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Research Paper

Determination of methadone in postmortem blood using QuEChERS extraction and HPLC-PDA

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ABSTRACT

Background: This study aimed to develop and validate a Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS)-based extraction method for determining methadone in postmortem blood using high-performance liquid chromatography (HPLC-PDA).

Methods: This study aimed to develop and validate a Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS)-based extraction method for determining methadone in postmortem blood using high-performance liquid chromatography (HPLC). The analytical performance of the method was validated in accordance with ICH-Q2 guidelines.

Results: The limit of detection (LOD) and limit of quantification (LOQ) were 0.05 and 0.14 mg L-, respectively. In addition, the environmental sustainability and practical applicability of the method were assessed using the Analytical Greenness metric for sample preparation (AGREEprep) and the Blue Applicability Grade Index (BAGI), yielding scores of 0.415 and 52.5, respectively.

Conclusion: These findings demonstrate that the proposed QuEChERS-HPLC method is a reliable and suitable approach for the routine determination of methadone in postmortem blood in forensic toxicology.

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Introduction

Accurate determination and quantification of methadone in postmortem blood is of great importance in the field of forensic toxicology. Methadone, a synthetic opioid and μ opioid receptor agonist, is widely used for the treatment of chronic pain and opioid replacement programs to alleviate withdrawal symptoms. However, improper or excessive use or abuse can lead to poisoning and even death.

Drug detection and quantification in postmortem blood are highly challenging because factors such as postmortem decomposition, postmortem redistribution, cellular lysis, and the presence of interfering compounds complicate the analytical process [1]. Although conventional sample preparation methods such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are effective, they are time-consuming, require large volumes of organic solvents, and depend on skilled personnel, which limits their efficiency in routine forensic laboratory workflows [2, 3]. In recent years, microextraction techniques have emerged as attractive alternatives to traditional methods. These approaches reduce sample and solvent consumption while improving analyte preconcentration and analytical sensitivity [4]. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method was originally developed for pesticide residue analysis and has since been increasingly adapted for forensic applications, including extracting drugs from biological matrices such as urine and blood. Furthermore, the strong potential for automation makes it an attractive candidate for routine applications in forensic toxicology laboratories [5].

Materials and Methods

Calibration curve and Linearity

Calibration curves were constructed using seven concentration levels (0.25, 0.5, 1, 2, 3, 4, and 5 mg L^{-1}) prepared by spiking blank postmortem blood with methadone and processing the samples using the optimized QuEChERS procedure. Method linearity was evaluated over the range of 0.25–5.0 mg L^{-1} using least-squares regression analysis. Back-calculated concentrations for all calibration levels were within ±15% of their nominal values. Excellent linearity was achieved throughout the investigated concentration range ($R^2 \geq 0.99$). The limits of detection (LOD) and quantification (LOQ) were established based on signal-to-noise (S/N) ratios of 3 and 10, respectively. Table 1 presents the calibration equation and validation results for linearity, LOD, and LOQ.

Table 1. Calibration characteristics, linearity, LOD, and LOQ of the QuEChERS method.

Calibration matrix	Linear range (mg L^{-1})	Regression equation	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
Methanolic standard solution	0.10-5.0	y = 110951x + 3111	0.9998	0.03	0.10
QuEChERS	0.25-5.0	y = 56921x − 1629	0.9964	0.05	0.14

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Recovery and Precision

The analytical accuracy was expressed as the percentage recovery of the measured concentration relative to the nominal concentration .Recovery was evaluated at three quality-control concentration levels (0.25, 2.0, and 4.0 mg L^{-1}) using three replicate samples at each level. Intra-day precision was assessed by analyzing the three replicate samples within a single day, whereas inter-day precision was evaluated over three consecutive days. An acceptance range of 80–120% (±20% bias) was applied according to ANSI/ASB Standard 036. The data are summarized in Table 2.

Table 2. Recovery and precision of the optimized QuEChERS method at three quality control levels.

QC concentration (mg L^{-1}) (n=3)	Intra-day mean recovery (%) ± RSD	Inter-day mean recovery (%) ± RSD
0.25	87 ± 11	86 ± 8
2.0	103 ± 3	107 ± 7
4.0	94 ± 7	106 ± 8

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Specificity and Selectivity

The specificity and selectivity of the optimized QuEChERS method were evaluated using blank postmortem blood samples and methadone-spiked samples. Chromatographic analysis of blank matrix extracts showed no interfering peaks at the retention time of methadone, indicating no false-positive results. Likewise, no co-eluting endogenous or exogenous compounds were observed in the spiked samples, demonstrating that the method selectively extracted and quantified the target analyte and supporting the absence of false-negative results under the conditions evaluated.

Robustness

Environmental robustness was assessed by performing the extraction procedure at three ambient temperatures and blood sample temperatures. Under all

tested conditions, the method demonstrated no appreciable changes in methadone retention time, peak shape, or resolution. The results are summarized in Table 3.

Table 3. Effect of ambient and blood sample temperatures on methadone recovery using the QuEChERS method (n=3).

Ambient temperature (○C)	Recovery (%) ± RSD	Blood sample temperature(○C)	Recovery (%) ± RSD
18	113 ± 9	8	96 ± 8
25	98 ± 7	23	104 ± 7
30	97 ± 9	NT	NT

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NT = Not tested

Matrix effect

Matrix effect (ME) was evaluated according to Matuszewski et al.[6] using methanolic methadone standards and post-extraction spiked postmortem blood samples at 2.0 mg L^{-1}, prepared in triplicate. ME was calculated as the percentage ratio of methadone peak areas in the post-extraction spiked samples to those of the methanolic standards. The mean ME was 91.49% (SD = 6.27%, RSD = 6.85%, n = 3), indicating slight matrix-induced suppression. The results are summarized in Table 4.

Table 4. Evaluation of matrix effects in the QuEChERS method for the determination of methadone in postmortem blood (n=3).

Matrix effect (%)	Mean Matrix effect (%)	SD	RSD (%)
88.75	-	-	-
98.67	91.49	6.27	6.85
87.05	-	-	-

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Carryover

Carryover was assessed by analyzing a blank sample after injection of the highest methadone standard. A response below %15 of the LOQ was considered acceptable; however, no methadone peak was observed in any of the blank samples.

Seven authentic postmortem blood samples from deceased individuals referred to the Forensic Toxicology Laboratory of Tehran were selected based on the confirmed presence of methadone by a confirmatory qualitative GC-MS method and subsequently analyzed using the proposed QuEChERS-HPLC-PDA method. The results are presented in Table 5.

Results

pH

The highest recovery was obtained at pH 2.3. One-way ANOVA demonstrated a statistically significant effect of extraction pH on methadone recovery ($P < 0.05$). Therefore, pH 2.3 was selected as the optimum extraction condition for subsequent experiments. Tukey's HSD post hoc analysis confirmed that recovery at pH 2.3 was significantly higher than at pH 7.3 and pH 9.2 ($P < 0.001$), while the difference between pH 7.3 and pH 9.2 was not statistically significant.

Blood sample volume

The 0.5 mL blood sample volume showed slightly higher methadone recovery. No statistically significant difference in methadone recovery was observed between the two blood sample volumes evaluated (one-way ANOVA, $P = 0.31$). Therefore, a sample volume of 0.5 mL was selected for subsequent experiments because it reduced sample consumption without compromising extraction efficiency.

Table 5. Methadone concentrations and case characteristics of seven authentic postmortem blood samples analyzed using the proposed method.

Sex/Age	Other Findings	History	Blood Methadone Concentration (mg L^{-1})
M/35	Clonazepam; Sertraline	Sudden death	1.32
M/28	Morphine; EDDP	Death after discharge from a drug rehabilitation camp	0.53
M/57	Tramadol	Died during sleep	1.02
M/30	Morphine; Methamphetamine	Fall from height	0.63
M/50	EDDP	No history available	0.95
M/65	Methamphetamine; Morphine	Appearance suggestive of drug dependence; death at home; coronary artery stenosis	0.40
M/36	Amphetamine; Methamphetamine	Death at home	0.38

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Salt

The influence of different salts used in the salting-out step was evaluated, including NaCl (100 mg), Na_2CO_3 (200 mg), and a mixture of NaCl (100 mg) and $MgSO_4$ (200 mg). The highest recovery was obtained using the $MgSO_4$/NaCl combination, although differences among the three salt compositions were not statistically significant (one-way ANOVA, $P = 0.29$). Therefore, the $MgSO_4$/NaCl mixture was selected for subsequent experiments

Time of vortex

Vortex time significantly affected extraction recovery (one-way ANOVA, $P = 0.001$). Tukey's HSD post hoc analysis showed that recoveries after 60 s and 180 s of vortexing were both significantly higher than those after 30 s ($P < 0.05$), whereas no statistically significant difference was observed between 60 s and 180 s ($P = 0.129$). Therefore, the 60 s vortex time was selected for subsequent experiments because it provided comparable extraction efficiency while reducing overall sample preparation time.

solvent volume

No significant difference in recovery was observed between 1.5 and 2.0 mL. Therefore, 1.5 mL was selected as the optimum extraction solvent volume.

Optimized QuEChERS extraction procedure

0.5 mL aliquot of methadone-spiked postmortem blood was transferred into a 15 mL polypropylene centrifuge tube, and the pH was adjusted to 2.3 using 150 µL of 5% hydrochloric acid. A mixture of 200 mg anhydrous $MgSO_4$ and 100 mg NaCl was then added, followed by 1.5 mL acetonitrile. The sample was vortexed for 60 s and centrifuged at 2870× g for 5 min. The upper acetonitrile phase was collected and allowed to evaporate to dryness at room temperature under ambient atmospheric conditions. The residue was reconstituted in 500 µL methanol, vortexed for 15 s, and 30 µL of the resulting extract was injected into the HPLC system. The results are shown in Table 6.

Discussion

From a mechanistic perspective, the salting-out partitioning step plays a central role in extraction efficiency. Adding inorganic salts promotes phase separation between the aqueous blood matrix and the organic solvent, facilitating analyte transfer into the acetonitrile phase while reducing the solubility of matrix components. Methadone is a weak base (pKa = 9.2), and classical liquid–liquid extraction theory predicts improved extraction under alkaline conditions, where the non-ionized form predominates. However, acidic conditions consistently produced the highest mean recovery and were therefore selected for subsequent validation.

Orfanidis et al. [7] reported a similar trend, observing the highest methadone recovery using an acetonitrile extraction system buffered with potassium dihydrogen phosphate at pH 4, whereas lower recoveries were obtained at pH 10. Acidic conditions may also promote protein precipitation and reduce co-extraction of interfering matrix constituents, potentially

Table 6. Effects of pH, blood sample volume, salt, vortexing, time, and solvent volume on Methadone extraction recovery using the QuEChERS method (n=3).

Parameter	Condition	Mean Recovery (%)	SD	RSD (%)
pH	2.3	69.16	4.08	5.90
	7.3	44.18	1.06	2.41
	9.2	45.51	2.59	5.69
Sample volume (mL)	0.5	67.95	7.91	11.65
	1	62.57	1.56	2.49
Salt	$MgSO_4$ (200 mg) + NaCl (100 mg)	69.26	5.35	7.73
	NaCl (100 mg)	59.55	10.67	17.91
	Na_2CO_3 (200 mg)	66.99	3.09	4.61
Time of vortex (s)	30	51.13	5.63	11.01
	60	77.30	8.94	11.56
	180	88.65	4.23	4.77
Solvent volume (mL)	1	69.13	5.23	7.56
	1.5	85.40	13.08	15.31
	2	75.19	8.41	11.18

contributing to the higher recovery observed in the present study. However, these mechanisms were not directly investigated. Acetonitrile was selected as the extraction solvent, consistent with its widespread use in QuEChERS applications. It readily separates from the aqueous phase during salting-out and efficiently extracts a broad range of organic compounds while limiting co-extraction of lipophilic matrix components [5]. These characteristics may have contributed to the clean chromatographic baseline observed, allowing elimination of an additional clean-up step. This simplifies sample preparation, reduces analytical time and cost, and may minimize analyte loss associated with sorbent-based purification [8, 9]. Although the $MgSO_4$/NaCl mixture provided the highest mean recovery, sodium carbonate showed comparable extraction efficiency, with no statistically significant difference. Using a single salt could nevertheless simplify preparation by reducing weighing steps, preparation time, and potential technical errors. The analytical performance of the proposed QuEChERS-HPLC method was comparable to previously reported methods for methadone determination in biological specimens.

A modified one-step QuEChERS-LC-MS/MS method for whole blood reported methadone recoveries of 90.3% and 75.9% at low and high quality-control concentrations, respectively [10]. Also, a modified QuEChERS-UPLC-MS/MS method for simultaneous determination of drugs of abuse and benzodiazepines in whole blood reported recoveries of 43–75% for methadone [11]. In comparison, the present study yielded recoveries of 86–107%. Although cross-study comparisons should be interpreted cautiously because of differences in instrumentation and validation protocols, these findings indicate that optimization of extraction conditions can substantially influence methadone recovery from biological matrices. The analytical calibration range should also encompass concentrations encountered in routine forensic casework. A systematic review by Pelletti et al. reported median postmortem blood methadone concentrations of 0.630 mg L^{-1} in adult methadone-only deaths, 0.399 mg L^{-1} in adult polydrug deaths, and 0.300 mg L^{-1} in pediatric cases, with corresponding mean concentrations of 0.994, 0.524, and 0.385 mg L^{-1}, respectively [1]. Thus, the validated calibration range of 0.25–5.0 mg L^{-1} encompasses concentrations commonly encountered in postmortem forensic samples. All steps were performed manually. However, commercially available QuEChERS kits could reduce preparation time by eliminating repeated weighing steps. The procedure is also compatible with semi-automated or fully automated workflows, potentially reducing operator-dependent variability and increasing sample throughput in routine forensic laboratories [12-15].

Although analytical performance remains the primary requirement for forensic toxicology methods, solvent consumption, waste generation, and operator safety are increasingly important. QuEChERS offers several environmental advantages, including lower solvent consumption and elimination of additional clean-up steps. Acetonitrile is also less hazardous than the chlorinated solvents frequently used in conventional liquid–liquid extraction. As reported by Asl et al. [9]elevated temperatures during GC-MS analysis may promote the formation of the methadone metabolite EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine), potentially resulting in an analytical artifact. Therefore, using HPLC, which enables methadone determination at ambient temperature, may reduce the potential for temperature-related EDDP formation. However, EDDP formation was not investigated in the present study, and this consideration should therefore be regarded as a potential analytical advantage rather than a demonstrated finding. This is particularly relevant in forensic toxicology because metabolite detection may be interpreted as evidence of methadone metabolism before death. Artifact formation could therefore affect interpretation of survival time following methadone administration and, potentially, postmortem interval. In spite of these advantages, some operational limitations should be considered. Manual weighing and adding salts can become time-consuming when processing large numbers of samples. In addition, residual salts occasionally precipitate in the dried extract, requiring daily washing to prevent clogging of the injector, connecting capillaries, and chromatographic column during repeated HPLC analyses. Overall, the method provides a practical balance between analytical performance, operational simplicity, moderate solvent consumption, and suitability for routine forensic applications.

Limitation

Dedicated short-term, freeze–thaw, and processed-sample stability studies were not performed. The effect of different degrees of postmortem blood decomposition on method performance was not specifically evaluated, which is a limitation of the present study.

Conclusion

The optimized QuEChERS-HPLC-PDA method was successfully developed and validated for methadone determination in postmortem blood, demonstrating satisfactory analytical performance and practical advantages, including simple preparation, low solvent consumption, and a targeted quantitative method for methadone determination in postmortem blood.

Ethical Approval

This study used anonymized postmortem samples provided by the Legal Medicine Center of Tehran Province. Informed consent was not required for the use of de-identified forensic specimens under the applicable ethical and legal framework. The study was approved by the ethics committee of the Faculty of Veterinary Medicine, University of Tehran (Approval No. vetethic-202509-1017) and conducted in accordance with applicable ethical guidelines.

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Conflicts of Interest

The authors report there are no competing interests to declare.

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