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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

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ABSTRACT

A simple, rapid and efficient method for the preconcentration of methadone was developed using dispersive liquid–liquid microextraction (DLLME) followed by high performance liquid chromatography with ultra violet detection (HPLC–UV). The extraction method is based on the fast injection of a mixture of extracting and disperser solvents into the aqueous solution to form a cloudy ternary component solvent (aqueous solution:extracting solvent:disperser solvent) system. The extraction parameters such as nature and volume of extracting and disperser solvents, pH of sample, and extraction time were studied for optimization. Under the optimal conditions (extracting solvent: chloroform, 250 μL; disperser solvent: methanol, 2.5 mL and pH of sample: 10.0) a linear calibration curve was obtained in the range of 0.5–5000 ng mL^{-1} with r^2 = 0.9995. To demonstrate analytical performance, figures of merits of the proposed method in four different biological matrices (urine, plasma, saliva and sweat) spiked with methadone were investigated. The limits of detection and quantification in these matrices were ranged from 4.90 to 24.85 ng mL^{-1} and 16.32 to 82.75 ng mL^{-1}, respectively. The extraction recoveries were above 97% and the preconcentration factors of methadone in distilled water, urine, plasma, saliva, and sweat samples were 196.52, 10.03, 9.93, 1.97 and 1.99, respectively. While the precision for inter-day was ≤6.43 (n = 5), it was ≤2.26 (n = 5) for intra-day assay. Finally, the method was successfully applied in the determination of methadone in the human urine, plasma, saliva and sweat samples.

1. Introduction

In many countries of the world, the selected treatment for opiates dependence is methadone maintenance therapy (MMT). Methadone (Fig. 1), also known as *Methadose*, *Dolophine*, *Amidone*, *Symoron*, *Physeptone*, *Heptadon* and many other names, is a synthetic analgesic drug which is commonly used to treat dependence on heroin and other opioids since the mid-1960s [1]. Because methadone treatment replaces a short-acting opioid (heroin) with a long-acting opioid (methadone), it has been controversial since its inception [2,3], particularly with regard to adequate dose levels.

According to clinicians and researchers, adequate methadone dosage should be based on an individualized clinical process using the best judgment of a physician trained to administer methadone [4,5]. In fact, due to the differences in the pharmacokinetics of methadone among different people, it is particularly important to develop analytical methods which can determine the total methadone concentration to individualize doses for achieving optimum treatment. Because of this, many analytical methods have been applied to the quantitation of methadone [6–18]. These include several analytical methods based on gas chromatography (GC) coupled with mass spectrometry (MS) [6,7] and flame ionization detection (FID) [8], liquid chromatography (LC) coupled with ultra violet (UV) [9], coulometric [10] and MS [11] detection, capillary electrophoresis [12] coupled with UV [13], MS [14] and electrochemiluminescence detection (ECL) [15], flow-injection analysis (FIA) [16], radioimmunoassay [17] and potentiometry with ion-selective electrode [18].

So far, analysis of methadone was performed in several biological samples such as serum [19], plasma [20], urine [21], hair [22], sweat [23] and saliva [24]. Due to the complex matrix of the real samples and the low concentration of methadone, making efforts to develop a simple and reliable method for preconcentration and determination of the methadone is the main challenge and a very important step for the analysis of it. The preconcentration methods, which are commonly used to monitor methadone in biological samples, are liquid–liquid extraction (LLE) and solid phase extraction (SPE). The SPE procedures used were based on several solid sorbents such as C8-SPE cartridge [25], Oasis HLB

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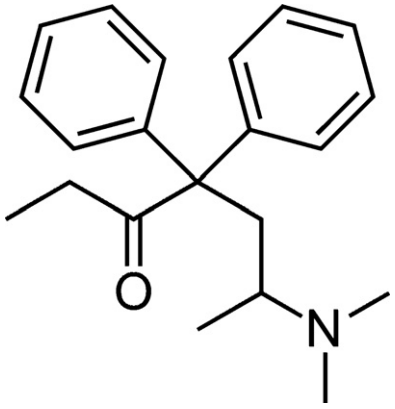

Fig. 1. Molecular structure of methadone.

96-well extraction plate [26], Bond Elut Certify cartridges [27] and Oasis cation-exchange cartridges (MCX) [28]. Recently, several solid sorbents comprise of mixed cationic exchange/lipophilic resin (BondElut Certify), hydrophilic/lipophilic balance cartridges (OASIS HLB), C8 cartridge and cyclohexyl (CH) were tested by Mercolini et al. [29], for the SPE, isolation and preconcentration of methadone and it was concluded that extraction using a C8-SPE cartridge provided a higher extraction yield with less interferences. Lucas et al. [30] employed solid-phase microextraction (SPME) as a rapid, solvent free and quicker procedure for the extraction of methadone from human hair. In 2002, Ho et al. [31] performed a comparison with liquid phase microextraction (LPME) and LLE for the extraction of methadone. LPME has been accomplished either by extraction into small water immiscible drops of organic solvents (two-phase LPME) or into small volumes of acceptor solution present inside the lumen of porous hollow fibers (three-phase LPME). Results confirmed that for the extraction of moderately or highly hydrophobic analytes, LPME provides higher analyte enrichment and superior selectivity as compared to LLE because the volumes of organic solvent used in both two- and three-phase LPME were very small.

However, each of these procedures has its own disadvantages; for instance, LLE and SPE methods are expensive, time-consuming and labor-intensive. The main drawback of two-phase LPME is the instability of the drop at high stirring rates or temperatures [32]. Three-phase LPME procedure suffers from manipulation of the hollow fiber at the time of placing it at the tip of the needle of the microsyringe before the microextraction process, because manipulation could be a source of contamination [32]. Drawbacks of SPME are mainly related to the polymeric extractant phase nature and the desorption process; in fact, the use of a polymer as extractant phase includes disadvantages such as batch-to-batch variation, artifact formation and low repeatability [32].

Despite the widespread usage of dispersive liquid–liquid microextraction (DLLME) in trace analysis of numerous materials in the variety samples [33–35], there is not any report about the extraction of methadone from the urine, plasma, saliva and sweat samples using DLLME. This method was introduced by Assadi and co-workers [33]. DLLME is based on a ternary component solvent system like homogeneous liquid–liquid extraction [36] and cloud point extraction [37] which the appropriate mixture of extracting solvent and disperser solvent is injected rapidly into a conical test tube containing aqueous solution; therefore, a cloudy solution is formed. At this time the analyte in the aqueous solution is extracted into fine droplets of extracting solvent. After centrifugation, the enriched analyte in the sedimented phase is withdrawn and is determined by chromatography or spectrometry methods.

Although, urine and plasma analysis is a commonly used method to detect drug abuse, it is impractical to collect urine or plasma samples under particular situations, such as in the monitoring of drivers, monitoring individuals in safety-related work, and surveying of drug use in the general population. For the first time, extraction of methadone was developed using DLLME–HPLC–UV which showed sufficient specificity and simplicity of operation for the measurement of trace amounts of methadone in urine, plasma, saliva and sweat.

2. Experimental

2.1. Chemicals

Methadone hydrochloride was purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, acetone (HPLC-grade), dichloromethane, chloroform, carbon tetrachloride and three flouroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). The water used for mobile phase was double distilled deionized which was produced by a Milli-Q system (Millipore, Bedford, MA, USA). A stock standard solution of methadone (100 mg L^{-1}) was prepared in methanol. The working solutions were prepared by appropriate dilution of the stock solution with double distilled/deionized water.

2.2. Instrumentation and operating condition

The chromatographic analysis was performed on an HPLC system equipped with a series 10 LC pumps, UV detector model LC-95 set at 205 nm, and model 7125i manual injector with a 20 μL sample loop all from Perkin-Elmer (Norwalk, CT, USA). Separation was done by an isocratic elution on a C_{18} (250 mm × 4.6 mm, 10 μm) column from Dr. Maisch GmbH (Beim Brueckle, Germany). Mobile phase was a mixture of 0.1% TFA in methanol:water (60:40, v/v) with flow rate of 1.0 mL min^{-1}. Adjustment of pH was done by model 3030 Jenway pH meter (Leeds, UK). A Denley bench centrifuge model BS400 (Denley Instruments Ltd., Billingshurst, UK) was used to accelerate the phase separation.

2.3. Dispersive liquid–liquid microextraction procedure

For DLLME, 10.0 mL aliquot of water sample containing 100 ng mL^{-1} of methadone was placed in a 15 mL conical glass test tube fitted with a plastic cap. A mixture of 2.5 mL of methanol (as disperser solvent) and 250 μL of chloroform (as extracting solvent) was injected into a sample solution using 5.0 mL syringe rapidly, so that a cloudy solution was formed. The cloudy solution was centrifuged for 3 min at 3000 rpm. After centrifuging, the sedimented phase was completely transferred into another test tube and was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 50 μL of HPLC grade methanol and injected into the HPLC using a 20 μL sample loop. All the experiments were performed in triplicates and average of the results was reported.

2.4. Sample collection and preparation

Blank urine and plasma samples were provided by healthy volunteer in our lab. According to the method of Shamsipur and Fattahi [38], for the sedimentation of undesirable compounds in the bottom of the conical test tube, these samples were kept frozen at −20 °C before extraction process. The frozen urine and plasma samples were thawed at room temperature and centrifuged for 10 min at 5000 rpm. Then, supernatants were decanted into clean glass tube and filtered through a 0.45 μm filter. 500 μL of filteration products were diluted to 10.0 mL and applied for extraction process as it was described in Section 2.3.

Collection of saliva was performed using a salivette (Sarstedt, Sevelen, Switzerland). After being chewed for about 2 min, the swab was placed in the container and centrifuged 5 min at 5000 rpm. 100 μL of saliva collected was diluted to 10.0 mL and applied for extraction process as it was described in Section 2.3.

Sweat samples were collected using sterile gauze pads. To remove all non-desirable compounds from the gauze pads, they were pretreated according to the method of Mebazaa et al. [39] (by soaking them in three successive baths of ethanol (30 min each bath) with subsequent drying for 2 h at 90 °C). The pretreated gauze pads were fixed under each armpit of the volunteer with hypoallergenic adhesive plaster. A piece of nylon was placed between gauze pad and adhesive plaster to avoid contamination of the sweat sample collected with compounds coming from the adhesive plaster. Sweating was induced with occlusive wrapping of the volunteer. Swabs of the salivette tubes were replaced with these gauze pads and the centrifugation was performed for 5 min at 5000 rpm. 100 μL of filterated sweat samples was applied for extraction process as it was described in Section 2.3.

3. Results and discussion

To obtain good sensitivity, precision and selectivity for extraction and determination of methadone, the various experimental parameters which influence the efficiency of DLLME procedure including extracting and disperser solvents as well as their volume, extraction time and pH of the solution were optimized using one variable-at-a-time optimization method.

3.1. Optimization of DLLME

In order to obtain the optimized extraction condition, extraction recovery (*ER*) was used to evaluate the optimum condition. *ER%* was defined as the percentage of the total analyte (n_0) extracted into the sedimented phase (n_{sed}). Accordingly, calculation of the extraction recovery, as analytical response, was carried out using the following equation:

$$ER\% = \frac{n_{sed}}{n_0} = \frac{C_{sed} \times V_{sed}}{C_0 \times V_{sam}} \times 100 \quad (1)$$

where C_{sed} and C_0 are the concentrations of analyte in sedimented phase and initial concentration of analyte in aqueous sample, respectively. C_{sed} is determined from a calibration curve which was obtained using direct injection of standard solutions. V_{sed} and V_{sam} are the volumes of sedimented phase and aqueous sample, respectively.

The preconcentration factor (*PF*) was defined as the ratio between the analyte concentration in the sedimented phase (C_{sed}) and the initial concentration of analyte (C_0) in the aqueous sample, as follows:

$$PF = \frac{C_{sed}}{C_0} \quad (2)$$

Combination of Eqs. (1) and (2) gives:

$$ER\% = PF \times \frac{V_{sed}}{V_{sam}} \times 100 \quad (3)$$

3.1.1. Selection of disperser and extracting solvent

To obtain a good extraction recovery for DLLME of methadone, the selection of an appropriate mixture of extracting and disperser solvents is very important. The extracting solvent has to meet four properties to extract the analytes efficiently comprising: (a) higher density than water (when we use a centrifuge tube with a conical bottom), (b) good chromatographic behavior or ease of evaporation, (c) extraction capability of interested compound, (d) low solubility in water. Hence, carbon tetrachloride (density, 1.59 g mL^{-1}), chloroform (density, 1.48 g mL^{-1}) and dichloromethane (density, 1.32 g mL^{-1}) were considered for this purpose.

In order to choose disperser solvent in DLLME, the miscibility in organic phase (extracting solvent) and aqueous phase (sample solution) is a key factor, which can disperse extracting solvent into very fine droplets in aqueous phase. Acetonitrile, acetone and methanol were tested as disperser solvent in the extraction of methadone.

For obtaining good efficiency, all combinations using CCl_4, $CHCl_3$ and CH_2Cl_2 (200 μL) as extractants with acetone, acetonitrile and methanol (2.0 mL) as disperser solvents were tried. Results showed that, methanol as disperser solvent and chloroform as extracting solvent provided maximum extraction recovery of 55.0%. Therefore, we selected methanol/chloroform as a suitable set for subsequent experiments.

3.1.2. Effect of extracting solvent volume

To consider the effect of the extracting solvent volume on extraction recovery, different volumes of chloroform were tested. Therefore, the volume of disperser solvent (methanol) was fixed at 2.0 mL and the volume of chloroform was changed from 100 to 350 μL. Under these conditions, the extraction recovery enhances by the increasing of chloroform's volume up to 250 μL but after this volume, the extraction recovery decreases slightly. As a result, 250 μL of chloroform was selected as the volume of extracting solvent in order to obtain the highest recovery.

3.1.3. Effect of disperser solvent volume

To obtain optimized volume of disperser solvent, extractions were carried out by changing the volume of methanol in the range of 0.5–3.0 mL. The obtained results showed that with increasing the volume of methanol, extraction recovery first increased till reached a maximum point at 2.5 mL and then became almost fixed. It can be attributed to the fact that, at lower volume of methanol consumption, cloudy state was not formed well and the extracting solvent (chloroform) could not be well dispersed among aqueous solution in the form of very little droplets, which resulted in poor extraction recovery. Therefore, in the following experiments, 2.5 mL methanol was used as optimal disperser solvent volume.

3.1.4. Effect of sample pH

pH of the sample is an important factor during liquid–liquid extraction (LLE) process involving analytes that possess an acidic or basic moiety. The ionic form of a neutral molecule formed upon deprotonation of a weak acid or protonation of a weak base normally does not extract through the organic solvent as strongly as its neutral form does. Thus pH should be adjusted to ensure that neutral molecular forms of the analytes are present prior to performing the microextraction step. Regarding to the structure of methadone (Fig. 1) and its dissociation constant ($K_a = 10^{-8.3}$), in the pHs lower than 8.0 this compound remains in its cationic form; therefore, the effect of pH solution on the amount of extracted methadone was investigated in the range of 6–12. As can be seen in Fig. 2, the best pH for extraction of methadone is 10.0, that methadone is completely in its molecular form.

3.1.5. Effect of extraction time

In DLLME, extraction time is defined as an interval time between injection of the mixture of disperser solvent (methanol) and extracting solvent (chloroform), before starting to centrifuge. The effect of time was examined in the range of 0–30 min and results showed that the extraction time was not effective on extraction recovery. In DLLME, the contact area between the water phase and organic phase is extremely large and equilibrium state is obtained rapidly; therefore, the time of extraction was very succinct because equilibrium state was obtained very fast. On the other hand, the

Table 1
Analytical performance of DLLME[a]–HPLC for determination of methadone in different biological samples.

Sample	Sample volume (μL)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LR (ng mL^{-1})	r^2	*ER*% ± RSD%	*PF*[b]
Distilled water	10^4	0.22	0.73	0.50–5000	0.9995	98.26 ± 0.92	196.52
Urine	500	4.90	16.32	10.00–5000	0.9988	100.34 ± 2.26	10.03
Plasma	500	7.30	24.31	20.00–5000	0.9986	99.26 ± 2.12	9.93
Saliva	100	25.12	83.65	75.00–5000	0.9991	98.58 ± 1.68	1.97
Sweat	100	24.85	82.75	50.00–5000	0.9992	99.70 ± 2.10	1.99

[a] Eextraction conditions: aqueous sample volume, 10 mL; disperser solvent (methanol), 2.5 mL; extracting solvent (chloroform), 250 μL; pH of sample solution, 10.0.
[b] All *PF*s were reported for an initial sample solution with concentration of 75 ng mL^{-1}.

Table 2
Inter- and intra-day precision and recovery of distilled water, urine, plasma, saliva and sweat spiked with methadone after DLLME (n = 5).

Sample	Concentration added[a]	Inter-day			Intra-day		
		Concentration found, mean ± SD[b]	*ER*% ± precision (RSD%)	Accuracy (E_r%)	Concentration found, mean ± SD	*ER*% ± precision (RSD%)	Accuracy (E_r%)
Distilled water	75	74.48 ± 2.16	99.31 ± 2.90	−0.69	73.69 ± 0.68	98.26 ± 0.92	−1.75
	500	488.70 ± 10.94	97.74 ± 2.23	−4.90	493.80 ± 3.77	98.76 ± 0.76	−1.24
Urine	75	75.94 ± 3.74	101.25 ± 4.94	1.25	75.26 ± 1.70	100.34 ± 2.26	0.34
	500	505.76 ± 28.16	101.15 ± 5.56	1.15	488.80 ± 5.2	97.76 ± 1.06	−2.24
Plasma	75	74.68 ± 4.80	99.57 ± 6.43	−0.43	74.45 ± 1.60	99.26 ± 2.12	−0.74
	500	508.22 ± 29.32	101.64 ± 5.77	1.64	494.10 ± 5.85	98.82 ± 1.18	−1.18
Saliva	75	74.29 ± 1.64	99.06 ± 2.20	−0.94	73.93 ± 1.24	98.58 ± 1.68	−1.43
	500	503.00 ± 19.37	100.60 ± 3.85	0.60	491.00 ± 1.39	98.20 ± 1.50	−1.80
Sweat	75	74.63 ± 1.32	99.50 ± 1.77	−0.50	74.78 ± 1.57	99.70 ± 2.10	−0.3
	500	498.04 ± 9.69	99.61 ± 1.95	−0.39	504.70 ± 7.36	100.94 ± 1.46	0.94

[a] ng mL^{-1}.
[b] ng mL^{-1}.

most time consuming step in DLLME is the centrifuging of sample solution in the extraction procedure, which was about 3 min.

3.2. Analytical performance of the DLLME–HPLC for determination of methadone

Under optimum condition, figures of merit of the proposed method consisting linear range (LR), determination coefficient (r^2), limit of detection (LOD), limit of quantification (LOQ), extraction recovery (*ER*) and preconcentration factor (*PF*) were studied in the distilled water. For evaluating the performance of DLLME method for the extraction of methadone from different biological samples, calibration curves were obtained by spiking the standards directly into urine, plasma, saliva and sweat samples (Table 1). Urine (500 μL), plasma (500 μL), saliva (100 μL) and sweat (100 μL) samples were diluted up to 10 mL (10^4 μL) and then extraction process was performed under the optimal conditions.

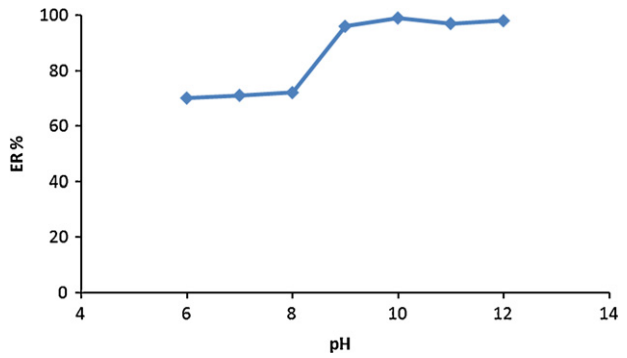

Fig. 2. Effect of pH of the sample solution on extraction recovery of methadone using DLLME. Extraction conditions: disperser solvent (methanol) volume, 2.5 mL; extracting solvent (chloroform) volume, 250 μL.

The linearity of the method was evaluated for each sample solution. In all cases, the least squares regressions were above 0.99. Limits of detection (LODs) and limits of quantification (LOQs) of the method for each matrix were determined by spiking samples with standard methadone at low concentrations, extracted by the described DLLME method and calculated as the concentration giving peaks for which the signal-to-noise ratio was 3 and 10, respectively. The *PF* was defined as the ratio of the concentrations of analyte in the sedimented phase (concentration after preconcentration) and in the initial aqueous sample solution (concentration before dilution).

As it is shown in Table 2, the intra- and inter-day precision of the method in different spiked real samples were determined as relative standard deviation (RSD%). Intra-day precision was assessed by five determinations per concentration in 1 day, while inter-day precision was evaluated by five determinations per concentration

Table 3
The application of presented method for determination of methadone in the urine, plasma, saliva and sweat samples (n = 3).

Actual samples	Methadone added (ng mL^{-1})	Methadone founded (ng mL^{-1})	*RR* (%) ± RSD (%)
Urine	–	117.75	–
	100	214.59	98.55 ± 1.63[a]
	300	409.23	97.96 ± 1.71
Plasma	–	291.00	–
	100	377.32	96.50 ± 1.97
	300	575.19	97.32 ± 1.03
Saliva	–	83.46	–
	10.0	93.17	99.69 ± 2.40
	30.0	112.41	99.07 ± 2.33
Sweat	–	35.78	–
	10.0	46.10	100.69 ± 1.89
	30.0	67.34	102.37 ± 2.16

[a] Mean value ± relative standard deviation.

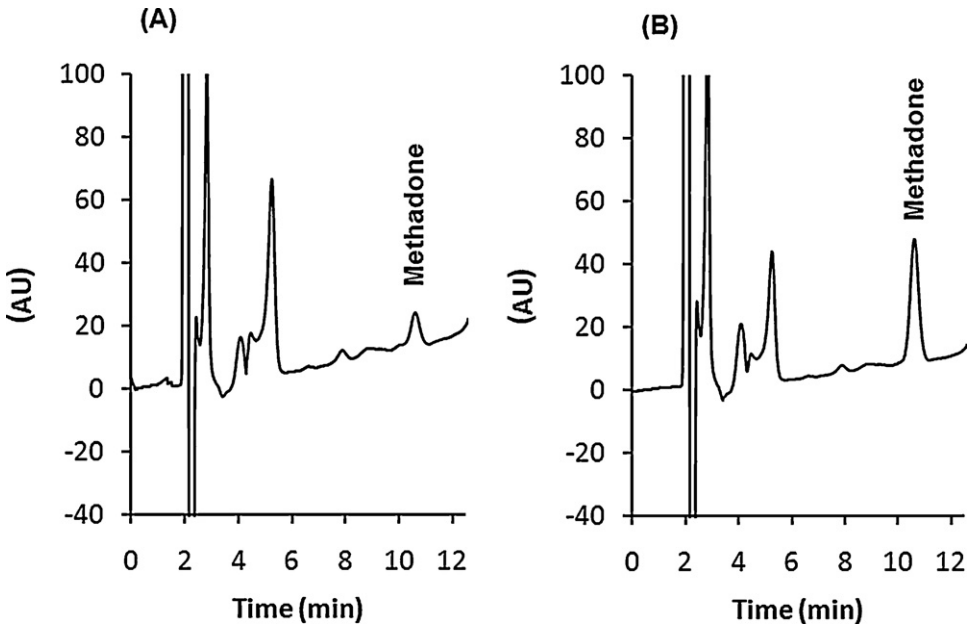

Fig. 3. Representative chromatograms of urine sample (A) and spiked urine sample (B). Experimental details are described in the text.

in different days. Accuracy of the method (E_r) was calculated as the percent difference from the expected concentration. The results of the validation studies in Table 2 demonstrate that the method has acceptable precision and accuracy.

3.3. Analysis of actual samples using DLLME–HPLC

Actual samples were obtained from a 25 year old male patient undergoing a methadone treatment after 3 weeks of administration, so that a steady state was acquired. Collection of the real samples was performed 6 h after the last intake of a tablet containing 40 mg methadone. After preparation of the samples for extraction process, as described in Section 2.4, determination of methadone was performed by standard addition method at two spiked levels.

For each concentration level, three replicate experiments with the whole analysis process were done and their experimental results are shown in Table 3. Relative recovery (*RR*) was calculated as follows:

$$RR(\%) = \frac{C_{\text{spiked}} - C_{\text{unspiked}}}{C_{\text{added}}} \times 100 \quad (4)$$

where C_{spiked}, C_{unspiked} and C_{added} represent the concentration of the analyte after adding a known amount of standard to the real sample, the concentration of the analyte in the real sample and the concentration of a known amount of standard that was spiked in the real sample, respectively. Table 3 shows that with respect to the complexity of the matrices studied, the average result of three replicate analysis of each biological sample obtained by the proposed method are in satisfactory agreement (relative recoveries between 96.50% and 102.37%) with the added amounts of

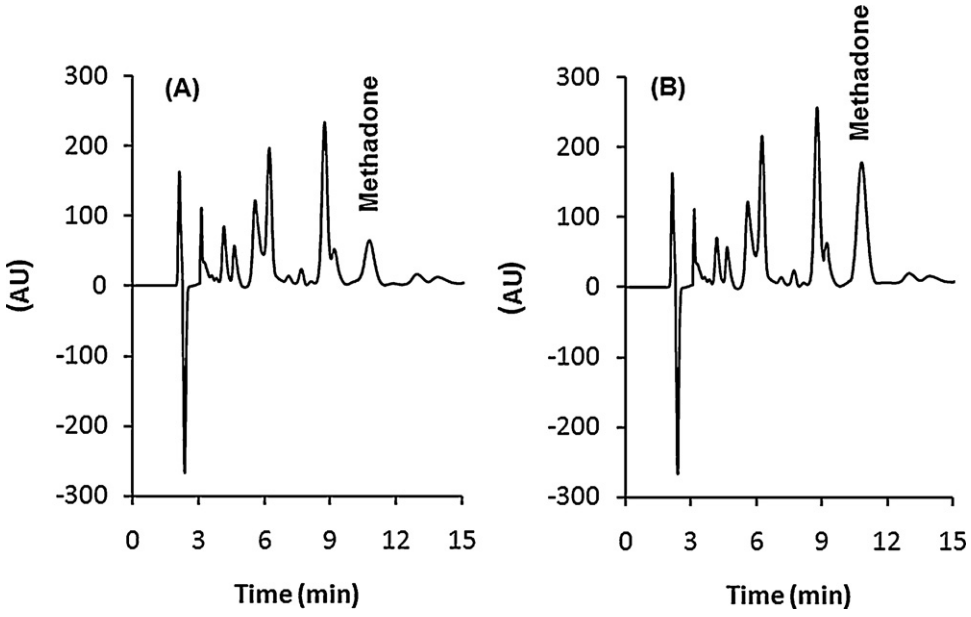

Fig. 4. Representative chromatograms of plasma sample (A) and spiked plasma sample (B). Experimental details are described in the text.

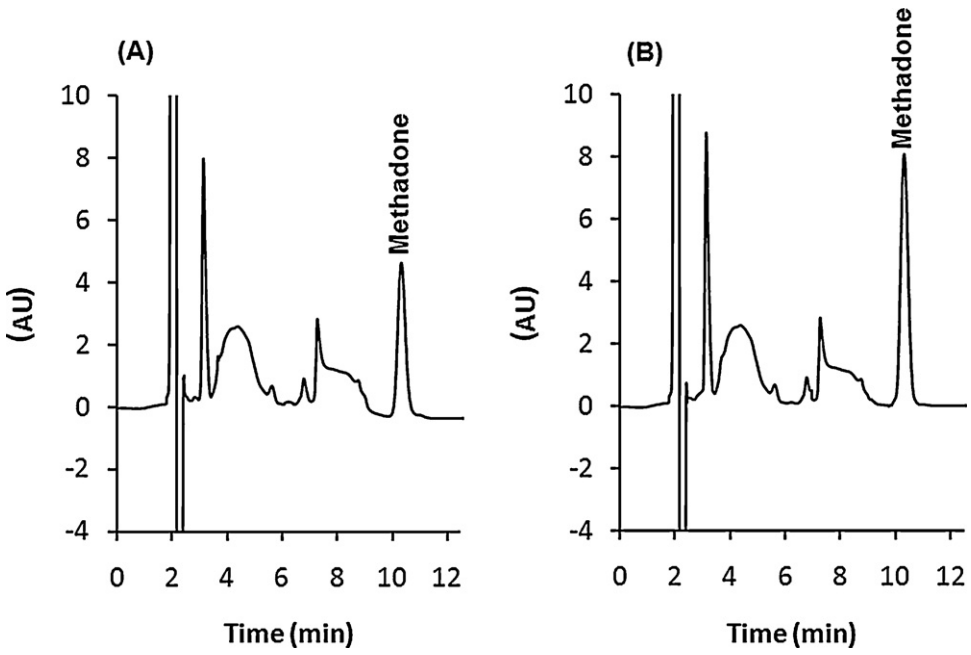

Fig. 5. Representative chromatograms of saliva sample (A) and spiked saliva sample (B). Experimental details are described in the text.

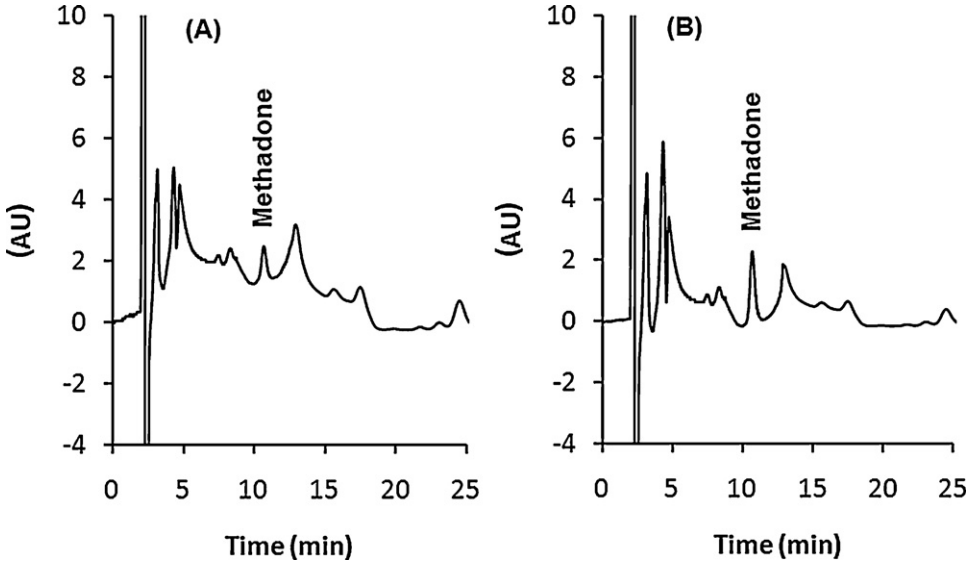

Fig. 6. Representative chromatograms of sweat sample (A) and spiked sweat (B). Experimental details are described in the text.

Table 4
Comparison of DLLME–HPLC–UV with previous methods.

Biological sample	Methods	LOD (ng mL^{-1})[a]	LR (ng mL^{-1})[b]	Intra-day precision%	References
Urine	SPE–HPLC–UV	20.00	500–10000	<4.50	[26]
	DLLME–HPLC–UV	4.90	10–5000	<2.50	Present work
Plasma	SPE–HPLC–DAD	49.00	100–10000	4.56	[27]
	DLLME–HPLC–UV	7.30	20–5000	<2.50	Present work
Saliva	SPE–GC–MS	0.70	5–200	<8.00	[40]
	DLLME–HPLC–UV	25.12	75–5000	<2.00	Present work
Sweat	LLE–GC–MS	20.00[c]	50–1000[d]	–[e]	[41]
	DLLME–HPLC–UV	24.85	50–5000	<2.20	Present work

[a] Except sweat sample which is in terms of ng patch^{-1}.
[b] Except sweat sample which is in terms of ng patch^{-1}.
[c] ng patch^{-1}.
[d] ng patch^{-1}.
[e] Not specified.

methadone standards, with RSD ($n = 3$) less than 2.5%. Figs. 3–6 show the chromatograms obtained from urine, plasma, saliva and sweat samples by DLLME–HPLC–UV, respectively.

4. Conclusion

For the first time, an identical procedure i.e., DLLME–HPLC has been successfully applied for preconcentration and quantitative determination of the methadone in four biological samples including: human plasma, urine, sweat and saliva. The method has highly useful advantages for detecting methadone; for instance, consumption of toxic organic solvents is very low (2.75 mL); extraction operation time (including sample preparation and centrifugation) is less than 5 min; the extraction procedure is very convenient and sample amount requirement is very low (urine and plasma, 500 μL; saliva and sweat, 100 μL) which makes the procedure appropriate for forensic investigations.

In a comparative study which is shown in Table 4, in all matrices, the precision of the represented method was higher than traditional methods [26,27,40,41] and the linearity was in a wide suitable range. In the cases of urine and plasma samples, lower LODs were attained using this method. However, the proposed analytical method can be used siolus for determination of methadone in four different biological matrices for monitoring of patients undergoing MMT, studying on correlation between urine, plasma, sweat and saliva methadone concentrations as well as pharmacokinetic and bioavailability studies of methadone with sufficient specificity, simplicity and sensitivity.

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