Extraction and Analysis of Methadone in Exhaled Breath Condensate Using a Validated LC-UV Method

Maryam Khoubnasabjafari[1], Khalil Ansarin[1], Vahid Jouyban-Gharamaleki[2], Vahid Panahi-Azar[3], Ali Shayanfar[4], Laya Mohammadzadeh[5], Abolghasem Jouyban[6]

[1] Tuberculosis and Lung Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; [2] Department of Mechatronic Engineering, International Campus, University of Tabriz, Tabriz, Iran; [3] Liver and Gastrointestinal Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; [4] Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; [5] Neurosciences Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; [6] Drug Applied Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

Received, November 29, 2014; Revised, January 27, 2015; Accepted, April 21, 2015; Published, May 28, 2015.

ABSTRACT – Purpose. A combined microextraction and separation method is presented for the determination of methadone in exhaled breath condensate (EBC) which is a promising non-invasive biological component for monitoring drug concentrations. **Methods.** In this work, dispersive liquid–liquid microextraction (DLLME) and ultrasonic liquid–liquid microextraction (ULLME) procedure coupled with a validated liquid chromatography method were used for analysis of methadone in EBC collected using an in-house cold trap setup. The method has been validated according to the FDA guidelines using EBC-spiked samples and tested on a number of EBC samples collected from patients. **Results.** The best DLLME conditions involved the use of a disperser solvent of methanol (1 mL), extraction solvent of chloroform (200 μL), EBC sample pH of 10.0 and centrifugation at 6000 rpm for 5 minutes. The conditions for ULLME were 150 μL of chloroform and the samples were sonicated for 4 minutes. The method was validated over the concentration range of 0.5–10 $\mu g/L^{-1}$ in EBC. Inter- and intra-day precision and accuracy were less than 5 % where the acceptable levels are less than 20%. Furthermore, the validated method was successfully applied for the determination of methadone in patients' EBC samples. **Conclusions.** The outcomes indicate that the developed LC-UV combined with DLLME and/or ULLME extraction methods can be employed for the extraction and separation of methadone in EBC samples.

INTRODUCTION

Methadone or 6-dimethylamino-4,4-diphenyl-3-heptanone (Fig. 1) is a synthetic analgesic drug with high affinity for μ–opiod receptors, which is used in the management of opiates withdrawal symptoms and also as an analgesic drug in patients with severe pain (1). Methadone is administered orally and/or parenterally as its hydrochloride salt. It possesses a chiral center with higher affinity of R-methadone at μ and δ opiod receptors (2, 3), whereas S-methadone is ineffective (4). It is used as a daily dosage of 80–120 mg and exhaled in breath through distribution of systemic blood circulation into the fluid of the epithelial lining (5) in a concentration range of 22–1147 pgL^{-1} (5). Analysis of volatile and non-volatile analytes in exhaled breath (EB) has attracted more attentions in recent years and a number of papers have reviewed the application of these analyses in experimental and clinical investigations (6-10).

Corresponding Authors: Khalil Ansarin, Tuberculosis and Lung Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; Email: kansarin@gmail.com; Abolghasem Jouyban, Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran; Email: ajouyban@hotmail.com

There are two potential mechanisms for the presence of analytes in EB, i.e. evaporation and through small droplets (aerosols) formed in the alveoli. The most widely accepted mechanism for appearance of the volatile organic compounds is the dehydration of these compounds in the process of exhalation where the vapor pressure of the analyte is the most important factor (11). The second mechanism is the exhalation of submicron droplets of epithelial lining fluid formed by the reopening of collapsed terminal airway structures (12). This hypothesis of droplet formation has been confirmed by experimental and computational data (13). The analyte's properties including lipophilicity, molecular weight, and protein binding are the main parameters for describing the concentrations of the analytes in the EB produced via this mechanism (14). In view of the low value of Henry constant of methadone, i.e. 6.3×10^{-9} atm·m^{3}mole^{-1} (14), the probability of excretion of methadone in EB using evaporation mechanism is very low and it is more likely to be explained by the droplet formation mechanism (15). A great quantity of water is released from the breath which is deposited on the walls of the cooling system of the EB collection device and dilutes methadone in the collected sample (8).

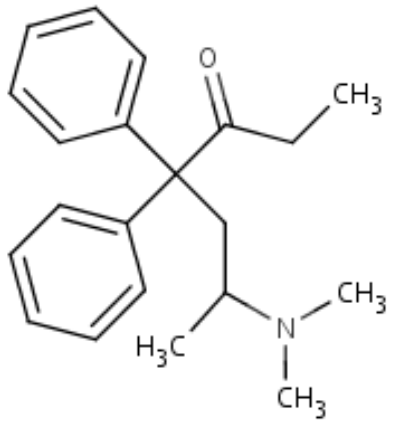

Figure 1. Chemical structure of methadone.

Methadone maintenance therapy (MMT) is a very common procedure for the management of opiate withdrawal symptoms. The pharmacokinetics of methadone varies among people (16) and to achieve the best MMT results, individualized dose adjustment is needed. Overdose of methadone causes respiratory depression, hypertension, coma and some other symptoms. Various biological samples have been analyzed to monitor methadone levels, including serum (17-20), plasma (21-25), whole blood (26), urine (17, 18, 21, 23, 24, 26-28, 31), saliva (18, 25), EB (14, 30) and sweat (25) samples. Due to depressed levels of methadone in biological fluids and especially in EB due to the further dilution of evaporating water which is entrapped in the cooling system of the EB sample collection device, solid phase extraction (14, 17, 20, 22, 23, 26, 27, 29, 30), liquid phase extraction (25, 31), or more sensitive detection like mass spectrometry (MS) method (14, 21, 22, 28, 30) has been applied for monitoring of methadone in biological samples. A brief summary of these methods is listed in Table 1. Regarding the instrumental techniques, liquid (14, 17, 19, 20, 21, 23, 25, 27, 29-31) or gas (18, 22, 24, 26, 28) chromatography methods are the most frequently used separation methods for methadone determination in biological samples. Capillary electrophoresis is another separation technique which has also been employed (32–34).

EB can be considered as a non-invasive method for monitoring of drug concentrations in clinical studies. The target analyte in EB can be trapped using adsorption, e.g., by solid phase extraction techniques, breath condensation and sampling in containers (14). Exhaled breath condensate (EBC) is collected using a cold trap setup and holds large quantities of water vapor. Moreover, the volume of EBC is affected by different parameters including; environmental temperature and relative humidity, time of sample collection and volume of inhaled air (8, 35–36). From an analytical point of view, EB provides a much simpler matrix when compared with routinely analyzed biological samples, i.e. serum or urine. On the other hand, due to lower concentrations of analytes in EBC and a simpler matrix in comparison with serum/urine, the analyst will be challenge with less matrix interference through the analytical process.

Table 1. Available methods for analysis of methadone in biological samples.

Matrix	Instrumental method	Pretreatment	Linear range	Ref
Urine	HPLC	SPE	-	17
Human plasma	LC/MS/MS	-	10- 1000 $ngmL^{-1}$	21
Urine	LC/MS/MS	-	20-2000 $ngmL^{-1}$	21
Plasma	GC/MS	SPME	50-2000 $ngmL^{-1}$	22
Urine	HPLC	LLE	0.125-12.5 µM	31
Serum, Urine	HPLC	SPE	0.02-5.0 μgmL^{-1}	27
Urine	GC/MS	-		28
Human plasma, Urine	HPLC	SPE	10.0-1500.0 $ngmL^{-1}$	23
Serum, Saliva, Urine	GC	-	0.05-2.0 μgmL^{-1}	18
Plasma, Urine	GC	HS-SPME	0.1-450 μgL^{-1}	24
Human urine	HPLC	SPE	-	29
Whole blood	GC	SPE	0-600 μgL^{-1}	26
Exhaled breath condensate	LC/MS	SPME	22-1147 pgL^{-1}	14
Serum	HPLC	-	5.8-25.9 $ngmL^{-1}$	19
Serum, Urine	HPLC	SPE	5.0-16.0 ngL^{-1}	20
Urine	HPLC	-	0.125-12.5 µM	31
Exhaled breath	LC/MS	SPE	100-2000 pg/sample	30
Urine	HPLC	DLLME	10-5000 $ngmL^{-1}$	25
Plasma	HPLC		20-5000 $ngmL^{-1}$	
Saliva	HPLC		75-5000 $ngmL^{-1}$	25
Sweat	HPLC		50-5000 $ngmL^{-1}$	

Despite the advantages of EB analysis for monitoring purposes, very low concentrations of the analytes in EB is the principal disadvantage of this sampling procedure which can be handled either by pre-concentration procedures and/or very sensitive detection systems such as MS detection. Most of the pre-concentration procedures are tedious and time-consuming and MS detection possesses its own restrictions.

The purpose of this work is to report a simple, accurate and low cost ultrasound-assisted liquid-liquid microextraction (ULLME) and/or compared with a dispersive liquid-liquid microextraction (DLLME) method for the pre-concentration of methadone in EBC samples. The extracted analyte using optimized extraction conditions was measured using a validated liquid chromatography (LC) method with UV detection system. Utility of the proposed method was demonstrated by examining a number of EBC samples collected from patients under MMT regimen admitted to a private MMT clinic in Tabriz.

METHODS

Chemicals

Methadone was provided as a gift from Temad Co. (Tehran, Iran). Dichloromethane, tetrachloroethylene, chloroform, carbon tetrachloride, methanol, acetone, acetonitrile, tetrahydrofuran, hydrochloric acid, and sodium hydroxide were purchased from Merck KGaA (Darmstadt, Germany). Distilled water was used for the preparation of aqueous solutions.

Apparatus

The liquid chromatography system consisted of a 1100 series pump, a 2-channel ERC-3315 degasser, a 1200 CE detector (UV-Vis) and an interface box, all from Cecil Instruments (Cambridge, UK) and the reversed-phase HPLC column was Nova-Pak C_{18} with dimensions of (3.9 × 150 mm) from Waters Co. (Massachusetts, US). The mobile phase consisted of 25 mM ammonium acetate/acetonitrile (10/90, v/v) with a flow rate of 1.2 $mLmin^{-1}$ (37). The pH of solutions was measured with a Metrohm 654 pH meter

(Herisau, Switzerland). A Hettich (EBA-20) centrifuge (Tuttlingen, Germany) and a Labtron (LS-100) vortex shaker (Tehran, Iran) were used for centrifugation and for shaking the solutions, respectively.

Sample Preparations

A standard stock solution of methadone (1000 mgL^{-1}) was prepared in methanol and stored at 4 ºC. Working solutions were prepared by diluting with methanol. EBC samples were obtained from healthy volunteers (for validation and calibration purposes) and patients receiving MMT (to show the applicability of the proposed method) using a lab-made setup based on a cooling trap system patented in the national patent office (38). All sample donors signed a consent form approved by the Ethics Committee of Tabriz University of Medical Sciences (approval number of 5/4/3822, dated 20 July 2014). The setup cools the EB down to -25 ºC and condenses the EBC with acceptable efficiency. Commercially available EBC collection devices provided the low temperature of -20 °C (39). Since there is a relationship between temperature and EBC collection efficiency and the lower the temperature, the better the efficiency (40), therefore, we are of the opinion that the efficiency of the developed setup is acceptable. Frozen (at -25 °C within the collection setup) EBC samples collected from healthy volunteers were thawed at room temperature and calibration standards in EBC were prepared by spiking 1000 µL of drug-free EBC with known amounts of the drug to achieve a concentration range from 0.5 to 10 μgL^{-1} and were kept at room temperature for 20 min before use. All EBC samples were diluted with water (for DLLME) and solution pH's was adjusted to 10.0 using 0.1 M NaOH.

Analytical Procedure

One millilitre of EBC was diluted to 10 mL and was rapidly injected into a coned bottom test tube containing 200 µL chloroform (extraction solvent) and 1 mL methanol (disperser solvent) for DLLME. Another 1 mL EBC sample was injected into a coned bottom test tube containing 200 µL chloroform and sonicated under the optimized ultrasonic condition for ULLME. The formed cloudy solutions from both procedures were centrifuged at 6000 rpm for 5 min, the organic phase settled to the bottom of the tube and was transferred to a microtube after discarding the supernatant. After evaporation of the solvent under a nitrogen stream, the rest was dissolved using 50 µL of methanol and injected into the LC system using a 20 µL injection loop. The mean of three replicates was used to plot a calibration curve and other charts reported in the following sections.

Assay Validation

The analytical method specificity was assessed by comparing the chromatograms of blank EBC and spiked EBC after DLLME and ULLME procedures. Linear range and correlation coefficient of the obtained calibration curve was investigated. The lowest and the highest concentrations of the standard solutions of methadone used for construction of the calibration curve are defined as the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) according to FDA validation guideline (41). Moreover, the standard addition method was also utilized in three concentration levels of methadone for the determination of intra-day and inter-day accuracy, precision and relative recovery.

RESULTS

Extraction efficiency of DLLME and ULLME depends on various parameters. One variable at a time optimization procedure was applied to study the affecting parameters on the extraction efficiency. In other words, the effect of one affecting parameter on the performance of the method was investigated and the rest of analytical parameters were kept constant. More or less important parameters such as types of extraction and dispersive solvents or time of ultrasonication and the volume of solvent, pH, sample volume, optimization of centrifugation rate and time were investigated.

Selection of the Extraction Solvent

Dichloromethane, tetrachloroethylene, chloroform, and carbon tetrachloride were used as extraction solvents. DLLME or ULLME procedures were performed using different volumes of selected extraction solvents mixed with 1 mL methanol to give an equal volume of organic phase (DLLME) or 4-min ultrasonication (ULLME). The results (Fig. 2) revealed that methadone was better extracted into chloroform than the other investigated solvents, therefore, chloroform was selected as the extraction solvent.

Selection of the Disperser for DLLME

To find out the appropriate disperser solvent, 1 mL of methanol, acetonitrile, acetone and tetrahydrofuran mixed with 200 µL of chloroform in separate experiments and was rapidly injected into the aqueous samples. As shown in Fig. 3, the highest response was observed when methanol was used.

Optimization of Extraction Solvent Volume

The extraction solvent volume effect was evaluated by injecting 1 mL of methanol (DLLME) to coned bottom test tubes containing different volumes of chloroform (50, 100, 150, 200 and 300 µL) in DLLME procedure. In case of ULLME, 4 min sonication was used instead of methanol addition. The results (Fig. 4) show that the analytical signal was increased by the addition of chloroform, then reached to a maximum value followed by a gradual decrease with further increase of extraction solvent volume. Therefore, 200 µL for DLLME and 150 µL for ULLME were chosen as volumes of extraction solvent for further experiments.

Optimization of Disperser Volume for DLLME

Different volumes of methanol (0.25–2 mL) containing 200 µL of chloroform were investigated. The results are illustrated in Fig. 5. For this reason, 1 mL methanol was selected as the optimum volume of disperser in subsequent experiments.

Optimization of Ultrasonication Time for ULLME

The time of ultrasonication was investigated by injecting 150 µL of chloroform for ULLME and the results are illustrated in Fig. 6. According to these results, 4 min was selected as the best time for sample ultrasonication.

Optimization of Sample Volume

The sample volume effect was studied using EBC ten times diluted (for DLLME) at four levels varying from 0.25 to 1.0 mL containing 20 $\mu g L^{-1}$ of spiked methadone. In general, peak areas should increase when the sample volume is increased. This is due to the existence of additional amounts of methadone in the aqueous solution. The peak area was increased with increasing sample size. Due to practical problems associated with collecting EBC samples of more than 1.0 mL, sample volumes were not further increased. Therefore, 1.0 mL of EBC was used as the optimum sample volume.

Optimization of Centrifugation Rate and Time

The extraction equilibrium can be attained quickly after adding a mixture of the extraction and disperser solvents. In the DLLME process, the time-consuming step is centrifugation. The effects of centrifugation rate and time were examined in the range of 3000–6000 rpm and 2–20 min, respectively. According to the results obtained (Figs. 7 and 8) 6000 rpm and 5 min were selected as centrifuge rate and time, respectively.

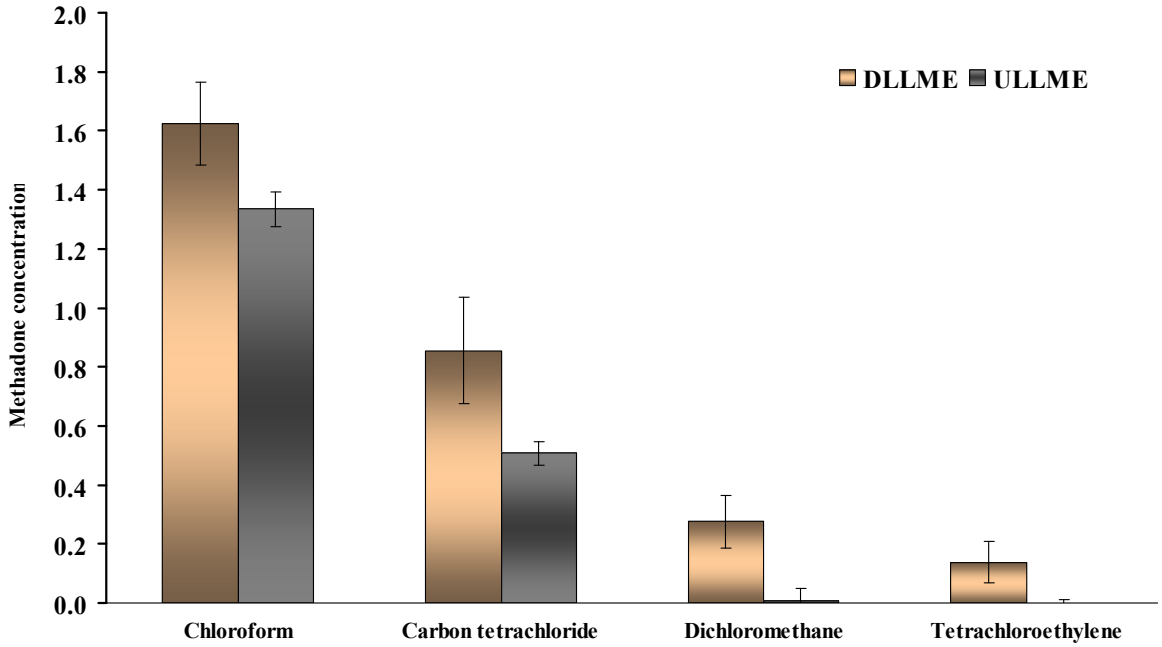

Figure 2. Effect of extraction solvent type on the extraction efficiency of DLLME and ULLME. Extraction conditions: extraction solvent, chloroform (200 µL for DLLME and 150 µL for ULLME), carbon tetrachloride (150 µL), dichloromethane (400 µL), tetrachloroethylene (100 µL); disperser solvent: methanol (1 mL) for DLLME or 4 minute sonication for ULLME; methadone concentration, 2 $\mu g L^{-1}$; pH 7.0; centrifugation time, 5 min and centrifugation speed, 5000 rpm. The bars indicate the standard deviations (N=3).

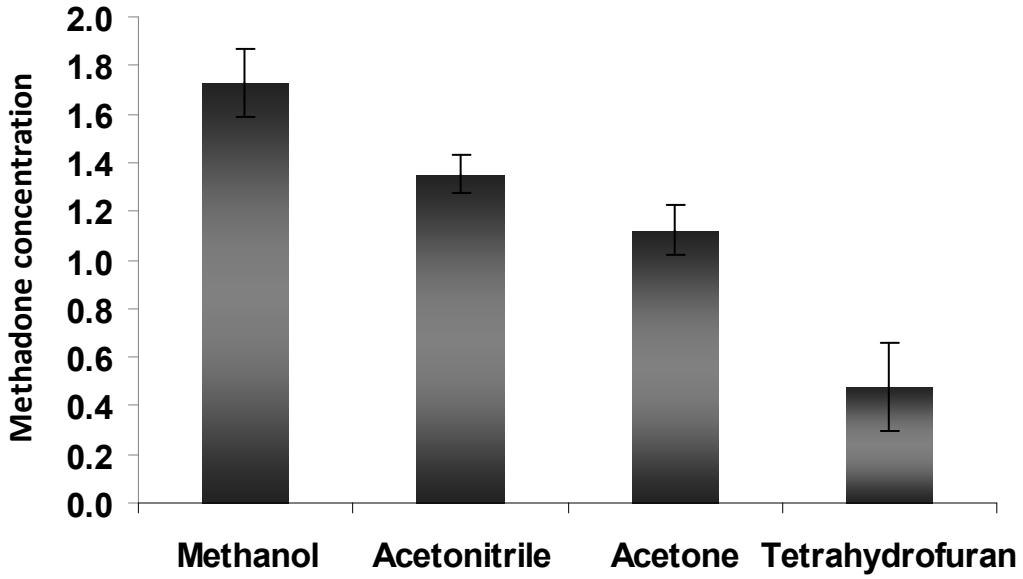

Figure 3. Effect of disperser type on the extraction efficiency of DLLME. Extraction conditions: extraction solvent, chloroform (200 µL); other conditions are the same as Fig. 2.

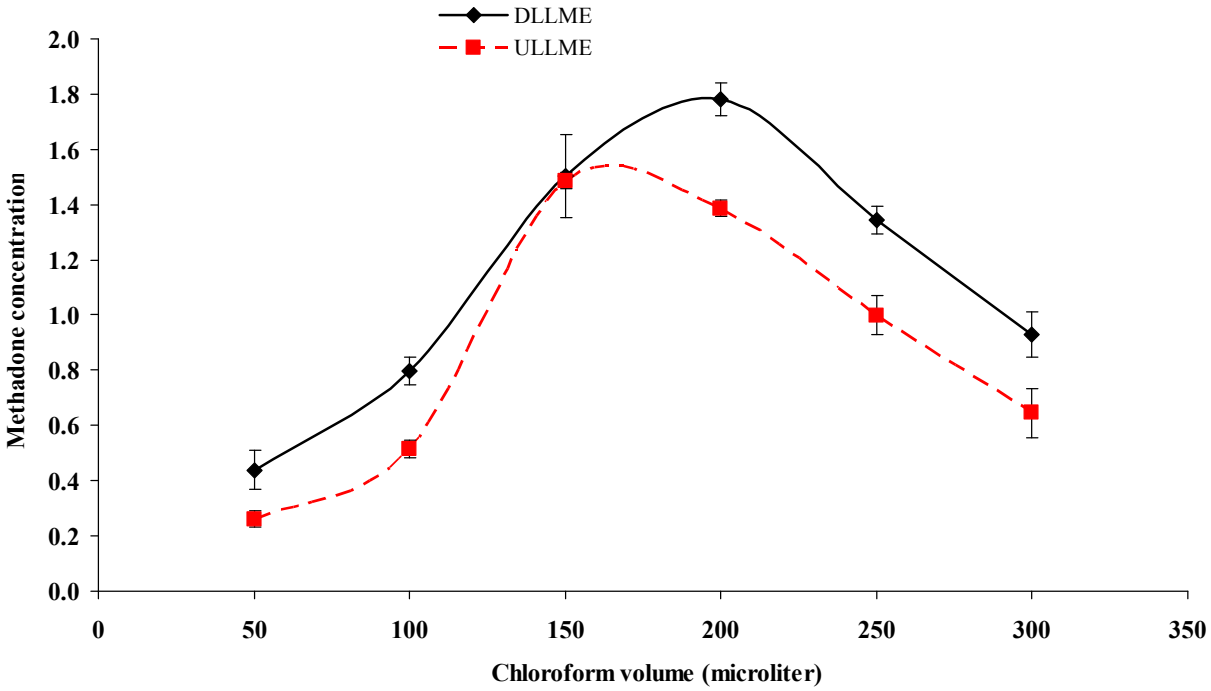

Figure 4. Effect of chloroform volumes on the extraction efficiency of DLLME and ULLME. Extraction conditions are the same as Fig. 2.

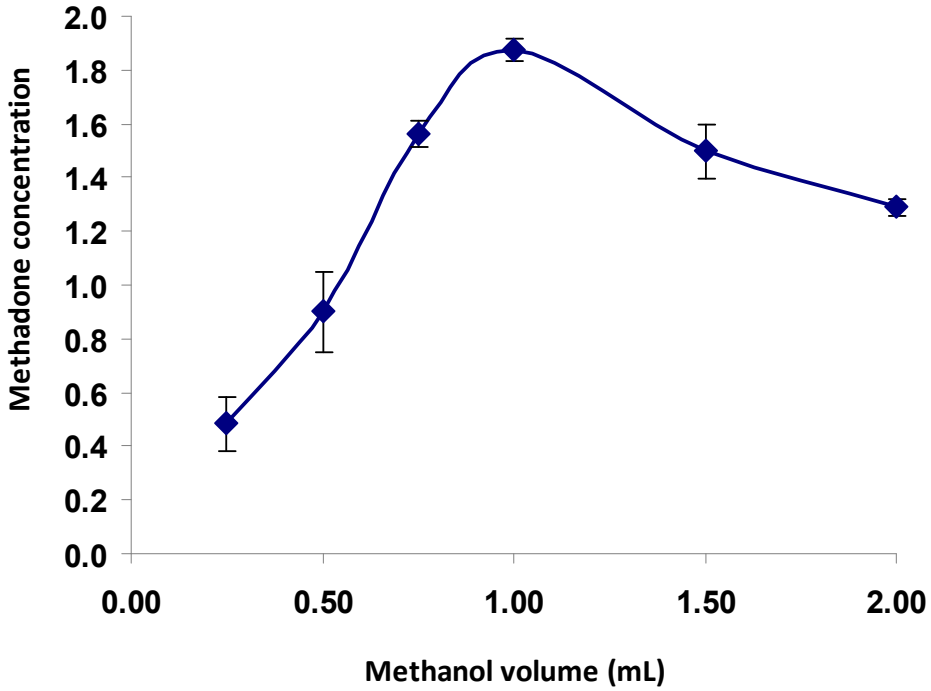

Figure 5. Effect of disperser volume on the extraction efficiency of DLLME. Extraction conditions are the same as Fig. 2.

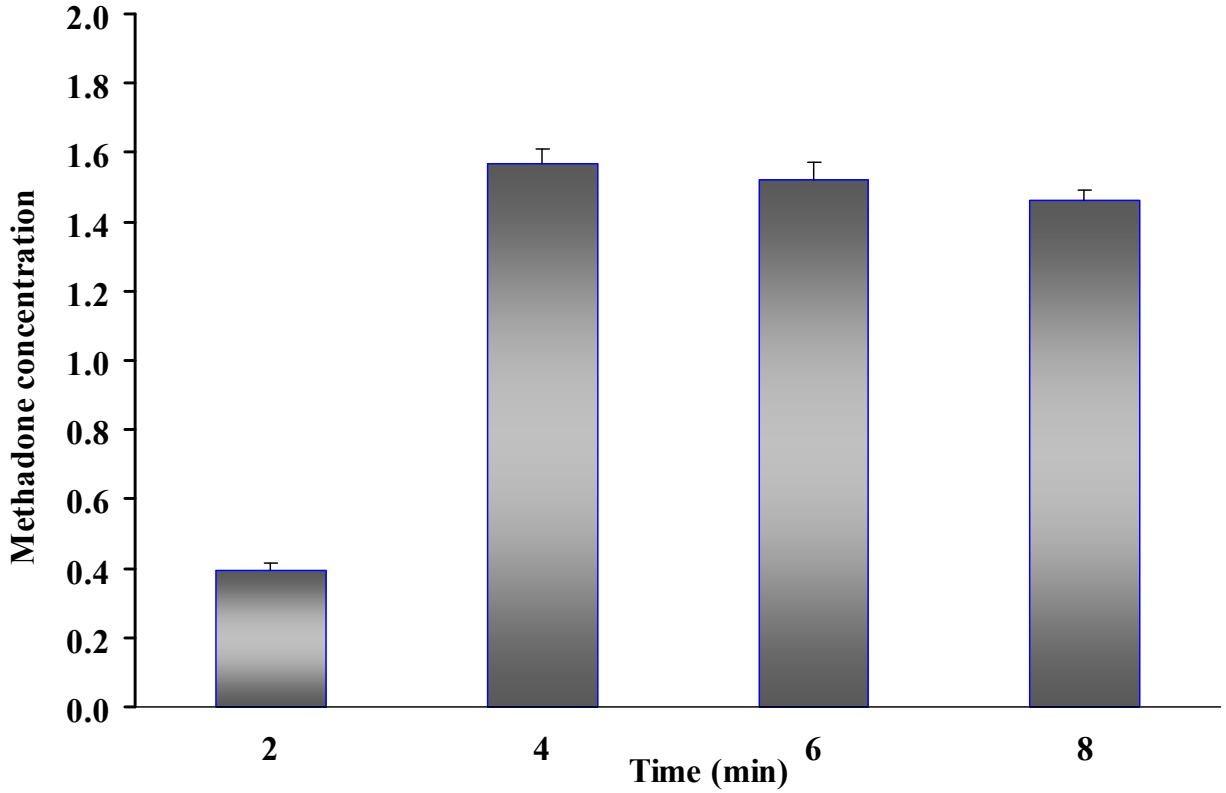

Figure 6. Effect of sonication time on the extraction efficiency of ULLME. Extraction conditions are the same as Fig. 2.

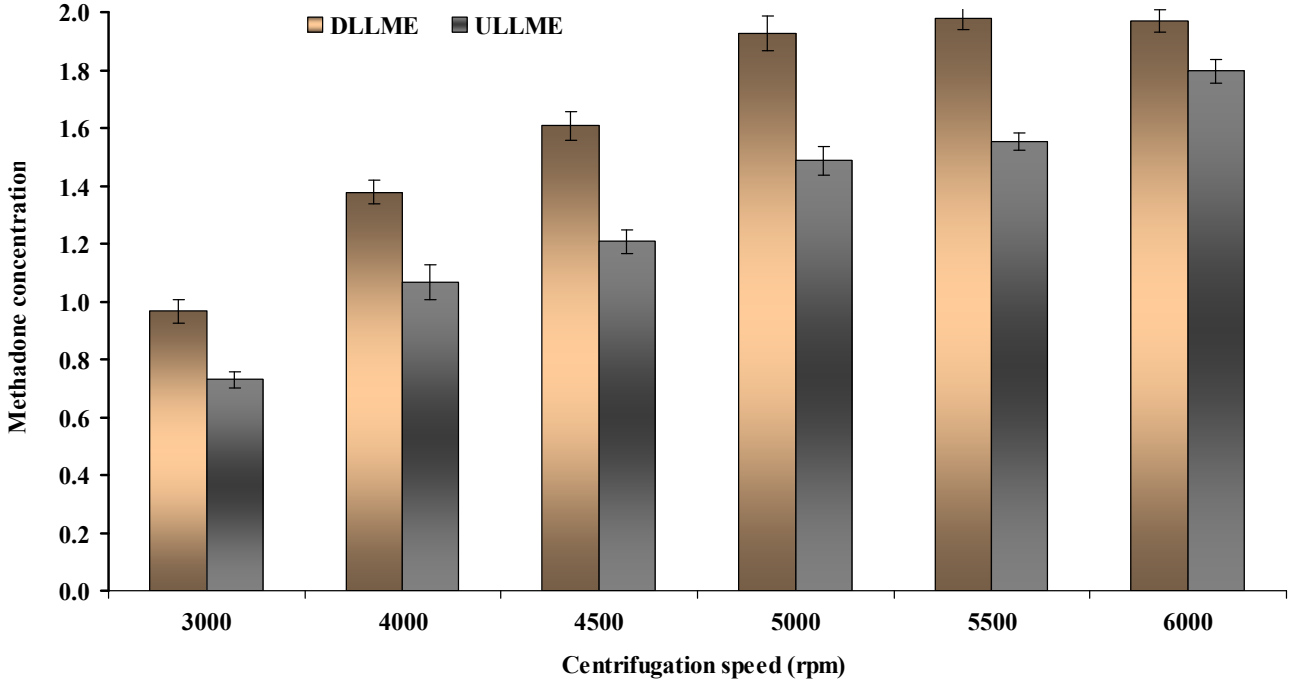

Figure 7. Effect of centrifugation speed on the extraction efficiency. Extraction conditions are the same as Fig. 2.

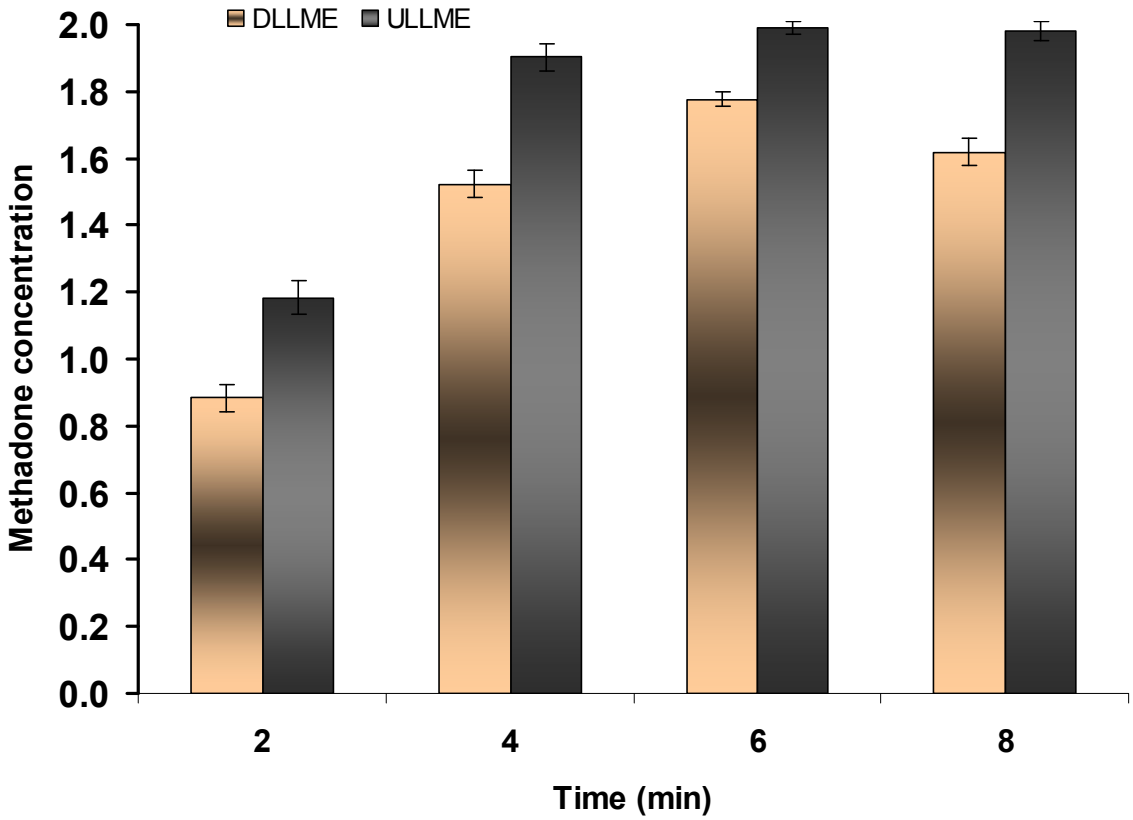

Figure 8. Effect of centrifugation time on the extraction efficiency of DLLME and ULLME. Extraction conditions are the same as Fig. 2.

Effect of pH

The effect of pH was studied ranging from 6.0 to 10.0 and using either HCl (0.1 M) or NaOH (0.1 M) for pH adjustment. More extracted methadone in organic phase is expected from alkaline solutions since the analyte is in un-ionized form. The extraction efficiency increases from pH 6.0 to 8.0 and reached a plateau at pH 10.0.

Validation

Under the optimized conditions, DLLME and ULLME procedures were performed on spiked EBC samples and the calibration curves were plotted using the peak area as a function of methadone concentration. Good linearity was obtained in the concentration range of 0.5–10 μgL^{-1} and the LLOQ and ULLOQ of both DLLME and ULLME were 0.5 and 10 μgL^{-1}, respectively. The correlation coefficients of 0.998 and 0.996 were obtained for DLLME and ULLME, respectively, as shown in Table 2. Table 3 listed intra day and inter day precision and accuracy for DLLME and ULLME methods for determination of methadone. The obtained RSDs are less than 5 % where the acceptable RSDs for biological samples are less than 20 % according to FDA guideline.

Analyses of Patient Samples

Some details of patients along with the measured methadone concentrations are described in Table 4. Fig. 9 shows the chromatograms of a blank EBC sample, methadone spiked EBC sample and an EBC sample of a patient admitted to the clinic. It is apparent (Fig. 9.c) that the principal metabolite of methadone, i.e. its N-demethylated form, could be analyzed utilizing the proposed extraction-LC method. The identification of the metabolite (retention time of 2.2 min) was not investigated in this work, however, it is similar to the previously reported

peak (37) concerning its retention time. The concentrations of methadone varied from 0.34 to 1.31 μgL^{-1}, and there is very good correlation (R=0.895, N=10) with daily intake of methadone (see Fig. 10). There was one outlier datum which belongs to a female donor. By excluding this data point, the correlation coefficient increases (R=0. 997, N=9). Although there are a number of confounding factors affecting the biological concentrations of methadone, the effect of gender requires further investigation.

DISCUSSION

Previous methods reported detection concentration values of 1 ngL^{-1} (14) and 3 pg/sample (30) using solid phase extractions followed by LC-MS analysis of methadone in EBC. A higher LLOQ value was expected for UV detection when compared with a sophisticated MS detection. One could easily transfer the developed DLLME-LC or ULLME-LC method to an LC instrument equipped with an MS detection system and improve the LLOQ values. It should be noted that the compatibility of the mobile phase components with an MS detector should be considered in the method transfer procedure. In such cases, the developed method of analysis on EBC samples could be employed in pharmacokinetic studies as well. Intra and interday analytical precision, accuracy and relative recoveries of methadone in three concentration levels are listed in Table 3. Although there is no significant difference between the findings from method validation procedures (or figures of merits) for DLLME and ULLME methods, in DLLME a disperser solvent (optimization the kind and volume of solvent are necessary) and dilution of samples are required for extraction of the analyte (42).

CONCLUSION

A simple, low cost, and easy to use analytical method is presented for the determination of methadone in EBC. Low levels of methadone in biological samples and its further dilution in the collection process makes its pre-concentration the most vital step prior to analysis. The developed method has been successfully applied to measure methadone concentrations in EBC of patients admitted to a private MMT clinic.

ACKNOWLEDGMENTS

This work is a part of the PhD thesis of M. Khoubnasabjafari. The authors acknowledge the partial financial support of the project by Research Affairs, Tabriz University of Medical Sciences. The authors would like to thank Dr Hushang Ghasemi, Temad Co. for providing methadone powder. We would also like to thank the Editor for his valuable comments on our submission.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

Table 2. Analytical and statistical parameters for the proposed DLLME-LC-UV and ULLME-LC-UV methods.

Parameter	DLLME-LC-UV	ULLME-LC-UV
Linear range	0.50-10 μgL^{-1}	0.50-10 μgL^{-1}
Slope	182034	214114
Intercept	61844	87814
Correlation coefficient	0.998	0.996
Number of data points	5	5
LLOQ	0.50 μgL^{-1}	0.50 μgL^{-1}
ULOQ	10 μgL^{-1}	10 μgL^{-1}

Table 3. Intra-day and inter-day analytical precision and accuracy for DLLME-LC-UV and ULLME-LC-UV determination of methadone in EBC sample.

Nominal concentration (μgL^{-1}; N=5)	*Found concentration (μgL^{-1})*	*Intra-day precision (RSD%; N=5)*	*Inter-day precision (RSD%; N=5)*
DLLME-LC-UV			
0	n.d	n.d	n.d
0.5	0.54	1.77	4.45
10	10.64	1.99	2.53
ULLME-LC-UV			
0	n.d	n.d	n.d
0.5	0.47	4.86	3.84
10	10.26	3.72	3.47

RE: Mean relative error; RSD: Relative standard deviation; n.d: not detected.

Table 4. Measured concentrations of methadone in ten patients using ULLME.

Patient No.	Age year	Gender	Daily dose mg	History of MMT	Conc. (μgL^{-1})
1	45	M	130	6	0.90
2	27	F	125	2	0.43
3	32	M	200	2	1.31
4	36	M	145	3	0.98
5	60	M	15	5	0.34
6	32	M	50	2	0.46
7	35	M	145	3	1.03
8	28	M	150	5	1.07
9	52	M	145	3	1.03
10	44	M	50	2	0.49

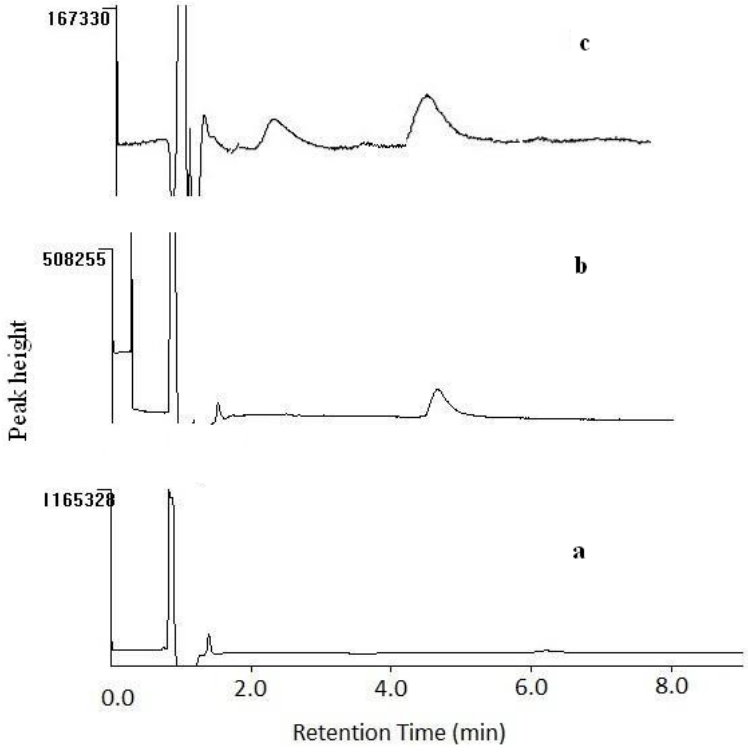

Figure 9. Sample chromatograms of a) blank EBC, b) Drug free EBC sample spiked with 0.5 μgL^{-1} methadone and c) EBC sample a patient under MMT.

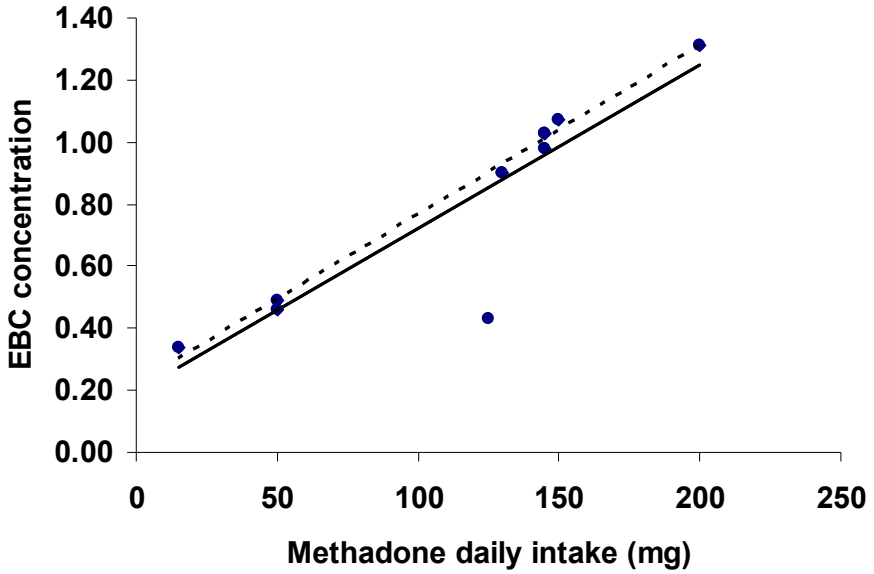

Figure 10. EBC concentration of methadone versus daily intake for 10 (——) and 9 (· · · · ·) patients under MMT.

REFERENCES

1. Foley KM. The treatment of cancer pain. New Eng J Med, 1985; 313:84-95.
2. Scott CC, Robbins EB, Chen KK. Pharmacologic comparison of the optical isomers of methadon. J Pharmacol Exp Ther, 1948; 93:282-286.
3. Kristensen K, Christensen CB, Christrup LL. The mu1, mu2, delta, kappa opiod receptor binding profiles of methadone stereoisomers and morphine. Life Sci, 1995; 56:45-50.
4. Isbell H, Eisenman AJ. The addiction liability of some drugs of the methadon series. Fed Proc, 1948; 7:162-170.
5. Beck O, Sandqvist S, Böttcher M, Erikson P, Franck J, Palmskog G. Study on the sampling of methadone from exhaled breath. J Anal Toxicol, 2011; 35:257-263.
6. Kuban P, Foret F. Exhaled breath condensate: Determination of non-volatile compounds and their potential for clinical diagnosis and monitoring: A review. Anal Chim Acta, 2013; 805:1-18.
7. Natale CD, Paolesse R, Martinelli E, Capuano R. Solid-state gas sensors for breath analysis: A review. Anal Chim Acta, 2014; 824:1-17.
8. Dodig S, Cepelak I. Exhaled breath condensate – from an analytical point of view. Biochem Med, 2013; 23:281-295.
9. Amann A, Costello BDL, Miekisch W, Schubert J, Buszewski B, Pleil J, Ratcliffe N, Risby T. The human volatilome: Volatile organic compounds (VOCs) in exlahed breath, skin emanations, urine, feces and saliva. J Breath Res, 2014; 8:034001.
10. Khoubnasabjafari M, Ansarin K, Jouyban A. Review of exhaled biomarkers in different pulmonary diseases. Med J Tabriz Uni Med Sci Heath Serv, 2013; 35:96-105 (in Persian).
11. Risby TH. Critical issues for breath analysis. J Breath Res, 2008; 2:030302.
12. Johnson GR, Morawska L. The mechanism of breath aerosol formation. J Aerosol Med Pulmon Drug Deliv, 2009; 22: 229-237.
13. Haslbeck K, Schwarz K, Hohlfeld JM, Aeume JR, Koch W. Submicron droplet formation in the human lung. J Aerosol Sci, 2010; 41: 423-438.
14. Berchtold Ch, Bosilkovska M, Daali Y, Walder B, Zenobi R. Real –time monitoring of exhaled drugs by mass spectrometry. Mass Spectrom Rev, 2013;9999: 1-20.
15. Beck O, Sandqvist, S., Dubbelboer, I., Franck, J., Demonstration that methadone is being present in the exhaled breath aerosol fraction. J Pharm Biomed Anal, 2011; 56:1024-1028.
16. Hanna J, Foster, DJR, Salter, A, Somogyi AA, White JM, Bochner F. Within- and between- subject variability in methadone pharmacokinetics and pharmacodynamics in methadone maintenance subjects. Br J Clin Pharmacol, 2005; 60:404-413.
17. Cheng YF, Neue UD, Woods LL. Novel high-performance liquid chromatographic and solid – phase extraction methods for quantitating methadone and its metabolite in spiked human urine. J Chromatogr B, 1999; 729:19-31.
18. Chikhi-Chorfi N, Pham-Huy C, Galons H, Manuel N, Lowenstein W, Warnet J, Claude J. Rapid Determination of methadone and its major metabolite in biological fluids by gas-liquid chromatography with thermionic detection for maintenance treatment

of opiate addicts, J Chromatogr B, 1998; 718:278-284.
19. Norris R, Ravenscroft P, Pond S. Sensitive high-performance liquid chromatographic assay with ultraviolet detection of methadone enantiomers in plasma, J Chromatogr B, 1994; 661:346-350.
20. Samanidou VF, Anastasiadou K, Papadoyannis IN. Development and validation of a rapid HPLC method for the determination of methadone and its main metabolite EDDP in biological fluids, following SPE. J Liq Chromatogr Rel Tech, 2006; 29:889-902.
21. Laurian V, Daniela-saveta P, Sorin E, Leucuta L. Bioanalysis of methadone in human plasma and urine by LC/MS/MS. Rev Roumanie Chim, 2008; 53:1157-1164.
22. Bermejo AM, Seara R, Dos Santos Lucas AC, Tabernero MJ, Fernandez P, Marsili R. Use of solid-phase microextraction (SPME) for the determination of methadone and its main metabolite, EDDP, in plasma by gas chromatography-mass spectrometry. J Anal Toxicol, 2000; 24:66-69.
23. Mercolini L, Mandrioli R, Conti M, Leonardi C, Gerra G, Raggi MA. Simulltaneous determination of methadone, buprenorphine and norbuprephine in biological fluids for therapeutic drug monitoring purposes. J Chromatogr B, 2007; 847:95-102.
24. Ebrahimzadeh H, Mehdinia A, Kamarei F, Moradi E. A sensitive method for the determination of methadone in biological samples using nano-structured α-carboxy polypyrrol as a sorbent of SPME. J Chromatogr B, 1994; 661:346-350.
25. Ranjbari E, Golbabanezhad AA, Hadjmohammadi MR. Preconcentration of trace amounts of methadone in human urine, plasma, saliva and sweat samples using dispersive liquid-liquid microextraction followed by high performance liquid chromatography. Talanta, 2012; 94:116-122.
26. Cooper GAA, Oliver JS. Improved solid-phase extraction of methadone and its two major metabolites from whole blood. J Anal Toxicol, 1998; 22:389-392.
27. He H, Sun Ch, Wang X, Pham-Huy Ch, Chikhi-Chorfi N, Galons H, Thevenin M, Claude JR, Warnet JM. Solid- phase extraction of methadone enantiomers and benzodiazepines in biological fluids by two polymeric cartridges for liquid chromatographic analysis. J Chromatogr B, 2005; 814:385-391.
28. Moore C, Guzaldo F, Hussain MJ, Lewis D. Determination of methadone in urine using ion trap GC/MS in positive ion chemical ionization mode. Forensic Sci Int, 2001; 119:155-160.
29. Cheng YF, Neue UD, Woods LL. Novel high-perfomance liquid chromatographic and solid-phase extraction methods for quantitating methadone and its metabolite in spiked human urine. J Chromatogr B, 1999; 729:19-31.
30. Beck O, Sandqvist S, Eriksen P, Franck J, Palmskog G. Determination of methadone in exhaled breath condensate by liquid chromatography-tandem mass spectrometry. J Anal Toxicol, 2011; 35:129-133.
31. Foster D, Somogyi AA, Bochner F. Stereoselective quantification of methadone and its major oxidative metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, in human urine using high-performance liquid chromatography. J Chromatogr B, 2000; 744:165-176.
32. Thormann W, Lanz M, Caslavska J, Siegenthaler P, Portmann R. Screening for urinary methadone by capillary electrophoretic immunoassays and confirmation by capillary electrophoresis-mass spectrometry. Electrophoresis, 1998; 19:57-65.
33. Kelly T, Doble P, Dawson M. A fast CE method for the achiral separation of methadone and its major metabolites, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline. Electrophoresis, 2007; 28:3566-3569.
34. Wang, CC, Chen, CC, Wang, SJ, Wu, SM. Cation-selective exhaustive injection and sweeping micellar electrokinetic chromatography for the analysis of methadone and its metabolites in serum of heroin addicts. J Chromatogr A, 2011; 1218:6832-6837.
35. Ahmadzai H, Huang S, Hettiarachchi R, Lin JL, Thomas PS, Zhang Q. Exhaled breath condensate: A comprehensive update. Clin Chem Lab Med, 2013, 51:1343-1361.
36. Mutlu GM, Garey KW, Robbins RA, Danziger LH, Rubinstein I. Collection and Analysis of Exhaled Breath Condensate in Humans. Am J Respir Crit Care Med, 2001; 164:731-737.
37. Samanidou VF, Anastasiadou K, Papadoyannis IN. Development and validation of a rapid HPLC method for the determination of methadone and its main metabolite EDDP in biological fluids, following SPE. J Liq Chromatogr Rel Tech, 2006; 29:889-902.
38. Jouyban A, Khoubnasabjafari M, Ansarin K, Jouyban-Gharamaleki V. Breath sampling setup. Iranian Patent, 81363, 2013.
39. Carter SR, Davis CS, Kovacs EJ. Exhaled breath condensate collection in the mechanically ventilated patients. Respir Med, 2012; 106: 601-613.
40. Vyas A, Zhang Q, Gunaratne S, Lee W, Lin JL, Lin JS, Warwick G, Thomas PS. The effect of temperature on exhaled breath condensate collection. J Breath Res, 2012; 6: 036002.
41. US Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, Center for Veterinary Medicine. Guidance for Industry Bioanalytical Method Validation (2001).
42. Andruch V, Burdel M, KocuovaL, Sandrejova J, Balogh JS. Application of ultrasonic irradiation and vortex agitation in solvent microextraction. Trend Anal Chem, 2013; 49:1-19.