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Analysis of opioids in postmortem urine samples by dispersive liquid-liquid microextraction and high performance liquid chromatography with photo diode array detection

Elham Alahyari[1], Mehrdad Setareh[2,3], Ahmad Shekari[2,3], Gholamreza Roozbehani[2,3] and Kambiz Soltaninejad[3*]

Abstract

Background: Opioids abuse and related deaths are increasing in the world. Therefore, the design of new analytical methods for detection of opioids in biological samples is necessary for clinical and forensic settings.

Methods: In this study, dispersive liquid-liquid microextraction (DLLME) combined with high-performance liquid chromatography with photo diode array detector (HPLC-PDA), as a new and sensitive method were examined for the extraction and determination of morphine, codeine and methadone in postmortem urine samples. Effective factors on DLLME were optimized. The extracts were analyzed by HPLC-PDA using a Eurospher® C_{18} column (250 mm × 4.6 mm, particle size: 5 μm).

Results: The volumes of chloroform as the extraction solvent and acetone as the dispersive solvent were selected 300 μl and 500 μl, respectively. The optimum pH 9.8 and extraction time was 0.5 min were selected. Under optimum condition, the enrichment factor and the recovery of morphine, codeine, and methadone spiked into postmortem urine samples were in the range of 175–215.8 and 87.5–107.9%, respectively. Calibration curves for each analyte are linear in the range of 0.5–100 μg ml^{-1}. Limit of detection (LOD) for the analytes was in the range of 10–25 μg l^{-1}. Finally, the proposed method was successfully applied to 50 postmortem urine samples for determination of the opioids.

Conclusions: The proposed method is an easy, fast, low cost and efficient for the extraction and determination of opioids in postmortem urine samples and should be considered as analytical method for determination of opioids in forensic and clinical toxicology labs.

Keywords: Opioids, Urine, Postmortem, DLLME, HPLC-PDA

Background

In recent years, substance abuse has been widely spread in the world and has social, economic, cultural and political dimensions in the society and considered as a major health threat (UNODC 2016). Opioids are a class of analgesics commonly used in clinical medicine for treatment of moderate and sever pain (Gergov et al. 2009; Pathan and Williams 2012). Also, they have the high potential for abuse (UNODC 2016). Therefore, analysis of the opioids in biological samples has been considered as an important issue in the forensic and clinical toxicology (Gergov et al. 2009; Shamsipur and Fattahi 2011). The determination of abused drugs in postmortem samples can provide some special challenges in comparison with clinical samples (Drummer 2004). The variety and quality of the biological samples such as decomposed tissues, instability and degradation of drugs of abuse in the postmortem conditions and drug redistribution are the some special features in analysis of drugs in postmortem forensic toxicology (Drummer 2004). Furthermore, the development of new analytical

* Correspondence: kamsoltaninejad@gmail.com
[3]Department of Forensic Toxicology, Legal Medicine Research Center, Legal Medicine Organization, Tehran 1114795113, Iran
Full list of author information is available at the end of the article

methods for the qualitative and quantitative analysis of opioids in postmortem biological samples is an important concern in the forensic toxicology (Drummer 2004). A fast, easy and effective method for sample preparation is a key role for achieving to the better analytical procedures. Some traditional analytical techniques such as liquid-liquid extraction (LLE) and solid-phase extraction (SPE) have been developed for extraction and determination of drugs including opioids in biological specimens (Wey and Thorman 2001; Whittington and Kharasch 2003; Mabuchi et al. 2004). There are some limitations on using of these methods of sample preparation. LLE method is time-consuming and requires the use of large volumes of high purity and toxic organic solvents. The SPE is a method with relatively good efficacy, but it is relatively time-consuming for some long processes such as the washing and evaporation of the solvents. Also, in some cases, the method recovery is not enough for trace analysis (Shamsipur and Fattahi 2011). Therefore, the development of rapid, easy and environment– friendly analytical methods is encouraged.

Recently, microextraction procedures are the most effective sample preparation methods prior analysis. For example, liquid-phase microextraction (LPME) was successfully used for extraction of analytes from aqueous samples (Jeannot and Cantwell 1996; He and Lee 1997). Hollow fiber LPME (HF-LPME) is another easy and low-cost sample preparation method in order to extraction of analytes from complex samples (Shen and Lee 2002; Lee et al. 2008; Saraji et al. 2011). The combination of ultrasound with microextraction and solvent drop solidification (LPME-SFO) are the two examples of developed methods based on microextraction (Leong and Huang 2008; Ma et al. 2009; Cheng et al. 2011; Zhang and Lee 2012).

Dispersive liquid-liquid microextraction (DLLME) is another type of microextraction method that consists of a trinary system of solvents including a high-density and water- immiscible extraction solvent (extractant), a dispersive solvent highly miscible with the extraction solvent and aqueous sample, and an aqueous sample (Rezaee et al. 2006). The method based on the formation of very small droplets of extraction solvent in the sample solution after injection of extractant and dispersive solvent into aqueous sample (Shamsipur and Fattahi 2011). The large contact surface area between the extraction solvent and aqueous sample forms a cloudy mixture. This phenomenon facilitates a rapid equilibration. When the cloudy solution is centrifuged, the extractant forms the sediment phase and removed with a microsyringe for later analysis (Yan and Wang 2013; Saraji and Boroujeni 2014).

The DLLME is a simple, fast, efficient, environmentally-friendly and economic method for sample preparation (Rezaee et al. 2006; Nagaraju and Huang 2007; Shamsipur and Fattahi 2011). It has been used for various types of biological matrices (Li et al. 2008; Xiong et al. 2009; Mashayekhi et al. 2010; Rezaee et al. 2010a; Rezaee et al. 2010b; Fernández et al. 2013). DLLME could be combined with a variety of chromatography techniques such as Gas chromatography- Mass Spectrometry (GC-MS) (Leong and Huang 2008; Meng et al. 2015), High Performance Liquid Chromatography (HPLC) (Ahmadi-Jouibari et al. 2013; Fernández et al. 2015) and capillary electrophoresis (Kohler et al. 2013).

Although, there are few studies about the analysis of opium alkaloids and opioids drugs in clinical biological samples (Wey and Thorman 2001; Whittington and Kharasch 2003; Saraji et al. 2011; Shamsipur and Fattahi 2011; Ranjbari et al. 2012; Ahmadi-Jouibari et al. 2013), but there are scant data about the analysis of opioids by DLLME-HPLC-PDA in postmortem urine samples. Therefore, in this study, we optimized a DLLME-HPLC-PAD for the extraction and determination of morphine, codeine, and methadone in postmortem urine samples.

Methods

Chemicals

Standard morphine, codeine and methadone were obtained from Darou Pakhsh Pharmaceutical Co. (Tehran, Iran). HPLC grade solvents including acetonitrile, methanol, acetone, chloroform, water, phosphoric acid, potassium dihydrogen phosphate, sodium carbonate were purchased from Merck Co. (Darmstadt, Germany). To prepare the 0.05 M phosphate buffer, 16.65 g potassium dihydrogen phosphate was dissolved in 2.5 l of HPLC-grade water and the pH of the buffer in the mobile phase was adjusted to pH 2.3 using phosphoric acid 85% *w*/*v*. Stock standard solution with concentration level 1 mg ml^{-1} were prepared for morphine, codeine and methadone in methanol was prepared. Working standards were made by dilution of stock solution to final concentrations in urine. All solutions were stored at 4 °C.

Instrumentation

An HPLC system including pump (Smartline, Model 1050) and Smartline PDA 2850 (multi wavelength) detector with RP column Eurospher® (250 mm × 4.6 mm, particle size: 5 μm) was used in this study. Data processing was performed with ChromGate® software (version 3.1.7), all from Knauer Co. (Berlin, Germany). The mobile phase consisted of acetonitrile (A) and 0.05 M phosphate buffer at pH 2.3 (B). Buffer and the mobile phase flow rate of 1 ml min^{-1} was used in gradient elution mode: 0–7 min, A% 10 and B % 90; 7–8 min, A% 20 and B% 80; 8–15 min, A% 20 and B% 80; 15–16 min, A% 37 and B% 63; 16–40 min, A% 37 and B% 63; 40–45 min, A% 10 and B% 90.

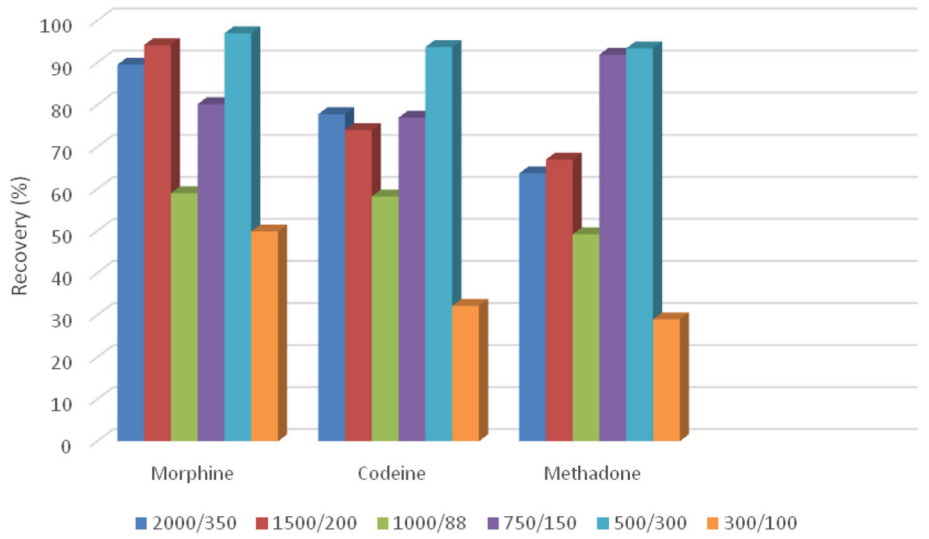

Fig. 1 Effect of the volume of acetone as disperser solvent and the volume of chloroform as extraction solvent on the recovery of morphine, codeine and methadone. Extraction conditions: sample volume, 5 ml, volumes of acetone: 300, 500, 750, 1000, 1500 and 2000 μl with containing 100, 300, 150, 88, 200 and 350 μl of chloroform, respectively, pH 9.8; extraction time, 0.5 min and the spiked concentration of morphine, codeine and methadone was 10μgml^{-1}

Extraction of opioids in postmortem urine samples with DLLME

Blank postmortem urine samples (drug-free) were obtained during the autopsy of cadavers without any drug abuse/poisoning history. The blank samples tested by routine postmortem toxicological analysis (Thin layer chromatography (TLC) for screening and GC-MS for confirmation). Also, postmortem urine samples were collected from the cadavers with opioids abuse/poisoning which have been transferred to forensic toxicology laboratory of Zanjan legal medicine center (Zanjan, Iran). The samples were stored at − 20 °C until analysis. The ethical committee of the Legal Medicine Research Center (Tehran-Iran) approved this project (Grant No. 20726).

Initially, the frozen urine samples were thawed at room temperature and then were centrifuged for 15 min at 4000 rpm. The supernatant was transferred into clean 15 ml conical test tube and filtrated by a 0.22 μm filter, then 2 ml of the sample was transferred to a 10 ml test tube and 3 ml distilled water was added (to reduce matrix effects).

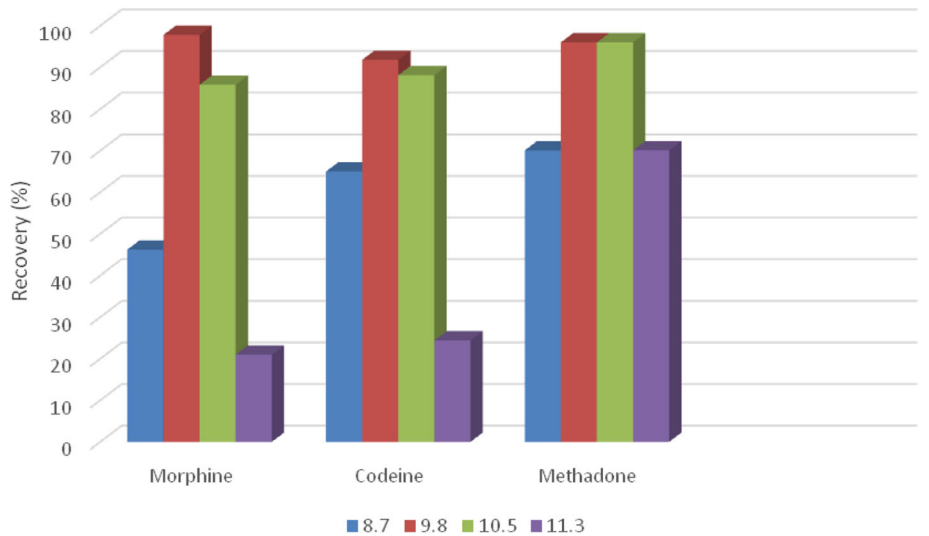

Fig. 2 Effects of the pH on the recovery of morphine, codeine and methadone from postmortem urine samples. Extraction conditions: sample volume: 5 ml, volume of acetone: 500 μl and volume of chloroform: 300 μl, extraction time: 0.5 min and the spiked concentration of morphine, codeine and methadone was 10 μg ml^{-1}

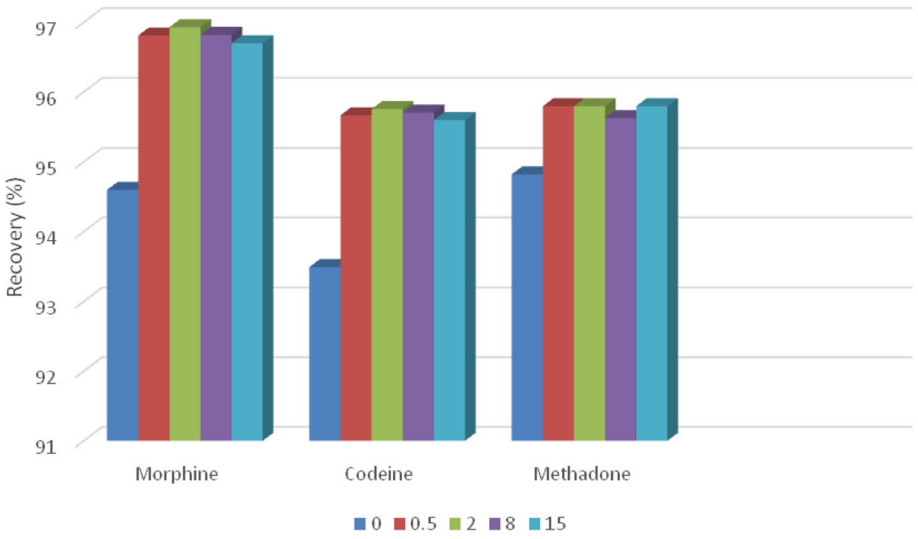

Fig. 3 Effect of extraction time (minutes) on the recovery of morphine, codeine and methadone. Extraction conditions: sample volume: 5 ml, volume of acetone: 500 μl and volume of chloroform: 300 μl, pH 9.8, the spiked concentration of morphine, codeine and methadone were 10 μg ml^{-1}

For the DLLME, an aliquot of 5 ml samples containing 2, 10 and 30 μg ml^{-1} of morphine, codeine and methadone were prepared and pH of the samples was adjusted at 9.8 by adding appropriate amounts of sodium carbonate (10%*w*/*v*). 300 μl of chloroform (extraction solvent) and 500 μl of acetone (disperser solvent) were mixed well together then this mixture was injected rapidly by using a 2 ml syringe into the sample solution and a cloudy mixture has been formed. In this step, the analytes were extracted into the tiny droplet of chloroform, in a very short time. Then the samples were vortexed for 30 s and centrifuged at 4000 rpm for 10 min. After the centrifugation, fine droplets of extraction solvent were sediment at the bottom of the test tube. The sediment phase removed with a 100 μl microsyringe (Hamilton, USA) and replaced to a 1 ml glass vial. After evaporation of the solvent under stream of nitrogen gas, the residue was dissolved in 50 μl methanol and injected into the HPLC-PDA.

Table 1 Quantitative results of morphine, codeine and methadone in spiked postmortem urine samples by DLLME-HPLC-PDA

Analyte	Linearity	R^2	LOD (μgl^{-1})	LOQ (μg l^{-1})
Morphine	y = 22,063 x + 1135.8	0.9991	25	100
Codeine	y = 669,350 x - 217,207	0.9995	9	30
Methadone	y = 892,444 x - 50,709	0.9989	10	35.5

Extraction condition: sample volume, 5 ml, pH = 9.8, volume of acetone as disperser solvent: 500 μl; volume of chloroform as extraction solvent: 300 μl and extraction time of 0.5 min
LOD (Limit of detection) for an S/*N* = 3
LOQ (Limit of quantification) for an S/*N* = 10

Optimization of DLLME

Affecting factors the DLLME procedure including the type and volume of extraction solvent, type and volume of disperser solvent, pH and extraction time were optimized in this study. Optimization of these factors was done by using postmortem blank urine samples spiked with morphine, codeine, and methadone.

Validation of DLLME- HPLC-PDA method

Limit of detection (LOD), limit of quantification (LOQ) and linearity

The limit of detection (LOD) and the limit of quantification (LOQ) were considered as the lowest concentration of the analytes corresponding to relationship of signal to noise ratio 3:1 and 10:1, respectively (SWGTOX 2013). The linearity of the method determined in the concentration ranges of 0.5–100 μg ml^{-1} of morphine, codeine, and methadone. The calibration curves were drawn for morphine, codeine and methadone into blank postmortem urine samples spiked with concentrations of 0.5, 2, 5, 10, 20, 30, 40, 50 and 100 μg ml^{-1} for each analyte. All concentrations were analyzed in triplicate.

Precision, accuracy, enrichment factor, recovery, and relative recovery

Inter-day and intra-day precisions method, and the enrichment factors and recovery for morphine, codeine and methadone were studied by extracting the spiked blank postmortem urine samples with 2, 10 and 30 μg ml^{-1} concentrations. In order to evaluate the accuracy of the method acting to prepare three urine samples with concentrations of 2, 10 and 30 μg ml^{-1} of morphine, codeine, and

Table 2 Validation parameters of analytes in postmortem urine samples

Analyte	Added concentration ($\mu g ml^{-1}$)	Intraday precision RSD (%) ($n = 3$)	Interday precision RSD (%) ($n = 3$)	Accuracy (Relative Error %) (SD, $n = 3$)	Recovery (%) (SD, $n = 3$)	EF
Morphine	2	4.1	2.8	−4.5 (0.12)	95.5 (0.05)	191
	10	1.63	3.04	−2.7 (0.09)	107.9 (0.28)	215.8
	30	5.6	4.05	−2.6 (0.23)	101.87 (0.04)	203.74
Codeine	2	3.8	4.1	−51 (0.23)	87.5 (0.09)	175
	10	3.9	6.07	−2.3 (0.37)	93.5 (0.4)	187
	30	3.8	1.17	3.9 (1.11)	101.5 (0.83)	203
Methadone	2	4.9	4.4	−8 (0.1)	89.5 (0.14)	179
	10	5.7	5.7	1.7 (0.26)	97 (0.12)	194
	30	3.4	3.6	−4.8 (0.44)	98.6 (0.73)	197.2

Extraction condition: Sample volume, 5 ml, pH 9.8, volume of acetone as disperser solvent: 500 μl; volume of chloroform as extraction solvent: 300 μl and extraction time: 0.5 min
EF Enrichment Factor

methadone (control urine samples). Then each of the three control samples was divided into three equal parts and was extracted by DLLME process. Accuracy in the format of the relative error (RE%) and precision to form of the relative standard deviation (RSD %) were reported.

The enrichment factor (EF) is the analytes concentration in the sediment and initial concentration of analytes within the sample and calculated according to previous study (Rezaee et al. 2006).

The extraction recovery (%ER) was defined as the ratio between the amount of the analyte in the sediment and the initial amount of the analyte within the sample and determined as previous method (Rezaee et al. 2006).

The relative recovery was studied by extracting the spiked postmortem urine samples (with suspected drug abuse) with two concentrations of morphine, codeine, and methadone (5 and 20 $\mu g\ ml^{-1}$). And then the relative recovery (%RR) was calculated according to the previous method (Rezaee et al. 2006).

Table 3 Relative recoveries and standard deviations of opioids in actual postmortem urine samples

Analyte	Sample number	Concentration of analyte ($\mu g\ ml^{-1}$)	Added concentration ($\mu g\ ml^{-1}$)	Founded concentration ($\mu g\ ml^{-1}$) $N = 3$ (SD)	Relative recovery
Morphine	1	2.5	5	7.5 (1.05)	100
			20	23.6 (1.19)	105.5
	2	17.8	5	21.4 (0.84)	88
			20	38.4 (1.57)	103
	3	8.7	5	13.5 (0.41)	96
			20	26.1 (0.77)	87
Codeine	1	2.2	5	7.12 (0.03)	98.4
			20	21.05 (0.53)	94.3
	2	5.3	5	9.98 (0.36)	93.6
			20	27.24 (1.84)	109.7
	3	0.9	5	5.34 (0.62)	88.8
			20	17.76 (1.10)	84.3
Methadone	1	1.9	5	5.96 (1.09)	82
			20	23.70 (1.31)	109
	2	3.2	5	8.35 (1.2)	103
			20	24.76 (1.29)	107.8
	3	10.4	5	14.87 (0.57)	89.4
			20	28.79 (0.12)	91.95

Extraction condition: sample volume: 5 ml; pH = 9.8, volume of acetone as disperser solvent: 500 μL, volume of chloroform as extraction solvent, 300 μl and extraction time: 0.5 min

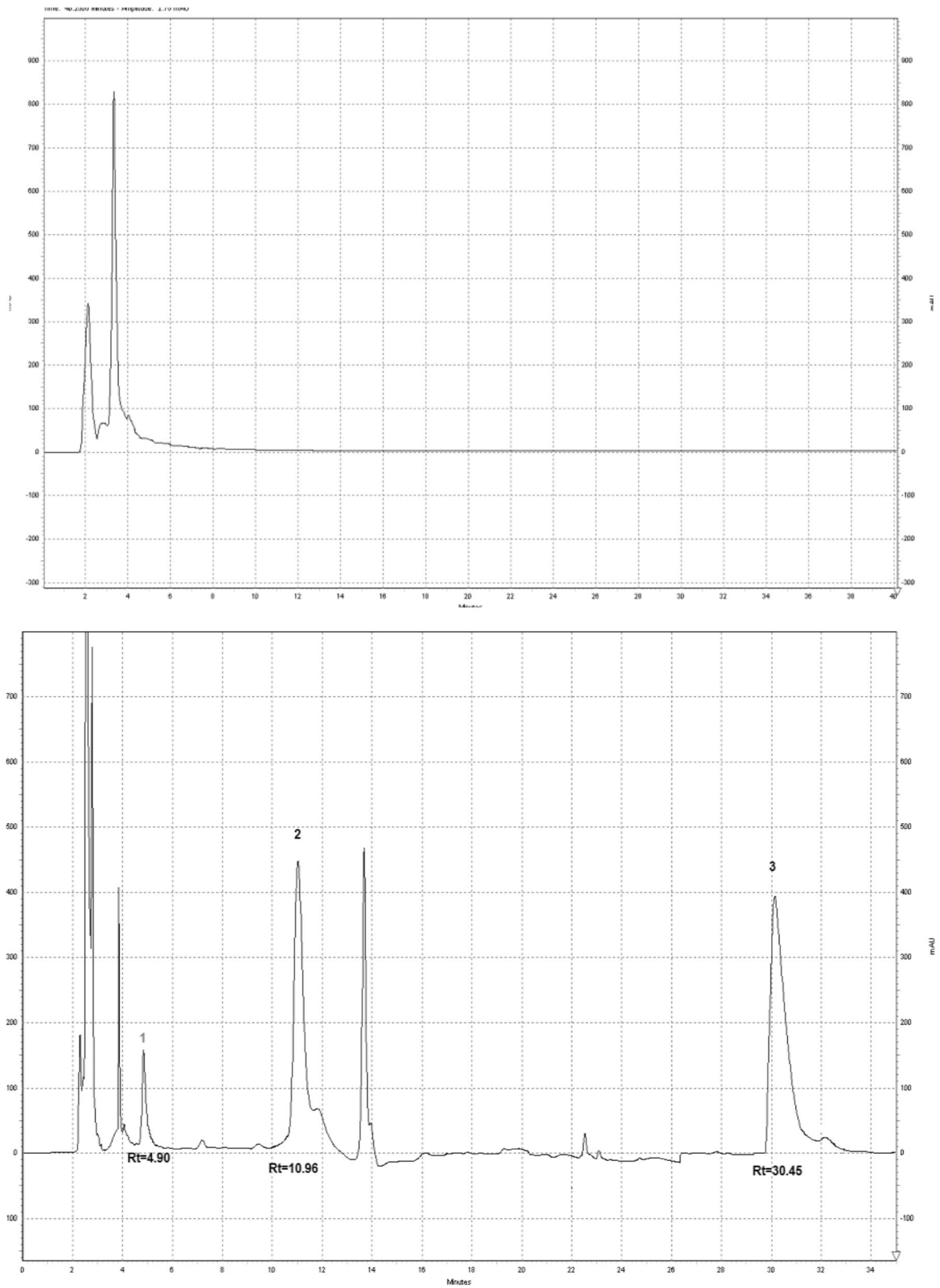

Fig. 4 Chromatogram of blank postmortem urine sample (upper) and spiked postmortem urine sample with drugs (Lower, 1: morphine, 2: codeine, 3: methadone) and extracted under the optimum condition of dispersive liquid –liquid microextraction (DLLME). Spiked concentration, 10 μg ml^{-1}

Results and discussion

Selection of extraction solvent

Selection of a suitable extraction solvent is one of the key steps in the DLLME procedure that direct impact on the efficiency of method (Rezaee et al. 2006). The extraction solvent should be have a characteristics such as higher density and low solubility in water, miscible with disperser solvent and capability for extraction of target analytes (Saraji and Boroujeni 2014; Sharifi et al. 2016). Chloroform due to have characteristics such as the higher density than water, the boiling point and the solubility in water, was used as appropriate extraction solvent to extract opioids from the postmortem urine samples (Shamsipur and Fattahi 2011).

Selection of disperser solvent

The disperser solvent should be miscible in the water and dissolve in the extraction solvent and capable to form a cloudy solution (Saraji and Boroujeni 2014; Sharifi et al. 2016). In this study, acetone was selected as dispersive solvent due to the highest recovery of opium alkaloids, lower toxicity and cheaper than methanol and acetonitrile.

Optimization of extraction solvent volume

The effect of extraction solvent volume in the recovery of morphine, codeine and methadone in postmortem urine samples in the DLLME process, was investigated using different volumes of chloroform (88, 100, 150, 200, 300 and 350 μl). All concentrations were analyzed in triplicate. With increase the volume of chloroform from 88 to 350 μl, increased the volume of the sediment phase. With the increasing volume of chloroform from 88 to 300 μl, for each analyte, extraction efficiency were increased, while a further increase in the volume of chloroform (higher than 300 μl) were given a small reduction in extraction efficiency and a reduction in the enrichment factor of these three compounds. At low volumes of chloroform (88 and 100 μl) a significant reduction in analyte extraction efficiency were observed. Therefore, based on the results, 300 μl was selected as the optimal volume of extraction solvent (Fig. 1).

Optimization of disperser solvent volume

Performance of DLLME method directly influences by the volume of dispersive solvent. Changes in the volume of dispersive solvent cause changes the volume of the sediment phase. To obtain an optimal volume of acetone, was performed by several experiments using different volumes of acetone contains (300, 500, 750, 1000, 1500 and 2000 μl) and various volumes of chloroform (88, 100, 150, 200, 300 and 350 μl). All concentrations were analyzed in triplicate. The results showed that at low volume of acetone (300 μl) dispersion of the chloroform did not complete and a decrease in extraction recovery was observed. Also, in the high volume of acetone (1500 and 2000 μl), extraction recovery of morphine, codeine, and methadone decreased due to the increased solubility of the analyte in the sample solution and reducing entry them into the organic phase. Therefore, based on the results, 500 μl of acetone was selected as the optimal volume of disperser solvent in this study.

Optimization of pH

Addition of adequate amount of sodium carbonate to the sample solutions, in order to adjust the pH and ionic strength of solutions, directly effect of the extraction efficiency. In this study, the extraction recovery of morphine, codeine, and methadone was examined in different pH in the range of 8.5–11.5 (an average of 8.7, 9.8, 10.5 and 11.3). The results obtained showed that very low pH in the range of 8.5–9 (average 8.7) and as well as in high pH in the range of 11–11.5 (average 11.3), extraction efficiency for all three compounds in the study, significantly decreased. Also, in the pH 10–11 (average 10.5) is a very small reduction in the extraction efficiency. So, with the view of increasing the extraction efficiency of morphine, codeine, and methadone in the pH 9.8, this pH was selected as optimum for extraction of the opioids from postmortem urine samples (Fig. 2).

Optimization of extraction time

Optimization of extraction time in a variety of liquid-phase microextraction, is a critical factor that plays a great impact on the extraction efficiency. In DLLME method, the extraction time is defined as the interval time between fast injecting a mixture of extraction and dispersive solvents into the sample solution before the start of centrifuges (Rezaee et al. 2006; Shamsipur and Fattahi 2011). In this study, the effect of extraction time on the enrichment factor and extraction efficiency was determined. A range time from 0 to 15 min (0, 0.5, 2, 8 and 15 min) were evaluated. The results showed that extraction time has no significant effect on the extraction recovery for morphine, codeine and methadone. Thus the extraction time in all experiments carried out in this study was 30 s (Fig. 3).

Validation of method

The characteristics of the calibration curves were summarized in Table 1. All results were obtained under the optimized conditions and repeated in triplicate. The calibration curves were linear over the concentration ranges of 0.5-100 μg ml^{-1} for morphine, codeine, and methadone. The values of the correlation coefficients (R^2) ranged from 0.9989 to 0.9995. Inter-day and Intra-day precision results that were studied by extracting the spiked samples with 2, 10 and 30 μg ml^{-1} of morphine,

Table 4 Comparison of DLLME-HPLC-PDA with other analytical methods for determination of morphine, codeine and methadone in biological samples

Method	Sample	Analyte	LOD (μg l^{-1})	%RSD	Recovery (%)	Extraction Time(min)	Author
SPE-FLC/DAD	Urine	Morphine	7.6	5.5	72.83	> 40	Dams et al. 2002
	Blood	Codeine	6.3	5.4	85.39		
SPE-HPLC/DAD	Plasma	Morphine	24	4.02		> 20	Fernandez et al. 2006
		Codeine	32	3.18	–		
DLLME - SFO –HPLC-UV	Plasma	Morphine	5	7.4	55.2	0.5	Leong and Huang 2008
		Codeine	5	6.5	66.3		
DLLME –HPLC-UV	Urine	Morphine	7	6.1	31.5	4	Shamsipur and Fattahi 2011
		Codeine	10	5.7	42.7		
DLLME –HPLC-UV	Urine Plasma	Methadone	4.9	2.26	100.34	3	Ranjbari et al. 2012
DLLME-CE-ESI-TOF-MS	Urine	Codeine	0.5	74	74	–	Kohler et al. 2013
		Methadone	0.25	–	90		
DLLME – HPLC-PDA	Plasma	Morphine	28.5	3	> 84	3.5	Fernández et al. 2013
		Methadone	13.9	1.2			
DLLME-UA-LDS- GC/MS	Urine	Methadone	1.5	4.7	86.5	3	Meng et al. 2015
DLLME – HPLC-PDA	Urine	Morphine	25	3.04	101.87	0.5	Present Study
		Codeine	9	3.8	93.5		
		Methadone	10	4.4	98.6		

LOD Limit of detection, *RSD* Relative standard deviation

codeine, and methadone, was reported (Table 2). Also, the accuracy of the method was evaluated by calculating of relative error. Relative error is not more than % ± 15. In this study, the relative error for morphine, codeine and methadone in the ranges of − 8 to 3.9% was obtained (Table 2). LOD and LOQ for all the analytes were in the ranges of 9-25μgl^{-1} and 30-100μgl^{-1}, respectively (Table 1). The enrichment factor, recovery and relative recovery for morphine, codeine and methadone, was calculated and showed in the tables (Tables 2 and 3).

The simplest method for evaluation of selectivity of an analytical method is checking of absence of response in blank samples. There were no interferences at the retention times of three target analytes in the method (Fig. 4).

Application of DLLME-HPLC-PDA procedure

After the optimization of the effective factors on DLLME and achieving to good and satisfactory results from the validated method, the DLLME-HPLC-PDA was used successfully for extraction and determination of morphine, codeine, methadone in 50 actual postmortem urine samples. Based on the obtained results, morphine was found in 22 samples, codeine was detected in 17 samples and methadone was detected in 27 samples. Some of the opium alkaloids such as papaverine, thebaine and noscapine were identified at 2, 1 and 3 samples, respectively. Also, 6-monoacetylmorphine and tramadol were determined in 8 and 6 samples, respectively. Concentration of morphine, codeine and methadone in postmortem urine samples were calculated in the range of: 0.28-26 μg ml^{-1} (mean: 6.7 μg ml^{-1}) for morphine, 0.9–25.4 μg ml^{-1} (mean: 13.52 μg ml^{-1}) for codeine and 0.4–43.8 μg ml^{-1}(mean: 33.5 μg ml^{-1}) for methadone.

Comparison of DLLME-HPLC-PDA method with other methods

Table 4 summarized the comparison of the proposed method for the determination of morphine, codeine, and methadone in postmortem urine samples by the DLLME-HPLC-PDA with previous methods such as SPE-HPLC-DAD, SPE-FLC-DAD, DLLME-GC-MS, DLLME-SFO-HPLC and DLLME-HPLC-UV. The recovery and extraction time of the present method is better than other methods (Table 4). LOD in the proposed method is similar to the previous studies (Dams et al. 2002; Leong and Huang 2008; Shamsipur and Fattahi 2011; Ranjbari et al. 2012; Kohler et al. 2013; Fernández et al. 2013; Meng et al. 2015) (Table 4).

Conclusion

In this study, the efficiency and performance of DLLME process were assessed under optimum conditions for the extraction of opioids from postmortem urine samples. According to repeatability, linearity, high extraction efficiency and good enrichment factor, this method is

suitable for qualitative and quantitative analysis of opioids in postmortem urine samples. This is the first DLLME-HPLC-PDA method which optimized for postmortem urine samples and should be considered as an applied analytical method for determination of opioids in forensic toxicology laboratory.

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Availability of data and materials
Not applicable

Authors' contributions
EA attributed in method validation, analysis of samples, data gathering and analysis and writing of the draft of the manuscript. MS attributed in sample collection and data gathering. AS attributed in method validation and analysis of the samples. GR attributed method validation and data gathering. KS attributed in study design, supervision on all of the research's steps, revision the draft and writing the final version. All authors approved the final version of the paper.

Authors' information
EA (MSc in Toxicology) works as a technical expert in Laboratory of Toxicology. MS (MD, Forensic Medicine specialist) is head of the Zanjan Legal Medicine Center, Zanjan, Iran and member of the Legal Medicine Research Center, Legal Medicine Organization, Tehran Iran.AS (MSc in Toxicology) and GR (BS) are work as technical experts in Forensic Toxicology Laboratory of Zanjan Legal Medicine Center, KS (PharmD, PhD in Toxicology) is Associate Professor of Toxicology, Department of Forensic Toxicology, Legal Medicine Research Center, Legal Medicine Organization, Tehran Iran.

Ethics approval and consent to participate
All procedures performed in study involving human participants were in accordance with the ethical standards of the Legal Medicine Research Center's ethical committee (Grant No. 20726) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interest.

Publisher's Note

Author details
[1]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Pharmaceutical Sciences Branch, Islamic Azad University (IAUPS), Tehran, Iran. [2]Legal Medicine Center of Zanjan Province, Zanjan, Iran. [3]Department of Forensic Toxicology, Legal Medicine Research Center, Legal Medicine Organization, Tehran 1114795113, Iran.

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