Research Paper: Ultrasound-Assisted Liquid-Liquid Extraction for Analyzing Methadone in Urine Samples by Gas Chromatography-Mass Spectrometry

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ABSTRACT

Background: Methadone abuse and dependence are a growing concern in some countries. The present study developed and validated a rapid, simple, easy, and sensitive method for analyzing methadone in urine specimens. This approach was intended for use in clinical and forensic toxicology and drug screening laboratories.

Methods: We determined methadone in urine samples by Gas Chromatography / Mass Spectrometry (GC / MS). Besides, we used Ultrasound-Assisted Liquid-Liquid Extraction (UALLE) method for the extraction and preconcentration of methadone before analysis.

Results: The detection limit was 2.1 ng/mL and the limit of quantification equaled 7 ng/mL. The methadone calibration curve was linear and ranged from 7 to 10000 ng/mL, and the correlation coefficient was calculated as 0.9984. The tested method was accurate and precise. Recovery fell in the range of 81.3% to 97.4% and the enrichment factor was measured as 8.7. The method was successfully used for determining methadone in real clinical and postmortem urine samples.

Conclusion: The presented method was a rapid, easy, simple, and sensitive procedure; thus, it could be applied in clinical and forensic toxicology laboratories as a routine approach for analyzing methadone.

1. Introduction

Methadone is a synthetic long-acting opioid analgesic, i.e., used in the maintenance treatment of Opioid Use Disorder (OUD) and the relief of moderate to severe pain [1, 2]. Methadone, as a mu-opioid receptor full agonist, generates

effects, such as analgesia, euphoria, sedation, miosis, constipation, nausea, and respiratory depression [2]. Methadone Maintenance Therapy (MMT) reduces the risk of illegal drug use, criminal acts, hospital admissions, and death in individuals with OUD; however, MMT is associated with some adverse consequences, including accidental and deliberate methadone poisoning and mortality [3-5].

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Therefore, methadone abuse and dependence are a growing concern in some countries, like Iran [6]. Accordingly, the analysis of methadone in biological samples critically affects the diagnosis, treatment, monitoring, and prevention of methadone abuse/dependence in clinical, forensic, and workplace settings.

In clinical, forensic, and workplace settings, determining methadone in bio-samples, such as blood, plasma, serum, and saliva is correlated with limitations. Such restrictions include interferences, short detection window, invasive sample collection method, infection risk, and high costs. Thus, urine is a bio-sample choice for drug abuse screening tests in clinical and forensic laboratories. In comparison to blood and plasma, urine is a simple matrix with a longer duration of excretion (detection window). Furthermore, it provides higher concentrations of parent drugs and metabolites than blood; it is available in sufficient quantities as well as in point-of-care tests. Additionally, well-established and cost-effective methods for collecting the sample are accessible [7].

Preparing biological samples is the most significant pre-analytical step for the purification and concentration of the desired analyte(s). It also controls the interfering compounds. Rapid and appropriate sample preparation procedures crucially impact achieving robust analytical methods. Multiple conventional analytical techniques, such as Liquid-Liquid Extraction (LLE) and Solid-Phase Extraction (SPE) have been introduced for the extraction and analysis of drugs, like methadone in bio-samples [8, 9]. Implementing these techniques for sample preparation is associated with some disadvantages. Conventional LLE procedure is time-consuming and requires using plenty of volumes of pure and hazardous solvents. SPE technique has a relatively desirable efficacy; however, it is time-consuming due to the need for washing, elution, and evaporation of the solvents [10]. Therefore, it is necessary to design rapid, feasible, and environmentally compatible sample preparation methods.

Ultrasonic-Assisted Liquid-Liquid Extraction (UALLE) technique, compared with the conventional LLE method, presents several advantages. Such benefits include increasing the efficiency and decreasing the time of extraction and volume of used solvents [11]. In this method, a low-frequency ultrasound wave induces a shearing action. It enhances the mass transfer and produces cavitations (acoustic cavitations phenomenon). The sample with added solvent is incorporated in an

ultrasonic bath and exposed to ultrasonic waves for a few minutes [12-14].

Several analytical techniques have been established for analyzing methadone and its metabolites in bio-samples, such as gas chromatography and liquid chromatography with SPE or LLE, as sample preparation procedures [8-18]; however, data on the urine sample preparation with UALLE for determining methadone by Gas Chromatography/Mass Spectrometry (GC/MS) are scarce.

In this study, we validated and optimized a rapid, easy, and sensitive UALLE-GC-MS for the extraction, preconcentration, and analysis of methadone in human urine samples.

2. Materials and Methods

Methadone standard solution (100 µg/mL) was obtained from Sigma Aldrich (St. Louis, USA). Methanol, chloroform, sodium hydroxide, and double-distilled water were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

Methadone standard solutions were prepared by dilution with methanol to a final concentration of 10 µg/mL and were used for sample calibration. A calibration curve was drawn by analyzing drug-free urine samples spiked with methadone at concentrations of 7, 10, 500, 5000, and 10000 ng/mL.

Blank human urine samples (drug-free) were obtained from healthy volunteers in our laboratory and analyzed by the routine GC/MS method. The collected samples were stored in containers and were kept frozen at -20° C before analysis. The frozen urine specimens were thawed at room temperature. Additionally, we employed the optimized extraction method for analyzing 50 real urine samples from patients undergoing MMT; postmortem urine samples were also collected from the cadavers with positive opioids abuse/poisoning history. These samples were transferred to the forensic toxicology laboratory of the Zanjan legal medicine center (Zanjan City, Iran).

In this study, we used and modified our previously-applied method [18]. In the screw cap test tube, a 1mL urine sample for an appropriate amount of sodium hydroxide (4mol/L) was added and the pH of the samples was adjusted at 10 (780 pH Meter, Metrohm, Switzerland). Then, the samples were immersed in an ultrasonic bath (305W, 40KHz, Soltec, Italy) and ultrasonicated

for 3 min. After ultrasonication, 1mL chloroform was added to the sample. Next, the samples were vortexed (Velp ZX3, South Korea) for 1 min, followed by ultrasonication for 5 min, again.

The samples were centrifuged at 1500 g for 5 min. After centrifugation, the liquid phase was discarded and the sediment phase was removed with a microsyringe (Hamilton, USA) and transferred to a vial. After the evaporation of the solvent under the nitrogen gas stream, the residue was dissolved in 100 μ L methanol and filtrated by a 0.22 μ m filter (Macherey-Nagel GmbH, Germany) and injected into the GC-MS.

A gas chromatograph (7890, Agilent, USA) equipped with a split/splitless injector was used for GC-MS analysis. The GC column was HP-5MS (5% phenyl & 95% dimethyl polysiloxane, 30 m length x 0.25 mm ID x 0.25 μ m film thickness) (Agilent, USA). The mass analyzer (Quadrupole, Agilent, USA) was connected to the column. Helium 99.999% was used as the carrier gas. The injection port temperature and the transfer line temperatures were 250° C and 280° C, respectively. The initial column oven was set to 60° C and held constant for 1 min. The temperature program rate was 20° C/min and the final temperature was set to 280° C and eventual hold for 15 minutes. The mass spectrometer was operated by electron impact (70 eV) in positive full scan mode (50-550 m/z). Wiley and NIST libraries were used for the analysis of samples.

The optimization and validation of the UALLE-GC-MS procedure were as follows. The pH and ultrasonication time were regarded as procedure parameters affecting the UALLE procedure.

The validation parameters, including precision, accuracy, linearity, the limits of detection and quantification, recovery, enrichment factor, and selectivity were evaluated [19]. The method validation was performed on the human urine samples.

The calibration curve was drawn by analyzing 5 urine samples spiked with methadone in the concentration range of 7-10000 ng/mL. For each standard solution, we conducted triplicate measurements.

The Limit of Quantification (LOQ) was determined during the evaluation of the linear range of the calibration curve. The Limit of Detection (LOD) and LOQ were calculated according to $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$; where σ is the standard deviation of the response, and S demonstrates the calibration curve slope.

Accuracy and precision were evaluated based on 3 replicates of 4 urine samples spiked with methadone in the concentrations of 7, 10, 400, and 8000 ng/mL. Accuracy and precision were expressed as the percentage of the true value (relative error percentage).

Reproducibility was determined by the same aforementioned range of concentrations. Each concentration was measured in 3 replicates over 5 consecutive days. For each value, we statistically determined its relative standard deviation by Microsoft Office Excel (Microsoft, USA).

The extraction recovery was defined and determined as the ratio between the analyte amount in the sediment and the initial amount of the analyte within the sample [19]. The method recovery was measured at 3 concentration levels, including 1 μ g/mL, 1.5 μ g/mL, and 2 μ g/mL. The same value was reported as a percentage of the experimentally derived concentration to the nominal concentration. Furthermore, the Enrichment Factor (EF) was calculated according to a previous study [16].

Method selectivity was evaluated by the analysis of 6 blank urine samples to exclude potential interferences. Then, it was tested by analyzing urine samples spiked with a mixture of common licit and illicit drugs in concentrations ranged from 100-500 ng/mL.

The extraction method was determined and validated with the greatest response. The validated method was used for analyzing methadone concentrations on real urine samples.

3. Results

The variables of UALLE efficiency were selected based on preliminary experiments on the distinct responses of the variables to achieve maximum AUC. Figure 1 shows the effect of sonication time on the extraction efficiency. The relevant results suggested that 5 min is an optimum time for ultrasonication. According to the optimization results, pH=10 provided the optimum effect on the extraction of methadone from urine samples (Figure 2).

The standard calibration curve for methadone was linear in the concentration range of 7-10000 ng/mL; with a regression equation of $Y = 994.38X + 73553$, and a correlation coefficient of 0.9984. These results were generally considered as evidence for indicating desirable linearity in the concentration range.

AUC: Area Under the Curve.

Figure 1. The effects of ultrasonication times on methadone extraction efficiency in UALLE

The obtained data indicated that the LOD and LOQ approaches for methadone, using UALLE-GC-MS were 2.1 ng/mL, and 7 ng/mL, respectively.

Precision for the quality controls in the between- and within-run are presented in Table 1 and Table 2, respectively. All data fell in the acceptance criteria. These data indicated that the optimized method was accurate, reproducible, and reliable.

The results were expressed as a percentage of recoveries obtained for different methadone concentrations. Table 3 lists the percentage of recoveries with relative error percentage, compatible with the proposed acceptance criteria.

Recovery rate ranged from 81.3% to 97.4% (mean=88.1%). Moreover, EF was calculated to range from 8.1 to 9.7 (mean=8.7) (Table 4).

The selectivity of the method was assessed with the absence of response in blank samples. There was no interference at the retention time of methadone in the method (Table 5).

After the optimization and validation of the UALLE-GC-MS, the method was applied for the extraction and determination of methadone in 50 real human urine specimens.

AUC: Area Under the Curve.

Figure 2. The effects of pH on methadone extraction efficiency in UALLE

Table 1. The intra-day precision evaluation data

Methadone Concentration (ng mL ⁻¹)	Run	Measured Methadone Concentration (ng mL ⁻¹)	Mean±SD	RE (%)
7	1	8	7.6±1.1	-8.5
	2	7		
	3	8		
	4	9		
	5	6		
10	1	8	8.2±0.8	18
	2	7		
	3	9		
	4	8		
	5	9		
400	1	400	396±8.9	1
	2	408		
	3	398		
	4	390		
	5	385		
8000	1	8342	8147±212	-1.8
	2	8245		
	3	7936		
	4	8425		
	5	7789		

SD: Standard Deviation.

RE: Relative Error

International Journal of
Medical Toxicology & Forensic Medicine

According to the collected results, methadone was detected in 15 samples, and EDDP (2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, the main metabolite of methadone) was found in 13 samples. Table 5 summaries the detected licit and illicit drugs in the studied real samples. Besides, Table 6 demonstrates the measured concentration of methadone in actual urine samples.

4. Discussion

The proposed UALLE-GC-MS method was optimized and validated for the extraction and analysis of methadone in human urine specimens in clinical and

forensic toxicology laboratories. Table 7 summarizes the comparison of this method with previous techniques [16, 20-22].

Ultrasound waves could generate cavitations bubbles at high temperatures and in the pressure zone. This phenomenon suggests that the solvent would easily penetrate the materials; accordingly, it facilitates the mass transfer of analytes from the matrix into the solvent, resulting in a suitable efficiency [23]. Furthermore, ultrasound energy accelerates the dispersion and contacting surface between the organic and aqueous phases. Therefore, UALLE has some advantages, such as reducing the extraction time and solvent sample volume, as well

Table 2. The inter-day precision evaluation data

Methadone Concentration (ng mL ⁻¹)	Run	Measured Methadone Concentration (ng mL ⁻¹)	Mean±SD	RE (%)
7	1	8	8±1.2	-14
	2	6		
	3	9		
	4	8		
	5	9		
10	1	9	9.6±1.8	4
	2	8		
	3	11		
	4	12		
	5	8		
400	1	405	393±13.2	1.75
	2	410		
	3	380		
	4	383		
	5	391		
8000	1	8425	8164±426	-1.9
	2	8365		
	3	7658		
	4	8480		
	5	7896		

RE: Relative Error

International Journal of
Medical Toxicology & Forensic Medicine

as increasing the quality and yield of the preparation method [24]. This finding is in concordance with our previous study on UA Dispersive Liquid-Liquid Microextraction (UADLLME) on saliva for the analysis of methadone. The present study results revealed that the solvent and sample volume required for UADLLE were 1/200 and 1/20 of those in conventional LLE, respectively [18].

Extraction time is the main factor in extraction methods due to the distribution of analytes in aqueous and organic phases [25]. In comparison with handshaking, UA extraction provides a shorter extraction time and higher-yielding [25]. In this study, ultrasonication time was tested in the range of 0-10 min. The related findings

indicated that the highest recovery could be achieved at 5 min. The duration of the ultrasonication did not affect the extraction efficiency. Thus, 5 min was selected as the optimum ultrasonication time. This finding is in line with that of a previous study [18].

Additionally, pH value is another important parameter in the extraction of analytes with an acidic or basic moiety [21]. The ionic form of a molecule could not be extracted by the organic solvent; therefore, pH value should be adjusted to ensure the analytes are deionized. In this study, as methadone is a basic compound (pKa= 9.2), the pH value must have been kept higher than the pKa value to ensure that the methadone was deprotonated. Moreover, the optimum pH was selected at

Table 3. The accuracy evaluation data

Methadone Concentration (ng mL ⁻¹)	Measured Methadone Concentration (ng mL ⁻¹)	Mean±SD	RE (%)
7	9	8±1	-14.2
	8		
	7		
10	8	10.3±2	3.3
	11		
	12		
400	410	405±1.5	-1.3
	408		
	398		
8000	8342	8174±212	-2.1
	8245		
	7936		

RE: Relative Error

International Journal of
Medical Toxicology & Forensic Medicine

pH=10. Previous studies identified pH=10, as the best pH value for the extraction of methadone from urine and whole blood samples [16, 18, 20].

Compared to previous methods, LOD and the recovery of the presented method were similar or better than that of previous techniques (Table 7). For example, Lin et al. documented similar accuracy, recovery, and

precision of methadone analysis in whole blood using UALLME/GC-MS [21].

For example, Meng et al. used the Hollow Fiber Liquid-Phase Microextraction (HF-LPME) and Ultrasound-Assisted Low-Density Solvent Dispersive Liquid-Liquid Microextraction (UA-LDS-DLLME) approaches for the analysis of abused drugs. Such medications consisted of Amphetamine Type Stimu-

Table 4. Recoveries and the enrichment factor evaluations of methadone in urine samples

Sample Number	Added Concentration (µg mL ⁻¹)	Initial Concentration (µg mL ⁻¹)	Mean	Founded Concentration (µg mL ⁻¹)	Mean	EF	Recovery (%)
1	1	0.95	0.95	7.8	7.73	8.1	81.3
2	1	0.97		7.5			
3	1	0.93		7.9			
1	1.5	1.6	1.7	14	14.6	8.5	85.8
2	1.5	1.8		14.8			
3	1.5	1.7		15.1			
1	2	1.95	1.96	19.1	19.1	9.7	97.4
2	2	1.98		19.8			
3	2	1.96		18.5			

EF: Enrichment Factor.

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Table 5. Detected drugs in the real urine samples using UALLE-GC-MS

Drugs	RT (min.)	Samples (No.)
Methadone	11.22	15
EDDP	10.72	13
Lidocaine	9.91	11
Tramadol	10.26	10
Methamphetamine	5.551	7
Amitriptyline	11.49	5
Caffeine	9.82	3
Phenytoin	12.36	3
Nicotine	6.63	2
Pethidine	9.20	2
Cyproheptadine	12.24	2
ketamine	9.14	1
Diphenhydramine	9.97	1
Carbamazepine	12.30	1
Imipramine	11.62	1
Codeine	12.40	1
Morphine	12.60	1
Sertraline	12.32	1

International Journal of
Medical Toxicology & Forensic Medicine

RT: Retention Time.

UALLE-GC-MS: Ultrasound-Assisted Liquid-Liquid Extraction-Gas Chromatography/Mass Spectrometry.

lants (ATS) and methadone in blood and urine samples by GC-MS. In optimized conditions, linearity was observed in the range 0.0030-10 µg/mL with the correlation coefficient of the R ranging from 0.9985-0.9994 for DLLME. The recovery of 79.3%-103.4% was obtained for DLLME. The LODs were determined to be in the range 0.5-4 ng/mL. Scholars concluded that compared with HF-LPME, the UA-LDS-DLLME method had the advantages of less extraction time and higher extraction efficiency [22].

In another study, SPE-LC-MS-MS method was developed for the quantification of methadone enantiomers and its metabolite in postmortem blood and brain tissue samples. The LOQ of methadone in the brain tissue and blood was equal to 0.005 mg/kg and 0.001 mg/kg, respectively. The accuracy varied from 95% to 109% [8].

A limitation of the present study was using only chloroform (as a low-price solvent). However, it is a toxic solvent and we should evaluate other safe solvents in a further study.

5. Conclusion

The UALLE-GC-MS method was developed and validated for the extraction and determination of methadone in human urine samples in clinical and forensic toxicology settings. The presented method is a rapid, simple, easy, sensitive, and cost-effective procedure. This approach could be implemented in clinical and forensic toxicology and drug screening laboratories, as a routine method for the qualitative and quantitative analysis of methadone.

Table 6. The concentrations of the methadone in the real urine samples by the presented method

Sample Number	Concentration (ng ml ⁻¹)
1	133
2	1471
3	92
4	3143
5	6572
6	4971
7	88
8	2511
9	440
10	5719
11	3012
12	1014
13	112
14	12
15	7914

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Table 7. Comparing the proposed method with previous techniques

Method	Sample	LOD (ng/mL)	LOQ(ng/mL)	R2	Recovery (%)	Reference No.
SPE-LC-MS-MS	Blood Brain	5 5	1 5	0.997 0.985	67-78	[8]
DLLME-HPLC-PDA	Urine	10	35.5	0.9989	98.6	[16]
DLLME-HPLC-UV	Urine Plasma	4.90 7.30	16.32 24.31	0.9988 0.9986	100.34 99.26	[20]
UA-DLLME-GC-MS	Whole Blood	10	40	0.9986	81.9	[21]
UA-LDS-DLLME-GC-MS	Urine Blood	1.5	5	0.9991	91.1	[22]
UA-DLLME-GC-MS	Saliva	50	150	0.9995	105	[18]
UA-LLE-GC-MS	Urine	2.1	7	0.9984	88.1	Present study

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Ethical Considerations

Compliance with ethical guidelines

All procedures performed in the study involving human participants were following the ethical standards of the Legal Medicine Research Center's ethical committee.

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Author's contributions

All authors contributed in preparing this article.

Conflict of interest

The authors declared no conflicts of interest.

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