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Development and validation of a reliable method for studying the distribution pattern for opiates metabolites in brain

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ABSTRACT

Brain distribution pattern of "street" heroin metabolites (morphine and codeine) was investigated in two fatalities due to "acute narcotism". A suitable sample pretreatment prior to solid-phase-extraction was developed to achieve a good recovery of the analytes and to eliminate the interfering species. After derivatization with MSTFA, samples were analyzed by GC/MS. Specificity, accuracy, precision and linearity of the method were evaluated; LOD and LOQ were, respectively, 10 ng/25 ng for morphine and 5 ng/10 ng for codeine.

This method was applied to the analysis of six brain areas (hippocampus, frontal lobe, occipital lobe, nuclei, bulb and pons) coming from two cases of heroin-related deaths. No evidence of accumulation of metabolites in a specific brain region was found.

1. Introduction

After intravenous assumption, heroin quickly crosses the blood-brain barrier to produce a rapid onset of action. However, heroin is rapidly hydrolyzed to 6-monoacetylmorphine (6-MAM) and then to morphine (Fig. 1), which is in turn metabolized mainly (98%) via conjugation with glucuronic acid at its 3- and 6-positions to form morphine-3-β-D-glucuronide and morphine-6-β-D-glucuronide. Morphine-glucuronides are hydrophylic compounds, which are mainly excreted in urine and, in minor quantities, in bile.

Since heroin plasma half-life is very short, it is commonly held that morphine and 6-MAM are responsible for the protracted pharmacological actions of heroin and, moreover, can be detected in biological specimens after heroin assumption [1].

Furthermore, "street heroin" usually contains a 5–10% of acetylcodeine, as impurity deriving from the synthesis, which in the body is metabolized to codeine, so that traces of codeine (Fig. 1) and of its metabolites can be found in heroin users' urine [2].

The concentration of drugs in blood [3], which are susceptible to rapid chemical and metabolic hydrolysis, does not always reflect drug concentration at the site of action, especially in the case of supposed death for heroin overdose, because post-mortem redistribution or drug instability can result in misleading variations of plasmatic drug levels [4].

Direct measurements of heroin metabolites concentrations in the brain are useful in post-mortem forensic toxicology to substantiate fatal overdoses [5]. Brain samples show several advantages respect to other specimens as concerns psychoactive drugs, because brain is an isolate compartment, endowed with lower metabolic activity, resulting in slower decomposition and delayed process of putrefaction [6,7].

Moreover, drugs of abuse exert their effects via the central nervous system, so that it can be assumed that the encephalic concentration of these drugs, measured in post-mortem specimens, is close or equal to their peri-mortem concentration at their site of action [8].

Although the advantages of brain samples in determining fatal overdoses are obvious, sampling of the brain is crucial and demands particular care; to minimize degradation, specimens should be analyzed as soon as possible. Furthermore, appropriate sample preparation is one of the most important pre-requisite for the successful identification and quantification of psychoactive drugs in brain specimens, eliminating interfering species such as proteins and lipids. To this end several methods were proposed ranging from liquid/liquid extraction [9–12] to solid phase extraction (SPE) [5,13–15] and several detection and quantification techniques were applied, based on liquid chromatography–mass spectrometry (LC/MS) [15–17] or gas chromatography–mass spectrometry (GC/MS) [3,4,17,18].

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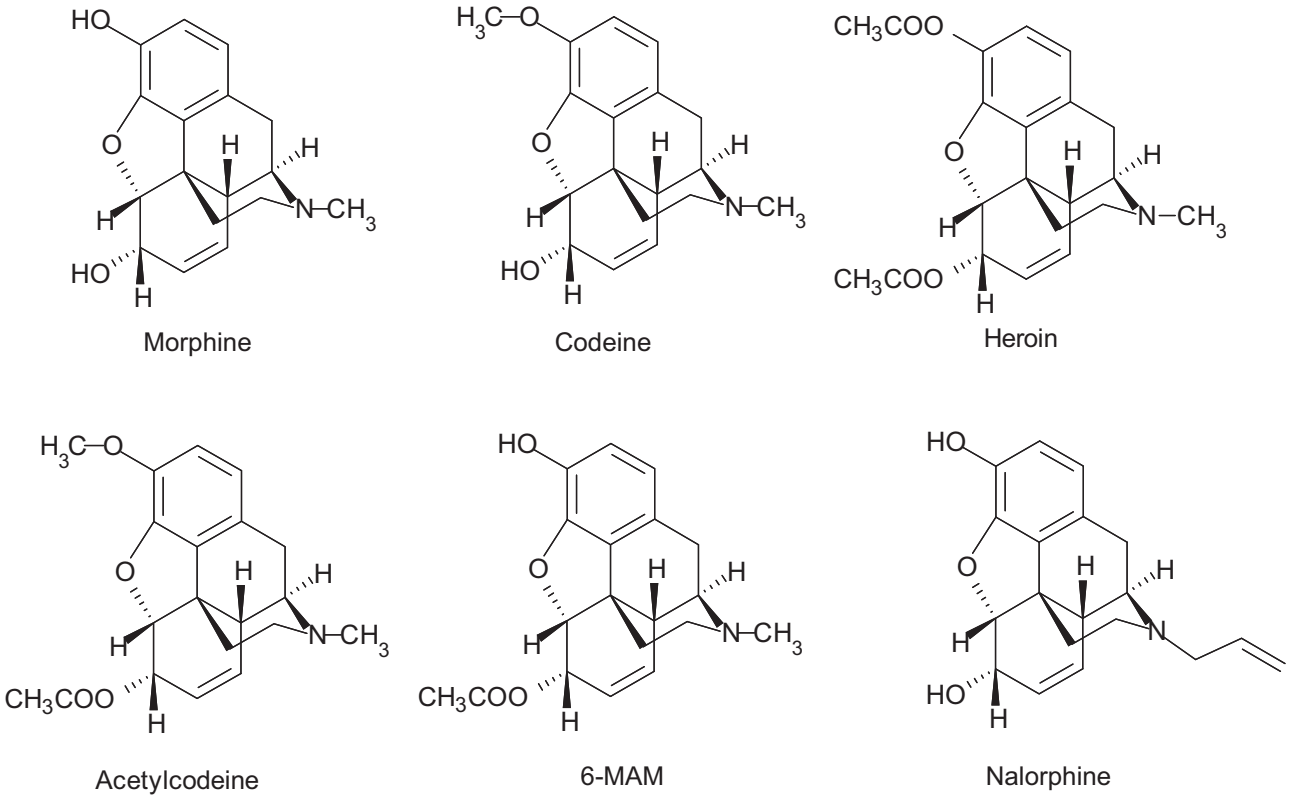

Fig. 1. Heroin, acetylcodeine and their principal metabolites.

The aim of this work was the determination of heroin metabolites concentration, i.e. morphine and codeine (the latter deriving from the impurities present in "street heroin"), in different brain areas collected from two cases of death (Table 1) attributed to heroin overdose, in order to evaluate the distribution pattern of the metabolites throughout the brain. To this end a GC/MS technique was applied and the pretreatment of the sample, especially the deproteinization step, carefully studied to optimize the recovery of the analytes and the elimination of the interfering species.

2. Experimental

2.1. Chemicals and reagents

Morphine base, codeine base and nalorphine (Internal Standard, IS) were purchased from S.A.L.A.R.S. (Italy). Methanol of analytical grade, 5-sulfosalicylic acid dihydrate, trichloroacetic acid and zinc sulfate were obtained from Sigma–Aldrich (Germany). Toluene, sodium tetraborate/hydrochloric acid pH 9 buffer solution and N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) were purchased from Fluka (Swiss), while dichloromethane and isopropyl alcohol from Prolabo (Italy). Ammonium sulfate was supplied from Carlo Erba (Italy) as well as glacial acetic acid. Water (18.2 mΩ/cm) was obtained with Milli-Q System (Millipore, France). All reagents were of analytical grade and stored as required by their specifics.

2.2. Sample preparation and deproteinization

Post-mortem encephalic samples were collected from two fatalities attributed to heroin overdose, and were supplied by the Institute of Legal Medicine and Assurance of the University of Palermo. The samples were collected from six different encephalic areas: nuclei, pons, frontal lobe, bulb, occipital lobe and hippocampus.

Each sample was homogenized with a blender or ball mill, depending on the quantity of material, then the deproteinization of the biological matrix was performed applying five different procedures.

2.2.1. Trichloroacetic acid

1 mL of trichloroacetic acid 10–15% (v/v) was added to 500.0 mg of homogenized brain previously added with 50 μL of IS. After 5 min centrifugation, a limpid supernatant was separated and extracted by SPE.

2.2.2. Zinc sulfate

1 mL of a mixture of methanol/zinc sulfate 0.2 M (8:2, v/v) was added to 500.0 mg of homogenized brain previously added with

Table 1
Overview of case studied.

Case	Case history	Detected substances	Alcohol in blood (g/L)
1	42-Year-old male, registered drug abuser, found dead at his home	Morphine, codeine, dextromethorfan, nicotine, caffeine	2.2
2	35-Year-old male, found dead in a public toilet, used syringe found at scene	Morphine, codeine, paracetamol, nicotine, caffeine	1.3

50 μL of IS. After 5 min centrifugation, the organic supernatant was evaporated to dryness with a stream of N_2 at 40 °C and the residue was reconstituted in 4 mL of water and 2 mL of pH 9 buffer solution before SPE.

2.2.3. Ammonium sulfate

500.0 mg of homogenized brain were treated with 50 μL of IS and 3 mL of H_2O and sonicated for 15 min before adding 1 mL of HCl 0.1 M. Then, $(NH_4)_2SO_4$ was added until saturation and the mixture was heated at 70 °C for 30 min. After cooling, centrifugation was carried out, but the biological matrix remained at the bottom of the tube, thus preventing the collection of the supernatant.

2.2.4. 5-Sulfosalicylic acid

200 mg of 5-sulfosalicylic acid were added to 500.0 mg of homogenized brain previously added with 50 μL of IS and 1 mL of H_2O. After 5 min centrifugation, a limpid supernatant was separated and extracted by SPE.

2.2.5. Ultrasonic bath

500.0 mg of brain previously added with 4 mL of water, 2 mL of pH 9 buffer solution and 50 μL of IS were sonicated for 15 min at room temperature. After 5 min centrifugation, a limpid supernatant was separated and extracted with SPE.

2.3. Extraction procedure

The homogenized and deproteinized encephalic samples were centrifugated at 4000 rpm for 5 min and extracted using Bond Elut-LRC Certify Solid Phase Extraction cartridges (Varian, CA, USA) with a Varian vacuum manifold (Varian, CA, USA). Cartridges were first conditioned with 2 mL of methanol and 2 mL of pH 9 buffer solution. Brain supernatants were loaded and allowed to absorb with gravity flow. The columns were washed with 2 mL of water, 3 mL of 1 M hydrochloric acid and 0.5 mL of methanol. The analytes were then eluted with 2× 1 mL of the elution solvent (dichloromethane:isopropyl alcohol:ammonium hydroxide, 8:2:0.2).

2.4. Chromatography

The extracts were evaporated to dryness under a stream of nitrogen at 40 °C and derivatized with 50 μL of a mixture MSTFA/toluene (1:4) at 70 °C for 30 min. Then GC/MS analyses were performed on a GC 6890 Plus with Mass Selective Detector and autosampler 6890, data were handled with a MSD Chemstation D.03.00 software (Agilent Technologies); chromatographic separation was carried out on a DB-5MS inert capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J.& W. Scientific).

The GC/MS system was operated under the following conditions: injection temperature 280 °C (splitless mode; 0.25 min splitless time); interface transfer line 280 °C; ion source 230 °C; initial column temperature 70 °C. Temperature was subsequently increased to 180 °C at a rate of 40 °C/min, then increased to 300 °C at a rate of 10 °C/min and finally held for additionally 5 min. Helium was used as the carrier gas at a flow rate of 1.2 mL/min. MS analysis was performed in SCAN (50/550 *m*/*z*) and SIM mode by a quadrupole mass detector operated in electron ionization mode, the beam energy being 70 eV. Injection volume was 1 μL.

2.5. Method validation

Specificity, accuracy, precision and linearity, as well as LOD and LOQ, were evaluated analyzing working standard solutions prepared with morphine and codeine at different concentrations (10, 25, 50, 100, 250, 500 and 1000 ng tot) and nalorphine as Internal Standard. Particularly, 100 μL of the working standard solutions (0.1, 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 μg/mL) were put in seven tubes, evaporated to dryness and then added with 500.0 mg of blank brain. After centrifugation for 1 min, 4.00 mL of H_2O, 50 μL of IS (4 μg/mL) and 2 mL of pH 9 buffer solution were added.

Table 2
Qualifier positive ions and retention times for derivatized morphine, codeine and nalorphine.

Analyte	Retention time (min)	Positive ions $(m/z)_{\text{relative intensity}}$
Codeine-TMS	11.99	371_{100}, 314_{21}, 343_{20}
Morphine-2TMS	12.35	429_{100}, 414_{52}, 401_{33}
Nalorphine-2TMS (IS)	13.30	455_{100}, 440_{44}, 414_{53}

Analytes retention times and their characteristic ions (*m*/*z*) used for GC/MS identification and quantification are listed in Table 2.

For quantitative analyses the following ions were monitored: *m*/*z* 371 for codeine, *m*/*z* 429 for morphine and *m*/*z* 455 for nalorphine.

The quantification of the two drugs was based on the response factor RR, defined as: $RR = (A_{analyte}/A_{IS})/C_t$. Where $A_{analyte}$ and A_{IS} are, respectively, the peak area of the analyte and the peak area of the IS and C_t is the analyte concentration in the working standard solution expressed as total ng of analyte. The response factor was calculated for all the working standard solutions described above and the averaged RR_{medium} was considered for the estimation of drugs concentration in brain specimens.

3. Results and discussion

3.1. Method development

Since brain is a complex matrix, we had to face several problems to obtain a homogenous and representative specimen with a good recovery of the analytes. To this end it was necessary to develop a suitable sample pretreatment before SPE, in order to minimize interfering species and optimize recovery.

After homogenization, each sample was submitted to a deproteinization procedure. Five methods were tested, whose comparison was based not only on the best results in terms of recovery, but also on the easy handling and required time.

The pretreatment with ammonium sulfate was the most complex and time-consuming. As a matter of fact, this procedure required several steps that increased the time of analysis; moreover, despite the last step in the centrifuge, the matrix was not completely deposited at the bottom of the tube, thus making difficult the recovery the supernatant, which often showed matrix residues, that could clog the pores of the sorbent extraction columns.

The method with zinc sulfate and methanol was also dismissed, because of the increasing time of analysis due to the evaporation of the solvent.

To compare the five deproteinization procedures, beyond practical reasons, we evaluated the recovery of morphine and codeine analyzing five different samples from the same homogenated brain area belonging to a real case of heroin fatality. Each sample was added with 50 μL IS (4 μg/mL) and submitted to the five types of deproteinization. Once extracted and analyzed by GC/MS, we compared the ratios of the analyte areas and of the internal standard areas ($RA = A_{analyte}/A_{IS}$) taking into account the amount of the biological matrix. Internal standard added before sample preparation provided an element of control of the analytical process because it minimized the variability factors due to instrumental analysis and loss of sample. Results are shown in Table 3.

Table 3
Deproteinization methods and analytes recoveries.

Sample	Deproteinization	Sample (g)	RAmorphine (g)	RAcodeine (g)
1	Trichloroacetic acid 15% (v/v)	0.5040	0.3457	0.0700
2	Zinc sulfate and methanol	0.5012	0.3557	0.1611
3	Ammonium sulfate	0.5230	0.3225	0.2508
4	5-Sulfosalicylic acid	0.5107	0.3029	0.1482
5	Ultrasonic bath	0.5164	0.4490	0.2849

As evident, the ultrasonic bath led to the best results in terms of RA/g, both for morphine and codeine. Furthermore, this technique was definitely faster than the others, so it was applied for the analysis of all the samples under investigation. Once established the best deproteinization method, the influence of the time of sonication on the recovery of the analytes was evaluated. Comparable results were obtained by increasing the sonication time up to 60 min (Table 4), so that a time of 15 min was chosen, in order to shorten the time of analysis.

For every brain area only one sampling was carried out because, as demonstrated by Stimpfl et al. [4], the distribution of drugs of abuse within each cerebral area is homogeneous and only one specimen is sufficient to represent the whole part.

Another point we had to assess was the amount of brain to weigh for each specimen. We prepared three samples from the same homogenated brain, the first sample of approximately 200 mg, the second of approximately 500 mg and the third of about 1000 mg; we concluded that the optimum amount to use was 500 mg, since this quantity was representative of the matrix and analyte concentrations were above the limit of quantification.

After the pretreatment step, samples were submitted to SPE, derivatization and analysis by GC/MS, in the conditions described in Sections 2.3 and 2.4.

3.2. Method validation

3.2.1. Specificity

Specificity was assessed by extracting control blank brain samples in each validation run. The lack of interfering peaks at the same analyte retention times was considered as an acceptable selectivity.

3.2.2. Linearity

The linearity of the response of GC/MS analysis was checked for codeine and morphine by plotting drug/internal standard peak area ratios versus the total amount of drug in the standard solutions, in the interval 10–1000 ng. Calibration curves showed good correlation coefficients (Fig. 2) for both analytes over the whole range.

3.2.3. Accuracy

Accuracy was expressed as the recovery (%REC) evaluated by analyzing in triplicate six standard solutions of morphine ranging from 25.0 to 1000.0 ng_{tot} and seven standard solutions of codeine ranging from 10.0 to 1000.0 ng_{tot}. %REC was calculated according to: $\%REC = [A_{analyte}/(A_{IS}\, RR_{med}\, C_t)] \times 100$. Where C_t is the total amount of analyte in the standard solution and RR_{medium} is defined in Section 2.5.

The averaged results of the recovery studies for morphine and codeine are reported in Table 5.

Table 4
Influence of the sonication time on the recovery of the analytes.

Sample	Time (min)	Sample (g)	RAmorphine (g)	RAcodeine (g)
1	15	0.5082	0.3789	0.3080
2	30	0.5076	0.3349	0.2907
3	60	0.5053	0.3114	0.2828

Table 5
Accuracy of the method.

Samples	%REC	SD	%RSD
Morphine: ACCURACY			
18	100.1	6.39	6.38
Codeine: ACCURACY			
21	101.7	16.15	15.87

3.2.4. Precision

The same standard solutions were analyzed in triplicate during three different days (I, II and III) in order to evaluate the precision of the method. The RR value was calculated for every standard solution and the medium response ratios (RR_{med}) are reported in Table 6. Data obtained demonstrate an adequate reproducibility.

3.2.5. LOD and LOQ

Limit of detection (LOD) and limit of quantitation (LOQ) were also evaluated. A series of decreasing concentrations of drug-fortified homogenized brain samples was analyzed. LOD was determined to be the lowest analyte concentration with a S/N ratio of at least 3 and resulted 10 ng for morphine and 5 ng for codeine, while LOQ was defined as the lowest concentration with a S/N ratio of at least 10 at which the values of accuracy and precision had a coefficient of variation below 15%. The LOQ was 25 ng for morphine and 10 ng for codeine and was calculated by analyzing standard solutions of morphine 25 ng_{tot} and codeine 10 ng_{tot} during 3 days (Table 7).

3.3. Analysis of brain areas

The validated method was applied to the analysis of six different brain areas: hippocampus, frontal lobe, occipital lobe, nuclei, bulb

Table 6
Precision of the method.

ng_{tot}	RR I	RR II	RR III		
Morphine: PRECISION					
25.0	0.0120	0.0122	0.0117		
50.0	0.0107	0.0117	0.0109		
100.0	0.0099	0.0119	0.0101		
250.0	0.0103	0.0123	0.0104		
500.0	0.0103	0.0106	0.0107		
1000.0	0.0107	0.0110	0.0107		
RR_{med}	0.0106	0.0116	0.0108	RR_{med}	0.0110
±SD	0.0007	0.0007	0.0006	$\pm SD_{med}$	0.0007
%RSD	7.04	5.75	6.02	$\%RSD_{med}$	6.06
Codeine: PRECISION					
10.0	0.0402	0.0408	0.0484		
25.0	0.0444	0.0452	0.0328		
50.0	0.0406	0.0305	0.0349		
100.0	0.0324	0.0362	0.0354		
250.0	0.0303	0.0337	0.0423		
500.0	0.0307	0.0319	0.0324		
1000.0	0.0309	0.0347	0.0309		
RR_{med}	0.0356	0.0361	0.0367	RR_{med}	0.0361
±SD	0.0059	0.0052	0.0063	$\pm SD_{med}$	0.0058
%RSD	16.62	14.71	17.23	$\%RSD_{med}$	16,05

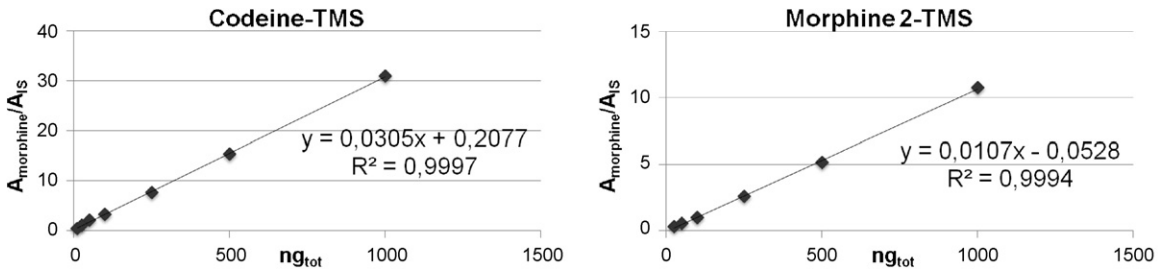

Fig. 2. Linearity of morphine and codeine.

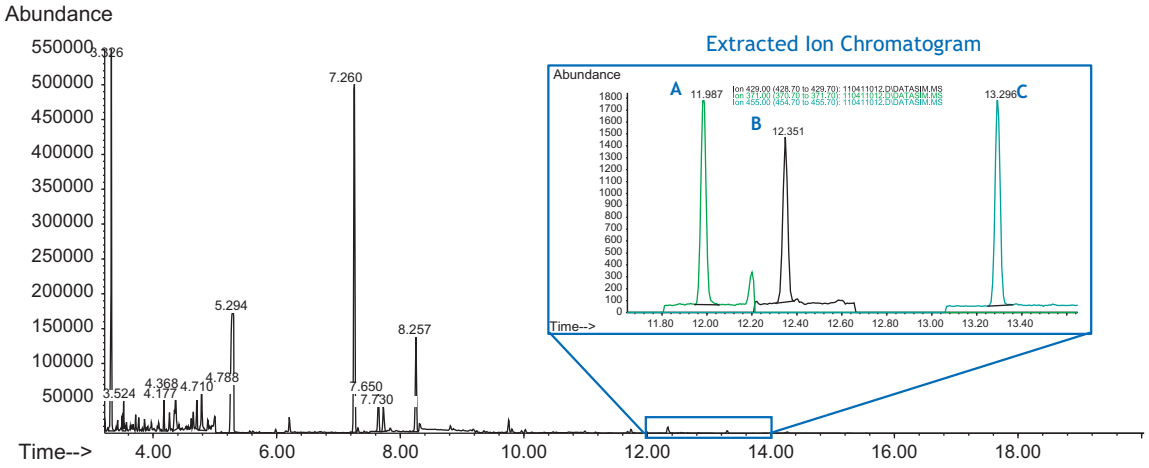

Fig. 3. SIM chromatogram of a specimen of case 2-(NUCLEI): (A) codeine-TMS; (B) morphine-2TMS; (C) IS.

Table 7
LOQ values.

	ng_{tot}	RR_{med}	±SD	%RSD
Morphine	25.0	0.0120	0.0003	2.10
Codeine	10.0	0.0431	0.0046	10.60

Table 8
Concentrations of drugs in case 1.

Brain areas	Morphine (ng/g)	Codeine (ng/g)
Hippocampus	106	34
Occipital lobe	71	32
Bulb	75	38
Frontal lobe	85	29
Pons	85	32
Nuclei	88	31

and pons, taken from two cases of heroin fatalities (Table 1), to evaluate brain distribution pattern of morphine and codeine.

A representative chromatogram obtained for the analysis of Case 2 nuclei is reported in Fig. 3.

The results demonstrated that morphine and codeine were homogeneously distributed in all the areas analyzed (Tables 8 and 9, Figs. 4 and 5) and the analyte concentration detected in the basal ganglia (nuclei), which is the area generally collected during an autopsy, was representative of the whole brain, showing an intermediate concentration of morphine and codeine in both fatalities.

Table 9
Concetrations of drugs in case 2.

Brain areas	Morphine (ng/g)	Codeine (ng/g)
Hippocampus	129	13
Occipital lobe	181	24
Bulb	114	14
Frontal lobe	147	16
Pons	159	25
Nuclei	149	28

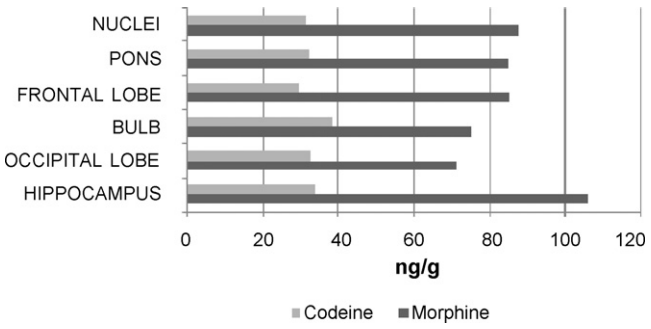

Fig. 4. Distribution of the analytes in case 1.

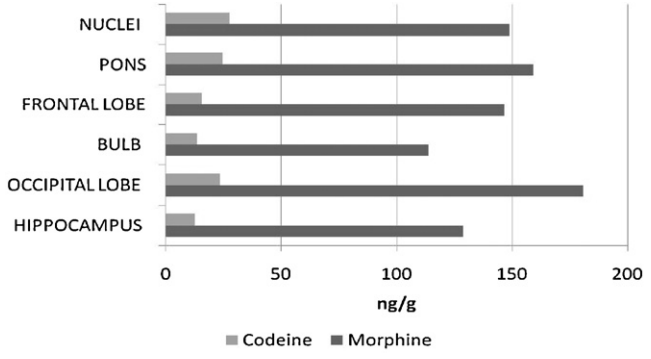

Fig. 5. Distribution of the analytes in case 2.

4. Conclusions

A simple and reliable GC/MS method for the quantification of heroine metabolites morphine and codeine in brain has been developed and validated. Specificity, linearity, accuracy, precision, LOD and LOQ were examined for the analytes and found satisfactory.

The pretreatment of the biological sample, especially the deproteinization procedure was studied in order to optimize the recovery of the analytes and the elimination of the interfering species in a complex matrix such as brain. Five methods were tested: pretreatment with trichloroacetic acid, ammonium sulfate, zinc sulfate, 5-sulfosalicylic acid and ultrasonic bath. The ultrasonic bath led to the best results in terms of recovery, both for morphine and codeine and was definitely faster and simpler than the others, so it was applied for the analysis of all the samples under investigation. After the pretreatment step, samples were submitted to SPE, derivatization and analysis by GC/MS.

The method was applied to the analysis of six brain area specimens coming from two heroin-related fatalities. Results showed that there was no evidence of accumulation of heroin metabolites in a particular brain area and that the analytes concentration in the nuclei was representative of morphine and codeine levels in brain tissue.

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