multiple reaction monitoring (MRM) transitions (Table 1), data acquisition, and elaboration were performed using the Agilent MassHunter Workstation software package (ver. B.04.01). Chromatographic separation was performed using a Zorbax Eclipse Plus C18 (2.1 × 50 mm, 1.8 µm, Agilent Technologies). The mobile phase initially consisted of 5 mM aqueous formic acid (A) and CH_3CN (B) 99:1.

The gradient of elution was carried out as follows: from 0–6 min, linear ramp from 0–10%B; from 6–10 min, ramp to 25%B; from 10–12 min, ramp to 70%B; from 12–13 min, ramp to 100%B and isocratic hold to 12.5 min. Post-time was set at 1.5 min. The flow rate was 0.4 mL/min. The injection volume was 5 µL.

Table 1. MRM (ESI+) transitions and retention time for each substance.

Compound	Ion Fragments (*m*/*z*)	Collision Energies (V)	Retention Time (min)
BDZ/Antidepressants			
7-aminoclonazepam	286: **222**, 121	25, 30	7.1
7-aminoflunitrazepam	284: **227**, 135	25, 28	8.5
7-aminonitrazepam	252: **208**, 121	35, 29	3.9
alprazolam	309: **281**, 205	40, 25	12.2
bromazepam	316: **209**, 182	25, 33	10.6
brotizolam	393: **314**, 279	21, 29	11.6
clonazepam	316: **270**, 214	25, 40	11.4
chlordiazepoxide	300: **283**, 282	9, 21	9.5
delorazepam	305: **165**, 162	29, 33	11.7
diazepam	285: **193**, 154	33, 25	11.8
flunitrazepam	314: **268**, 239	25, 37	11.6
flurazepam	388: **317**, 315	17, 25	10.7
lorazepam	321: **303**, 275	13, 17	11.4
lormetazepam	335: **317**, 289	9, 17	11.8
midazolam	326: **291**, 223	25, 40	10.4
nordiazepam	271: **165**, 155	29, 29	1.6
oxazepam	287: **269**, 241	9, 21	11.3
pinazepam	309: **269**, 241	29, 33	12.2
prazepam	325: **271**, 140	21, 40	12.4
temazepam	301: **283**, 255	9, 21	11.6
triazolam	343: **315**, 308	29, 25	11.5
α-hydroxyalprazolam	325: **297**, 216	30, 35	12.4
α-hydroxyflunitrazepam	330: **311**, 284	35, 30	11.8
α-hydroxymidazolam	342: **203**, 168	35, 45	10.6
Main drugs of abuse			
6-MAM	328: **211**, 165	25, 40	5.1
codeine	300: **165**, 58	40, 33	3.7
DXM	272: **171**, 147	42, 30	10.2
methadone	310: **265**, 105	13, 29	11.2
morphine	286: **165**, 155	40, 37	1.6
oxycodone	316: **298**, 256	17, 25	4.5
tramadol	264: **246**, 58	25, 30	7.7
Others			
MDA	180: **163**,105	9, 25	4.1
MDMA	194: **163**, 105	9, 25	4.6
methamphetamine	150: **119**, 91	21, 9	5.1
MDPV	276: **135**, 126	25, 25	8.2
ketamine	238: **179**, 125	13, 29	6.5
norketamine	224: **207**, 125	9, 25	6.2
cocaine	304: **182**, 82	17, 33	8.3
benzoylecgonine	290: **168**, 105	17, 29	6.8

Quantitative transitions highlighted in bold.

2.5. Validation Parameters

The method was validated following the American Academy of Forensic Sciences' (AAFS) standard practices for method validation in forensic toxicology [12].

2.5.1. Interferences Studies

Ten different blank cerebrospinal fluid samples were analyzed to check potential endogenous interfering peaks. The estimation of exogenous interferences was performed by spiking 10 different blank cerebrospinal fluid samples with 200 ng/mL of common drugs and their main metabolites (including barbiturates, cannabinoids, and antidepressants).

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

LOD is the lowest concentration producing a signal-to-noise ratio (SNR) $\geq$ 3 and meets the identification criteria. It was calculated by analyzing three replicates of blank specimens spiked with decreasing quantities of each substance.

LLOQ estimation was achieved using the same methodological approach, but its value had to provide an SNR $\geq$ 10 and had to meet identification, bias, and precision criteria.

2.5.3. Calibration Model

Due to the different LOQ concentrations, we were not able to set up the same calibration curve for all compounds. Calibration curves from LOQ to 100 ng/mL were prepared by adding appropriate amounts of the analytes of interest to 200 µL of a pre-checked drug-free CSF sample.

Five replicates of blank CSF spiked at the proper concentrations were analyzed, and the least-squares regression procedure was applied to the data. Linearity was evaluated by means of the coefficient of determination (R^2, acceptance criterion: $\geq$0.9900).

2.5.4. Bias and Precision

Three separated blank CSF samples spiked at different concentrations (quality control, QC) were analyzed five times: 0.15, 0.3, 1.5, or 15 ng/mL ($\leq$3 times the first calibration level, QC1) depending on the specific calibration curve, and 30 ng/mL (QC2) and 75 ng/mL (~80% of the highest calibrator, QC3). Bias had to be within $\pm$ 20% at each concentration level. Precision was measured as coefficient of variation (%CV). Three samples of QCs were analyzed five times. Within-run precision was calculated for each QC separately for each of the five runs. For between-run precision, the evaluation of each concentration was performed over five runs. %CV was accepted if <20%.

2.5.5. Recovery Rate (RR), Matrix Effect (ME) and Carry-Over

RRs were measured at QC1 and QC3 levels over 6 replicates. Slopes from the QCs spiked before and after the extraction were compared.

ME estimation was carried out following the post-extraction addition approach. Ionization suppression (IoS) or enhancement (IoE) was calculated by comparing the areas from set 1 (two neat standards at QC1 and QC3 concentrations) and set 2 (ten different blood samples extracted in duplicate and then spiked at QC1 and QC3 levels). Each neat standard was injected six times to establish the mean area of set 1. IoS or IoE should not exceed $\pm$25%. Calculation was performed as follows:

$$IoS\ or\ IoE(\%) = \left(\frac{\overline{A}_{set2}}{\overline{A}_{set1}} - 1\right) \times 100$$

Carry-over estimation was achieved by injecting the extracted blank samples into the LC-MS/MS system immediately after the highest calibrator over five runs.

3. Results

3.1. MRM Transitions and Chromatographic Separation

For each compound, the two most abundant MRM transitions were included (Table 1). The acquisition was performed in dynamic MRM mode (retention time window: 1.5 min; max concurrent MRM: 36; dwell range: 10.39–246.50 ms). In order to obtain the best

chromatographic performances, several gradients of elution were tested. The choice was achieved on the basis of the peaks' shapes and the number of co-eluting compounds.

The final chromatographic run was 12.5 min long (Figure 1), with the elution ranging from 1.650 (morphine) to 12.457 min (prazepam). The only co-eluting molecules were prazepam and α-hydroxyalprazolam (12.4 min). In this case, identification can be achieved on the basis of their transitions 325 > 271 m/z for prazepam and 325 > 297 m/z for α-hydroxyalprazolam.

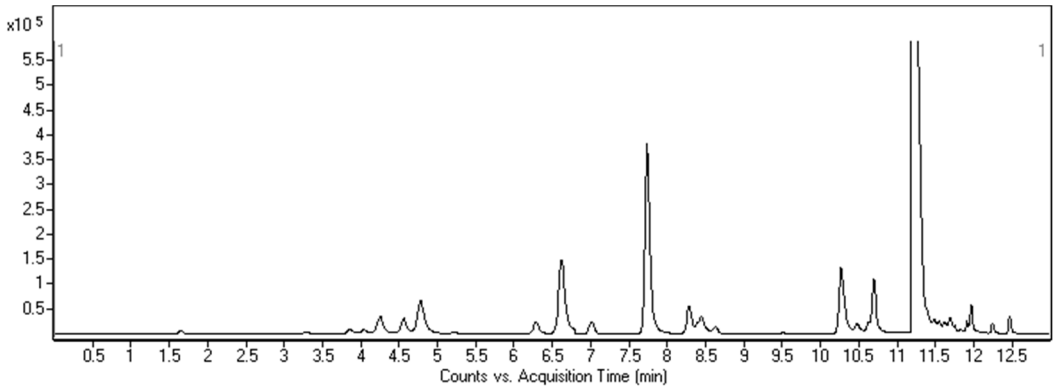

Figure 1. Chromatogram at QC2 level.

3.2. Sample Treatment Procedure

The sample treatment procedure was based on a previously published method for NPS and BDZ/antidepressant quantification in blood [8]. This consists of protein precipitation with ACN preliminary tested at QC levels, and the evaluation was based on RR (>75%), ME (from −30 to +30%), and time/resource consumption. This method provides the best outcomes for amphetamines/NPS/phencyclidine (RR > 80%; ME from 15 to 25%) but was not suitable for two BDZ drugs, namely, flurazepam and triazolam (RR < 59%), nor for methadone (RR: 55%). For this reason, several liquid–liquid (LL) extractions were also tested, using different organic solvents (dichloromethane, ethylacetate, iso-propanol, and their mixtures) at various basic conditions (phosphate buffer at pH 8 and at pH > 10 by NaOH solutions). However, due to the different chemical properties of the included compounds, the RR values varied depending on the extraction conditions. Overall, RRs were lower. Thus, although some RRs for PP were lower than the acceptance criteria (RR > 60%), it provided the best overall values; moreover, the procedure was less time- and resource-consuming. Subsequently, it was optimized at varying volumes (from 200 to 500 µL) and temperatures (from −25 °C to room temperature) of ACN. This method seemed to be easier and faster than the previously published procedures, which require single or double extractions (LLE or SPE) [10,13–15].

3.3. Methods Validation

The method was found to be highly specific and selective since neither endogenous nor exogenous interfering peaks were observed. For eight compounds, the coefficient of determination (R^2) was lower than 0.99 (but > 0.9850). This may be due to the large calibration range.

LOQ ranged from 0.05 ng/mL for most of the compounds to 5 ng/mL for 7-aminoclonazepam, bromazepam, and clonazepam (Table 2). The highest sensitivities were observed among amphetamines, NPS, and phencyclidines with a value of 0.05 ng/mL, except for ketamine's metabolite (0.5 ng/mL). Low LOQ levels were also registered for some BDZs such as flunitrazepam, diazepam, and their metabolites (0.05 ng/mL) and opioids (0.05 ng/mL, except for morphine and oxycodone at 0.5 ng/mL). The test was sensitive enough to detect all included substances at recreational or therapeutic concentrations. At the lowest QC level, the acceptance criteria for bias were not met by three substances (−20% < %MRE < 20%) with the highest value of −41% (clonazepam). Bias improved at

higher QC concentrations, and all compounds were within the acceptance criteria (QC2, −20% < %MRE < 20%; QC3, −10% < %MRE < 10%). None of the compounds showed high bias across all concentrations. %CV was always within the acceptance criteria suggested by the AAFS guidelines (%CV: <20%), except for methadone at the QC1 level (41%).

The mean ME was −2% for ketamine, with the highest values of ion suppression and ion enhancement at −37% (lormetazepam) and +20% (morphine), respectively. Carryover was not observed.

Table 2. Main validation parameters estimated for the new method.

Compound	LOD (ng/mL)	LOQ (ng/mL)	R^2	%MRE			%CV			ME (%)	RR (%)
				QC1 *	QC2	QC3	QC1	QC2	QC3		
6-MAM	0.01	0.05	0.9901	10	6	−20	13	12	5.5	−12	88
7-aminoclonazepam [1]	2	5	0.9877	13	2	−18	18	8	7.5	−15	87
7-aminoflunitrazepam	0.03	0.05	0.9900	3	2	1	3.7	5	1.3	−20	71
7-aminonitrazepam [2]	0.1	0.5	0.9899	−1	5	3	2	8	5	−25	61
alprazolam [2]	0.01	0.5	0.9989	−4	2	−4	5.2	7	5.2	−18	71
benzoylecgonine	0.01	0.05	0.9991	9	1	−1	13.3	10	1.5	−8.5	75
bromazepam [1]	1	5	0.9919	−1	7	4	1.7	8	5.3	−25	62
brotizolam	0.03	0.1	0.9875	13	10	1	18	16	1	−15	65
clonazepam [1]	1	5	0.9989	−41	−10	−25	59	18	6	−33	65
delorazepam	0.01	0.05	0.9940	3	1	1	5	1.7	0.4	−18	75
chlordiazepoxide [2]	0.3	0.5	0.9904	2	9	1	3.2	7	1.1	−30	68
cocaine	0.01	0.05	0.9859	5	1	0	7	10	0.3	−6	72
codeine [2]	0.1	0.05	0.9964	12	10	−18	15	12	10	16	70
DXM	0.02	0.05	0.9967	−13	−1	4	18	4	4	−4	76
diazepam	0.03	0.05	0.9999	−12	5	1	16	16	2	−9	83
flunitrazepam	0.01	0.05	0.9911	−4	−7	1	1	6	0.4	−32	66
flurazepam	0.01	0.05	0.9871	23	−7	15	10	12	1.8	−31	59
ketamine	0.01	0.05	0.9967	−5	5	2	1	1	3	−2	83
lorazepam [2]	0.1	0.5	0.9999	2	7	1	19	20	0.4	−33	64
lormetazepam [2]	0.2	0.5	0.9979	20	9	19	14	6.8	1.8	−37	67
MDA [2]	0.01	0.05	0.9981	2	12	15	8	5	2	10	81
MDMA	0.01	0.05	0.9999	10	3	6	2	4	3	−8	75
MDPV	0.01	0.05	0.9956	2	2	11	0.5	3	0.9	−15	80
methadone	0.03	0.05	0.9899	2	2	1	41	12	0.2	−35	55
methamphetamine	0.01	0.05	0.9990	2	5	8	10	5	1	14	80
midazolam [2]	0.1	0.5	0.9940	2	4	1	3.3	5.6	0.2	−25	74
morphine [2]	0.1	0.5	0.9995	−8	−3	−2	16	2.7	10	20	70
norketamine [2]	0.05	0.5	0.9998	2	−4	1	3	1	0.1	11	78
nordiazepam	0.01	0.05	0.9990	2	1	1	6	4.8	2.6	3	81
oxycodone [2]	0.1	0.5	0.9899	6	1	5	20	10	17	5	84
oxazepam [2]	0.1	0.5	0.9999	2	−1	1	9	2	4	−33	66
pinazepam	0.01	0.05	0.9932	2	5	1	5	1	5	−10	78
prazepam [2]	0.1	0.5	0.9964	2	9	1	9	1	5	−20	92
temazepam	0.05	0.1	0.9914	2	2	1	17	9	6.3	−18	79
triazolam	0.01	0.05	0.9899	2	2	1	11.8	3	0.2	−32	59
α-hydroxyalprazolam	0.05	0.5	0.9954	−4	7	4	16.4	9	15	−20	83
α-hydroxyflunitrazepam	0.1	0.5	0.9989	−5	−3	4	6.3	10	5	−15	70
α-hydroxymidazolam	0.02	0.05	0.9910	2	5	1	4.8	3	0.8	−20	69
tramadol	0.01	0.05	0.9917	−13	10	14	4	8	9	7	75

* 0.15 ng/mL except for brotizolam and temazepam, 0.3 ng/mL; 7-aminonitrazepam, α-hydroxyflunitrazepam, α-hydroxyalprazolam, alprazolam, chlordiazepoxide, codeine, lorazepam, lormetazepam, MDA, midazolam, morphine, norketamine, oxazepam, oxycodone, prazepam, 1.5 ng/mL; 7-aminoclonazepam, bromazepam, clonazepam, 15 ng/mL. Calibration curves: LOQ, 0.5, 5, 10, 50, and 100 ng/mL except for: [1] 5, 10, 50, and 100 ng/mL; [2] 0.5, 5, 10, 50, and 100 ng/mL.

4. Discussion

The aim of this study was to validate a sensitive analytical method using LC-MS/MS for the simultaneous quantification of therapeutic drugs and main drugs of abuse (including their main metabolites) in CSF. To the best of our knowledge, this is the first attempt to validate a multi-analyte analysis for CSF. Indeed, despite the fact that several CSF analyses are available in the literature, they are mainly related to specific fatal intoxication and have focused on single substances or classes. For example, Strehmel et al. detected the new

opioid U-47700 and oxycodone in the CSF and various matrices (i.e., blood, liver, urine, gastric contents, bile, and hair) using an untargeted LC-MS screening method [16]. MDMA and MDEA (3,4-methylenedioxy-N-ethylamphetamine) were quantified by Libiseller et al. in a case of suicide by oral ingestion of ecstasy [17].

In 2015, Tominaga et al. performed a large study on CSF use as an alternative matrix in PM analysis [13]. They analyzed 103 cases where various xenobiotics were quantified both in CSF and blood, stating that CSF demonstrated suitability in routine forensic toxicology and could be a valuable alternative to blood also for the estimation of pharmaco-/toxicokinetics and PM redistributions. In this research, 25 drugs (amphetamines, BDZs, antidepressants, anesthetics, and others) were detected by gas chromatography (GC) coupled to MS. The sample treatment consisted of a multi-step procedure with consequential phases of LL and solid-phase extraction, which resulted in being more time- and sample-consuming than ours. Regarding GC/MS-based methods, David A. Engelhart et al. and Fumio Moriya et al. reported the simultaneous determination of different classes of pharmacological substances in CSF [10,14]. Both researchers used different sample preparation procedures based on substance classes, i.e., David A. Engelhart et al. used three different LLE extraction methods based on basic drugs (10 mL of ethylacetate), acid-neutral drugs (7 mL of dichloromethane), and BDZs (2 mL of n-butyl chloride). Both methods were more expensive and time-consuming. In addition, the sensitivity of Fumio Moriya's method is lower than ours (i.e., 8 vs. 0.05 ng/mL for diazepam, 15 vs. 0.05 ng/mL for nordiazepam, and 138 vs. 0.5 ng/mL for codeine).

The main advantages of this new method lie in the simple and fast sample preparation and the low matrix volume consumption (200 µL). The limitations are the low RRs (<60%) for three compounds and a linearity <0.99. However, accuracy and sensitivity were not negatively affected, thus the procedure can be used in forensic casework.

This newly validated method was applied to a real case. A 40-year-old woman was found dead in a known drug-dealing area in Florence. To verify the cause of death and exclude third-party interference, a forensic autopsy and an additional toxicological investigation were ordered by the prosecution authority. Following the autopsy, sudden death from myocardial infarction (MI) was assessed. The woman, HCV+, was affected by psychiatric disorders and had a history of drug abuse, in particular heroin and crack; she was receiving treatment for BDZ and methadone. Samples of central blood (CB) and CSF were collected during the autopsy for toxicological investigation.

CB was analyzed following a previously published procedure [18–20]. The method proposed here was successfully applied to CSF analysis. Both samples were positive for lorazepam, methadone, cocaine, and benzoylecgonine with a high positive correlation between the two matrices (Table 3). No data are available on the PM blood/CSF concentration ratios for these substances, except for cocaine and its main metabolite [21,22]. The ratios were always >1, for both cocaine and benzoylecgonine. In our case, the ratios were ~0.5 and ~0.7, respectively, indicating higher concentrations in CSF than in blood. Future studies are necessary to better assess the distribution between them.

Table 3. Comparison between blood and CSF concentrations for a real case.

Substances	Blood (ng/mL)	CSF (ng/mL)
Methadone	280	460
Cocaine	69	125
Benzoylecgonine	3160	4640
Lorazepam	25	19

The current study supports these findings and expands the database to include not only therapeutic drugs but also illicit substances of forensic toxicological interest. It demonstrated how CSF can be useful to support evidence from classical matrices or even when these are unavailable. Due to this fact, we fully validated the first method for the simulta-

neous determination of BDZs, opioids, amphetamines, NPS, phencyclidines, and cocaine-based substances in CSF. The rapid and simple sample preparation (PP) and the small sample volume (200 µL) represent the main positive features of this method. Moreover, the LC separation was efficient and short (12.5 min + 1.5-min post-time). The high specificity and sensitivity make this methodology suitable for cases that require the identification and quantification of a wide range of compounds, i.e., in cases of uncertain death.

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