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Research Paper: Determination of Methadone and Tramadol in Vitreous Humor Specimens Using Dispersive Liquid Liquid Microextraction and Ultra High Performance Liquid Chromatography

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ABSTRACT

Background: Drug abuse is spreading rapidly all over the world. Methadone and tramadol are among not only the most abused opioids but also important from the forensic point of view. Therefore, we need to devise a simple and sensitive method for the sample preparation and identification of abused drugs in postmortem specimens.

Methods: A simple and rapid Dispersive Liquid-Liquid Microextraction (DLLME) technique coupled with Ultrahigh Performance Liquid Chromatography (UHPLC) was developed for the extraction and analysis of methadone and tramadol from postmortem vitreous humor samples. Different parameters affecting the extraction recovery, such as the type and volume of extraction and dispersion solvents, pH value, sensitivity, and specificity, were optimized and studied.

Results: Under optimized conditions, the recovery ranges were 82.3%-89.6% and 85.4%-87.1% for methadone and tramadol, respectively. The linear range was 25-100 ng/mL for both methadone and tramadol with a correlation coefficient (R^2) of more than 0.98. Limit of Detection (LoD) and Limit of Quantification (LoQ) were 3 and 8 ng/mL for methadone and 6 and 16 ng/mL for tramadol. The accuracy level of the methods for methadone and tramadol detection were 99.4%-100% and 99.7%-99.9%, respectively. The method was specific enough for the qualitative and quantitative determination of methadone and tramadol.

Conclusion: The obtained results showed that DLLME combined with UHPLC is a fast and straightforward method for determining methadone and tramadol in postmortem vitreous humor specimens.

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1. Introduction

Opioids such as methadone and tramadol are widely used to relieve pain. However, their abuse or misuse is prevalent due to their highly addictive properties. The high abusive potential of methadone and tramadol makes them essential targets in forensic and analytical toxicology as they are a frequent cause of death in many cases [1]. Systematic and postmortem forensic toxicological investigation is based on the analysis of multiple samples such as blood, liver, bile, urine, and Vitreous Humor (VH) specimens [2]. Medicolegal investigations of unnatural deaths, especially in burned, decomposed, and traumatized bodies, are complicated as a result of changes that occurs in autolysis of tissues and putrefaction [3]. Blood has been the primary matrix for quantitative analysis in postmortem toxicology. However, substantial issues with quantitative analysis of drugs and interpretation of results in blood specimens are multifactorial and subject to postmortem redistribution [4]. Postmortem redistribution is defined as artificial drug concentrations and significant site- and time-dependent variations in tissue drug concentration due to the anatomical and physiological changes that occur after death. The diffusion process and degradation by microorganisms change drug concentration in different parts of the body [5].

Drugs are detected in VH after crossing the selective blood-retinal barrier; therefore, VH is a useful alternative matrix for postmortem forensic toxicology analysis. VH analysis offers some advantages over common biological matrices. VH is easy to collect, less susceptible to postmortem redistribution due to anatomic remoteness from viscera, and has no vascularization. It is a simple matrix without interfering compounds that embarrass forensic toxicology analysis. Moreover, this sample shows stability over time after death and needs a simple pretreatment process for toxicology laboratory analysis [6].

Sample preparation is a crucial step in analytical toxicology for determining drugs and poisons in postmortem specimens. Classical sample preparation methods such as liquid-liquid extraction and solid-phase extraction are time-consuming and use large volumes of expensive solvents. Microextraction techniques such as those based on liquid-phase microextraction are suitable alternatives to classic methods with satisfactory extraction yields [7].

Previous studies had focused on the quantitative detection of drugs in various biological matrices, such as benzodiazepines [8], opiates, designer amphetamines [9, 10], and medications with toxicological relevance [11].

In the present study, Ultrahigh Performance Liquid Chromatography (UHPLC) was employed to quantitate methadone and tramadol concentrations in authentic VH specimens obtained from cases referred for postmortem toxicological analysis. Also, VH and urine samples of all cases were analyzed using GC/MS instrumentations.

2. Materials and Methods

Materials and reagents

Acetonitrile, chloroform, methanol, ethanol, dichloromethane (HPLC grade solvents), phosphoric acid, potassium dihydrogen phosphate (KH_2PO_4), hydrochloric acid, and sodium hydroxide were purchased from Merck Chemical Co. (Darmstadt, Germany). Buffers, mobile phase for UHPLC system, and eluents were prepared with HPLC grade water for chromatography (Merck Millipore). Drug standards for methadone and tramadol were prepared under the license of the Ministry of Health and Medical Education, Iran. Helium gas (99.99% purity) was supplied by Roham Co.,Tehran, Iran.

Solutions and samples

VH samples were collected in medicolegal autopsies from both eyes using a syringe and needle. The authentic VH and urine samples were obtained from dead cases with a history of methadone and tramadol use. It should be noted that liver, stomach content, and bile of all cases were analyzed in a systematic toxicological analysis. The samples were stored at 4ºC until further analysis. A pooled blank VH matrix was used for optimization and validation of the method.

Stock standard solutions of methadone and tramadol were prepared separately in methanol at concentrations of 1 mg/mL. Standard and quality control samples were freshly prepared by appropriate dilution of stock solutions. All stock solutions were stored at -20°C.

Instrumentation

The chromatographic analyses were performed using a UHPLC from KNAUER (Germany), equipped with a photodiode array detector and a cooling autosampler (PDA-1, 6 channels). Methadone and tramadol were separated on a Eurospher II 100 Å C-18 (100 mm × 3 mm) column. Two high-pressure pumps, one with a degasser module and the other with a mixing chamber,

Table 1. Method validation parameters and acceptance criteria for methadone and tramadol quantification

Method Validation Parameter	Analytical Features for Methadone	Analytical features for Tramadol
Linear range (ng/mL)	25-100	25-100
Correlation coefficient (R^2)	0.9890	0.9811
Regression equation	Y=864.5X-2065.8	Y=9507.6X+60065
LoD	3	6
LoQ	8	16
Inter-day RSD (%)	11.5-18.6	2.05-3.3
Accuracy (%)	99.4-100	99.7-99.9
Extraction recovery (%)	82.3-89.6	85.4-87.1

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R^2: Correlation Coefficient; LoD: Limit of Detection; LoQ: Limit of Quantification; RSD: Relative Standard Deviation.

were used. Data acquisition, integration, and processing were performed using the EZChrom chromatographic software. The mobile phase was a mixture of phosphate buffer (pH=2.32) and acetonitrile (63:37 v/v). Loop volume was 10 µL, tubing volume equal to 15 µL, and 250 µL syringe volume in autosampler AS-1. Tray configuration was 48 vials with tray cooling system.

GC/MS was carried out on an Agilent 5975 mass series coupled with Agilent 6850 gas chromatograph. Instrument control and data acquisition were performed using Agilent MS Chemstation software. Agilent 5-MS capillary column (30 m, 0.25 mm I.D, 0.25 mm film thickness) was employed to separate analytes throughout the study. The chromatographic conditions for the G.C. method were as following: helium (99.999%), a constant flow of 1.5 mL/min, the inlet temperature of 250°C, injection volume of 1 mL (splitless). The oven temperature was programmed at 90°C, held for 1 min, followed by 20°C/min ramp to 280°C and held for 5 min.

Mass source and quadrupole temperatures were set at 230°C and 150°C, respectively. The ion source was operated in full scan and Selected Ion Monitoring (SIM) mode both together. In full scan mode, scan range was 40–500 m/z, selected ions for methadone were fragment peaks at m/z 72 and 294 and 58 and 263 for tramadol.

Dispersive Liquid-Liquid Microextraction (DLLME) procedure optimization

For choosing the best extraction and dispersion solvents, and also optimum pH, different solvents in different pH values were evaluated, and the extraction recovery was calculated for each parameter. Chloroform and dichloromethane were used as extraction solvents, and the recovery from methadone and tramadol were evaluated in fixing conditions. Methanol, ethanol, and acetonitrile were chosen as dispersion solvents, and recoveries for both methadone and tramadol were assessed. To optimize the extraction and dispersion solvents volumes, we tested different volumes of chloroform (100, 200, and 300 µL) and methanol (500, 1000, and 2000 µL). To achieve the optimum pH, extraction of methadone, and tramadol from spiked VH samples were performed at different pH (pH=9, 10, and 11).

Sample preparation using DLLME procedure

DLLME was performed for VH and urine sample preparation. A diagram of the steps of the DLLME procedure as sample preparation phase is shown in Figure 1. Briefly, a pre-prepared mixture of extraction (chloroform) and dispersion (methanol) solvents were pushed by force into the sample. To improve the organic phase dispersion into the aqueous phase, the mixture was vortexed and converted to a cloudy solution. The extraction solvent was sedimented after centrifugation. The extraction product was withdrawn from the bottom of the conical tube, evaporated to dryness under a nitrogen stream, and dissolved in methanol to be analyzed using UHPLC and GC/MS instrumentations.

Bioanalytical method validation

Method validation was assessed to define selectivity, linearity, the Limit of Detection (LoD), Limit of Quan-

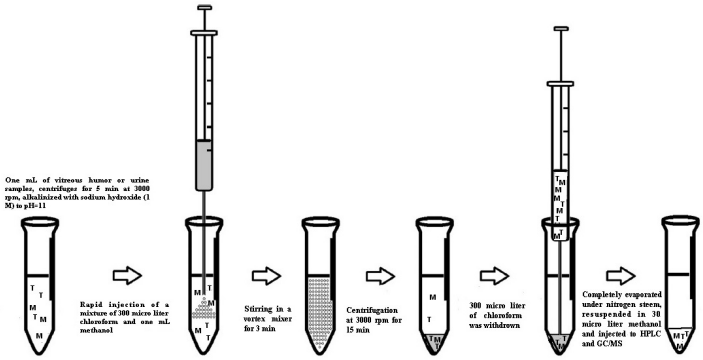

Figure 1. Steps for dispersive liquid liquid microextraction (DLLME) as sample preparation method for the determination of methadone and tramadol in vitreous humor samples

tification (LoQ), recovery, accuracy, and precision. Selectivity was assured by analyzing blank vitreous humor samples spiked with methadone, tramadol, codeine, and amitriptyline at 50 ng/mL concentrations, extracted and analyzed using UHPLC. To evaluate the cleanup power of DLLME procedure and the non-interference with endogenous materials in biological specimens, five blank VH samples were prepared using DLLME procedure and analysed using UHPLC.

Calibration curves were obtained to assess the linearity of the method. Blank VH samples were used to prepare 25, 50, 75, and 100 ng/mL of methadone and tramadol separately. Plots of peak area versus concentration were made, and the relationships were determined by linear regression. The least-square method was used for the regression line preparation and expressed a correlation coefficient (R^2).

The LoD was defined as the lower concentration of methadone and tramadol spiking in the VH sample that showed acceptable accuracy and precision (<20%) analyzed in triplicate against calibration curve concentrations. All experiments were performed under the same conditions. LoD was evaluated as the concentration with a signal/noise of 10. LoQ was calculated as the concentration of analyte with a signal/noise of 3.

Extraction efficiency (recovery) of the DLLME method for methadone and tramadol in VH has been evaluated considering the possible losses of analytes during sample preparation steps. For the estimation of recovery, concentrations of 50, 75, and 100 ng/mL of methadone and tramadol were made in blank VH samples and extracted using the proposed method. Peak areas of the spiked VH extracts were compared to peak areas acquired from unextracted standard solutions at the same concentrations. Data were expressed as the percentage of the amount of methadone and tramadol transferred to the extraction solvent.

Accuracy and precision were evaluated by inter-day (analyzing triplicate of samples in a single run, n=9) and intra-day (analyzing triplicate of samples over 3 consecutive days, n=27) by analyzing spiked VH specimens with 50, 75, and 100 ng/mL of methadone and tramadol.

Evaluation of the applicability of V.H. as a postmortem sample

The representativeness of drug detection was evaluated by analyzing autopsy urine and VH samples in parallel and comparing results in two samples. A total of 50 successive cases representing the routine laboratory procedures were selected for the study. All samples were analyzed using a validated method for the efficient extraction and detection of methadone and tramadol from urine and VH using HPLC and GC/MS instrumentations.

3. Results

The present study showed that the validated method was sensitive and specific for the quantitative determination of methadone and tramadol in VH specimens. In choosing extraction and dispersion solvents, chloroform and methanol had shown the best extraction recoveries for both methadone and tramadol. The optimum volumes for extraction (chloroform) and dispersion (methanol) solvents were 300 µL and 1000 µL, respectively, with the highest extraction recoveries. pH value for efficient extraction of methadone and tramadol was chosen at 11.

Under optimized conditions, UHPLC and GC/MS chromatograms had shown no interfering peaks for VH and urine endogenous compounds, showing good selec-

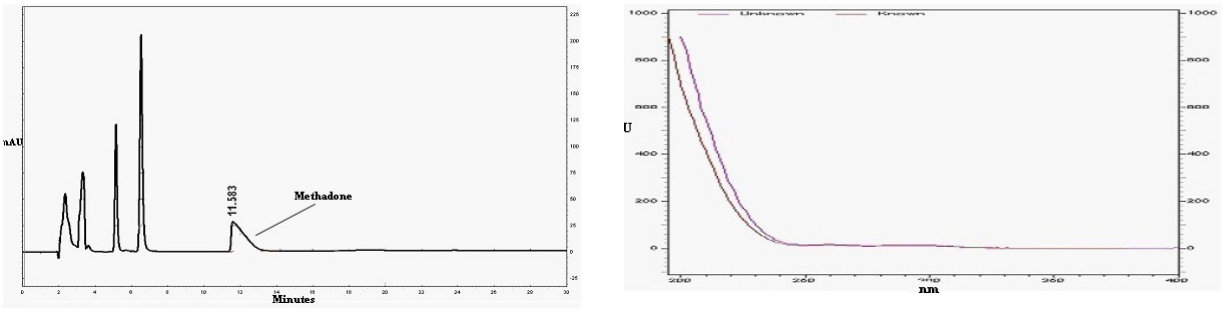

Figure 2. UV spectrum and chromatogram of methadone extracted from an authentic vitreous humor sample

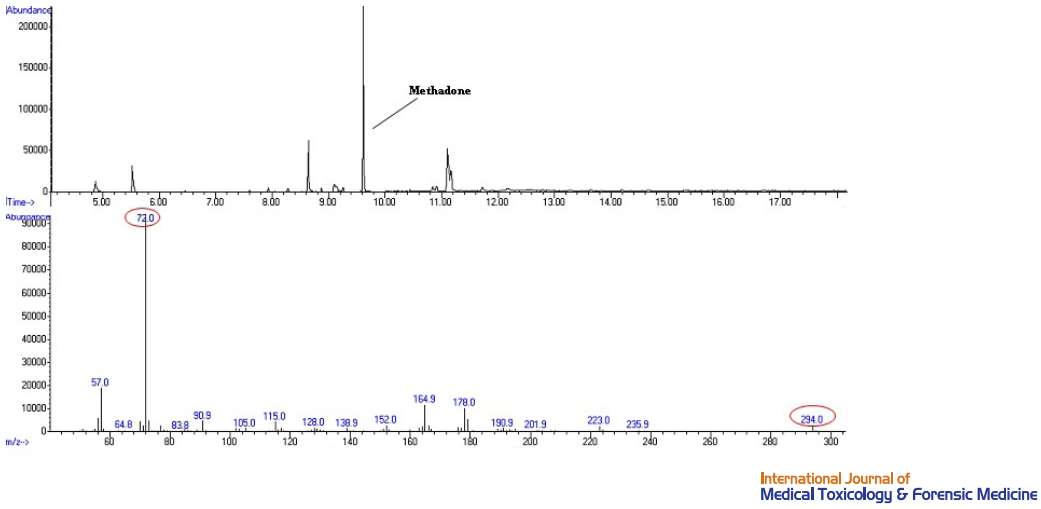

Figure 3. Gas chromatography/mass spectrometry chromatogram and spectrum of methadone extracted from an authentic vitreous humor sample

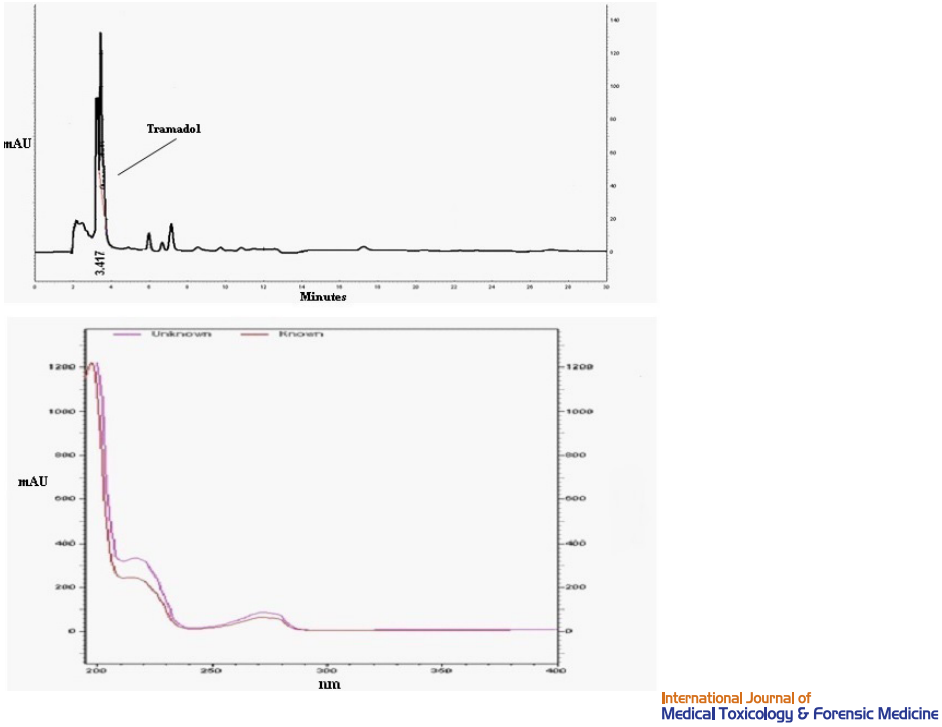

Figure 4. UV spectrum and chromatogram of tramadol extracted from an authentic vitreous humor sample

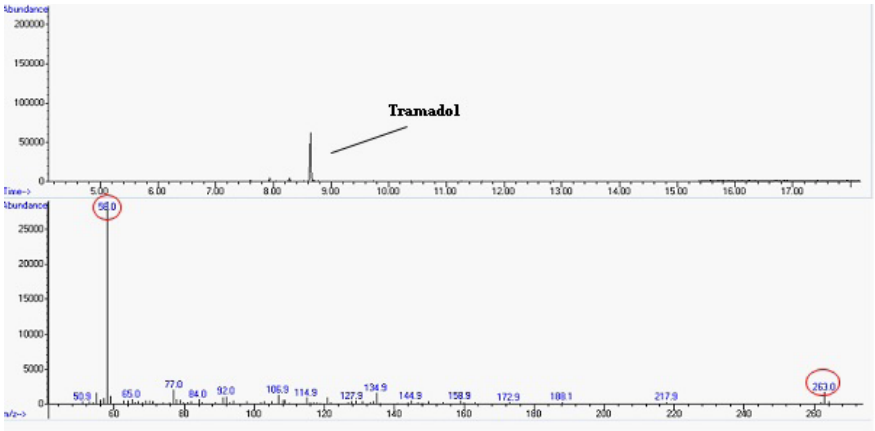

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Figure 5. Gas chromatography/mass spectrometry chromatogram and spectrum of tramadol extracted from an authentic vitreous humor sample

tivity and cleanup of the sample preparation step (Figures 2, 3, 4 and 5). The proposed method has proved to be sensitive and specific and also accurate and precise enough. Table 1 presents a summary of the validation parameters for methadone and tramadol detection in VH samples. The method was linear over the concentration range of 25-100 ng/mL (R^2>0.95) for methadone and tramadol. Furthermore, acceptable RSD values (<20%) were obtained for the within and between run precision and accuracy. Methadone and tramadol concentrations in authentic VH and urine samples are shown in Figures 6 and 7.

4. Discussion

The research aimed at assessing the validity of the DLLME method for sample preparation and the UHPLC technique for the detection of methadone and tramadol in postmortem VH specimens. Also, urine and VH samples of each case were analyzed simultaneously. The present study showed that the validated method was sensitive and specific for the efficient extraction of methadone and tramadol from postmortem VH samples.

Our results are consistent with previous studies, suggesting that VH can be an alternative matrix for the quantitative determination of methadone and tramadol. The use of VH as a reliable sample for detecting other drugs remains the subject of additional investigations [4].

Some important factors for the detection of drugs in VH specimen are drug concentration in VH, the ability of the drug to cross the blood-retina barrier, postmortem redistribution of medicines, and also the matrix effect. In the study of Metushi et al., methadone and tramadol were detected in postmortem blood and VH samples [4].

Choosing a suitable extraction solvent is essential to obtain good recovery of the analyte from biological ma-

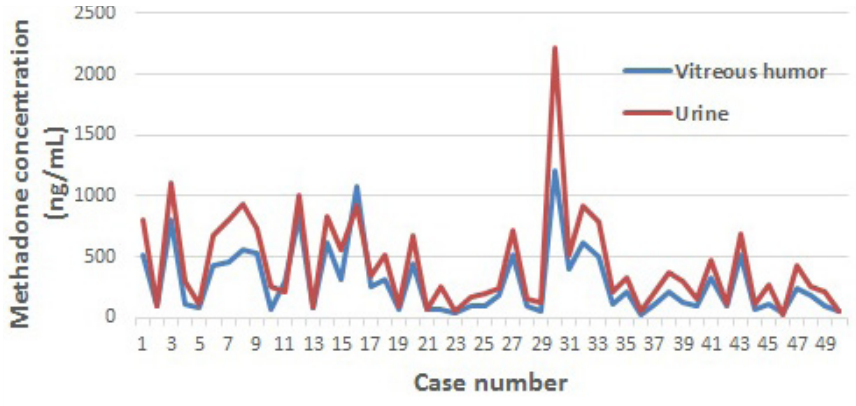

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Figure 6. Results for quantitative analysis of methadone in 50 authentic vitreous humor and urine samples using dispersive liquid liquid microextraction as sample preparation method prior to ultra high performance liquid chromatography

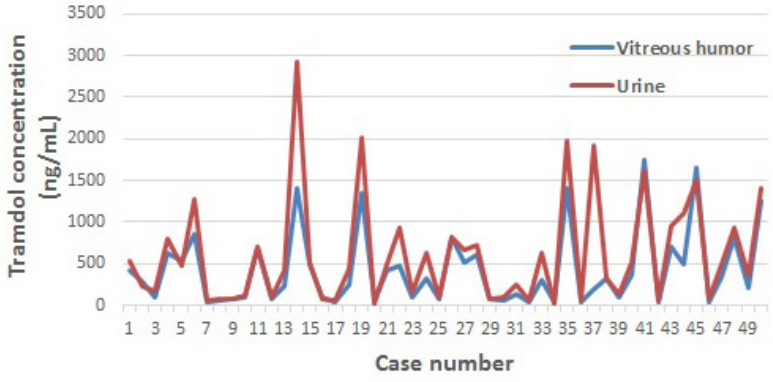

Figure 7. Results for quantitative analysis of tramadol in 50 authentic vitreous humor and urine samples using dispersive liquid liquid microextraction as sample preparation method prior to ultra high performance liquid chromatography

trices [12]. There are some crucial criteria in choosing extraction solvent in forensic toxicology analysis. For instance, the solvent should be water-immiscible, allowing the separation of aqueous and organic phases; it should be compatible with different steps in the analytical procedure, and be volatile enough to evaporate in a short period and also with density higher than water. Halogenated hydrocarbons such as chloroform and dichloromethane have the desired specifications. However, chloroform was chosen as the best extraction solvent in the present study. Dispersion solvents should be soluble in both aqueous and organic phases. Dispersion solvent allows better extraction efficiency by dispersing extraction solvent in the aqueous phase and increasing contact surface. The results showed that methanol was the best dispersion solvent.

The effect of extraction and dispersion solvents types and volumes on extraction efficiency has been discussed by other colleagues [11-15]. In their study to validate methadone and tramadol detection in VH samples, Badakhshan et al. chose 100 µL chloroform and 500 µL methanol in pH=10 as optimum extraction condition [16]. However, we had used different amounts of extraction and dispersion solvents in pH=11 and achieved better recoveries for both analytes. If the extraction solvent volume is chosen at an optimum dose, it can dissolve more analytes, and therefore a good recovery would be achieved. However, the increase in dispersion solvent volume can reduce extraction efficiency due to the increase in the solubility of the analyte in the aqueous phase and a reduction in moving to the organic phase [13]. In line with our study results, da Silva et al. chose chloroform as a suitable extraction solvent to determine cocaine adulterants in urine samples. But they had used acetonitrile as a dispersion solvent [12].

Changing the pH values in the experiment medium can convert water-soluble ionized forms of drugs to non-ionized organic soluble forms. In this context, the pH value was chosen as two units above the pKa of the analyte [12]. pKa is 9.2 for methadone and 9.41 for tramadol [14, 15]. Therefore, the optimum extraction pH was chosen at 11.

Some of the validation parameters are similar to the procedures previously described. Badakhshan and coworkers presented the linear range of 1-1000 ng/mL for methadone and tramadol [16]. Similar precision and accuracy, as well as better recovery, were obtained in the present study. In a study conducted by Matushi et al. for the detection of drugs in VH, it was concluded that VH assay was similar to blood samples and VH assay was based on the analytical method developed for the quantitative determination of drugs in whole blood and had achieved reliable response for drug detection [4].

Good recovery was obtained for the extraction of methadone and tramadol from VH samples. According to the FDA (2018), it is unnecessary to achieve 100% recovery. But it should be consistent, precise, and reproducible for a specific analyte [17]. As we did not have any pharmacokinetic information in dead bodies, we could not find any correlation between methadone and tramadol concentrations in VH and urine specimens obtained from 50 cases. When interpreting postmortem analytical toxicology results, we should be aware of general pharmacokinetic factors of a specific drug and its changes during the postmortem phase [18]. As stated by previous studies, the therapeutic blood range for methadone and tramadol are 0.1-0.5 and 0.1-1 ng/mL, respectively. In a study completed by Jennings on 47 methadone-positive medical examiner cases, the ratio of average VH to peripheral methadone concentration was 0.29 [19]. The results of the present study showed that methadone and

tramadol VH concentrations were far above therapeutic ranges. Most cases in the present study were drug abusers with very high drug concentrations in postmortem specimens. As shown by the results of the present study, methadone and tramadol concentrations were higher in urine samples compared to VH.

To quantify drugs in biological specimens, the drug had to meet the detection criteria outlined in a validated method. The concentration range of calibration should frame the drug concentration range in authentic specimens. Due to high concentrations of methadone and tramadol in urine and VH samples, the highest concentrations were obtained by serial dilution of samples to lie within the calibration curve concentration ranges.

5. Conclusion

The developed method shows significant features such as good recovery, precision, and low LoD. It can be used as a reliable sample preparation method in forensic toxicology analysis of biological matrices, affording high recovery with minimum organic solvent consumption.

Ethical Considerations

Compliance with ethical guidelines

The study protocol was in conformity with the ethical guidelines of the 1975 Declaration of Helsinki, as revised in 1983. The anonymity of cadavers was protected and approved by all participating persons in the study. This study has been approved by the Ethics Committee of the Legal Medicine Research Center, Tehran (Ethical Code: IR.LMO.REC.1396.063).

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Authors' contributions

Methodology, conceptualization, statistical analysis: Maryam Akhgari and Negin Mirahmadi Sani; Supervision: Maryam Akhgari; Investigation, Writing – original draft, and Writing – review & editing: Maryam Akhgari, Negin Mirahmadi Sani, and Zahra Mousavi.

Conflict of interest

The authors declared no conflict of interest.

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