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ORGINAL ARTICLE

Effects of Temperatures and Time on the Stability of Methadone in Urine Samples

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KEYWORDS

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ABSTRACT: Precise detection of drugs in urine samples is crucial in both clinical and forensic settings. However, the pre-analytical phase, which includes sample handling before testing, can significantly affect the results. Additionally, Forensic investigations may involve longer storage periods than traditional clinical scenarios with immediate testing, therefore, proper sample collection and storage are essential to ensure accurate analysis. This study aims to evaluate the impact of temperature and storage duration on the stability of target compounds in urine samples. Specifically, the research investigates how different storage conditions—namely freezing versus refrigeration—affect the detectability of methadone. A total of 240 urine samples were analyzed to assess methadone stability under various temperature conditions (room temperature, 40°C, 4°C, and -20°C), both with and without the use of preservatives. The findings underscore the complex interaction between storage temperature, duration, and methadone stability. The results demonstrate that the most stable condition was storage at -20°C with a preservative, where methadone exhibited relatively high stability after one month, a slight reduction in concentration after three months, and approximately 25% degradation after six months. These results emphasize the critical importance of temperature regulation in maintaining the integrity of urine samples for accurate drug testing. These findings hold significant implications for healthcare professionals, toxicologists, and laboratory analysts involved in urine drug testing. Ultimately, the present research seeks to minimize errors and misinterpretations caused by mishandling during the pre-analytical phase and storage, leading to more reliable drug testing outcomes.

INTRODUCTION

Opioid dependence treatment relies on methadone, a medication that binds to opioid receptors, mimicking the analgesic and sedative effects of natural opiates [1]. Typically administered orally, methadone effectively alleviates withdrawal symptoms and cravings, aiding patient recovery [1]. Despite existing research, a critical gap remains in our understanding of how methadone's chemical stability and effectiveness are influenced by storage conditions, particularly temperature and environmental factors. Elucidating the impact of these variables on methadone's preservation and biochemical degradation during storage is essential for optimizing analytical protocols and minimizing drug concentration loss, especially in high-throughput testing environments. This study aims to address this gap by systematically investigating the stability and detectability of methadone under various storage temperatures and environmental conditions. The stability of analytes within forensic samples is acknowledged as a fundamental prerequisite in the realm of forensic toxicological analysis. The

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presence of instability or the occurrence of artifactual formation can frequently be attributed to factors such as enzymatic degradation, chemical reactivity, autoxidation, and the decomposition of N-oxides. Urine is typically devoid of proteins, lipids, and enzymatic activities; however, it exhibits considerable variability in its composition and encompasses a broad spectrum of pH levels. Forensic toxicologists must take into account that the instability and the formation of novel compounds are predominantly influenced by the sample, the matrix, and the specific analyte involved [2]. A comprehensive understanding of the stability of analytes in samples is of paramount significance during quantitative analyses within the discipline of forensic toxicology. The degradation of analytes may lead to misinterpretation of results, which can result in either underestimation or overestimation of concentrations. Numerous investigations have demonstrated that analytes tend to exhibit greater stability at lower storage temperatures. Researchers have posited that the stability of analytes should be duly considered prior to any interpretation of concentration levels in forensic toxicological cases [3].

Accurate bioanalysis of drugs in urine samples relies heavily on analyte stability. While refrigeration or freezing was ideal for sample preservation, logistical constraints often necessitate alternative methods. Pellegrini and co-workers demonstrated the stability of various drugs of abuse at room temperature urine supplemented with a specific salt mixture [4]. Similar to preservative-coated oral fluid collection devices, additives can potentially maintain sample integrity [5]. Daeid and Houck highlighted the time- and temperature-dependent degradation of methadone in urine, underlining the need for further investigation [6]. However, their study was limited to a 6-month timeframe. The present research addresses this gap by evaluating methadone stability in urine under various storage conditions (temperature and duration). A wide range of temperatures (-20°C, room temperature, 40°C) and extended storage periods (1, 3, and 6 months), were explored. Additionally, the impact of a specific preservative cocktail on stability was assessed. Validated High-Performance Liquid Chromatography (HPLC) was employed to quantify methadone concentrations and analyze degradation profiles. The obtained data offered valuable insights for optimizing urine sample preservation for methadone analysis, particularly in settings with limited refrigeration or freezing capabilities. Dahlin and Petrides reported that certain benzodiazepines exhibited a range of degradation patterns over the course of their storage [7]. Nickley and team conducted an investigation into the bioanalytical and methodological challenges associated with detecting cocaine exposure, highlighting the intricacies of accurate quantification in forensic contexts [8]. Aldubayyan et al. performed a comprehensive study on the stability of selected synthetic cathinones (SCts) and their metabolites in human urine under various storage conditions. Their findings revealed that elevated temperatures accelerated analyte degradation, whereas freezing significantly enhanced stability. Notably, dihydro-metabolites demonstrated greater resilience across all conditions, suggesting their potential as reliable biomarkers for SCt detection. Based on these results, it was recommended that SCt-positive urine samples be frozen and analyzed promptly to minimize analyte loss [9].

Álvaro-Alonso and colleagues evaluated the physical and microbiological stability of methadone hydrochloride oral solutions—both with and without parabens—over a 91-day period under different temperature conditions. Their study supported the possibility of extending the beyond-use date (BUD), thus facilitating methadone maintenance therapy in clinical settings [10]. Similarly, Riggio et al. reported considerable degradation of amphetamines under various storage conditions, reinforcing the importance of controlled storage environments to preserve analyte integrity [11]. In a related study, Aldubayyan's team also examined the long-term stability of compounds in whole blood. Their results indicated that temperature had a more substantial impact on analyte degradation than the use of preservatives. While sodium fluoride (2% w/v) was effective in reducing degradation at room temperature, compounds containing aromatic halogens were found to be less stable compared to those with N-pyrrolidine structures. For accurate toxicological analysis, prompt testing is advised, and for long-term preservation, storage at or below –40°C is recommended [12].

Patient dosage

The variability in patient response to methadone underscores the importance of individualized dosing regimens. Analytical techniques, including capillary electrophoresis, radioimmunoassay, gas chromatography, liquid chromatography, and enzyme immunoassay can help in methadone quantitation throughout several samples and their testing [13].

Monitoring

Methadone maintenance treatment (MMT) requires meticulous monitoring for optimal outcomes. Urine drug testing, a non-invasive tool, assesses patient adherence, detects illicit drug use, and informs treatment decisions. While blood offers accuracy, urine was preferred due to ease of collection and higher drug concentrations [14]. Multivariate analysis validates the reliability of drug testing. This study utilizes validated analytical methods and explores patient characteristics to understand methadone pharmacokinetics and potential variations in patient responsibility [15].

Effects of temperatures and time on methadone stability in urine sample

Methadone stability in urine significantly decreases with higher temperatures and longer storage durations, potentially compromising detection accuracy in drug testing. Lower temperatures and shorter storage times improve stability. This highlights the importance of controlled temperature and storage practices for reliable methadone detection. Balancing proper supervision to prevent sample tampering with privacy concerns and operational efficiency remains a challenge. Understanding the impact of temperature and storage time on methadone stability was crucial for accurate drug testing and treatment monitoring in methadone maintenance therapy (MMT) programs. Furthermore, validated analytical techniques like direct extraction methods were necessary for accurate quantification of methadone, ultimately improving patient care and treatment outcomes [16].

Role of urine preservatives

The use of chemical preservatives in urine samples plays a crucial role in inhibiting bacterial proliferation, reducing analyte degradation, and preserving compound solubility. Moreover, preservatives can help prevent the oxidation of unstable analytes, thereby enhancing the overall integrity of the sample [13]. Although refrigeration is widely regarded as the gold standard for urine sample preservation, its effectiveness can be significantly improved through the concurrent use of appropriate chemical preservatives. Kolb and colleagues emphasized the critical role of chemical preservation in maintaining the stability of urine samples and preventing microbial contamination. Commonly employed preservatives include boric acid, hydrochloric acid, acetic acid, and oxalic acid. In addition, buffers and bicarbonate salts may be utilized, depending on the specific analytical requirements. It is imperative that laboratories thoroughly assess the efficacy of these preservatives and evaluate their potential to interfere with analytical outcomes [17].

Sample preparation using salt mixture and formaldehyde

Three types of urine samples were prepared for analysis: (i) samples treated with a salt-based preservative mixture, (ii) samples preserved with formaldehyde, and (iii) samples without any preservatives. The preparation process for each type is illustrated in Figures 1 and 2. The present study aims to assess the stability of methadone in urine samples stored under varying temperature conditions and time intervals, both with and without the addition of chemical preservatives.

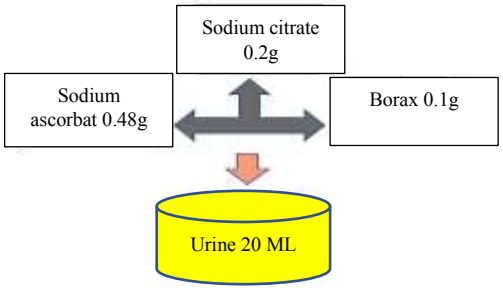
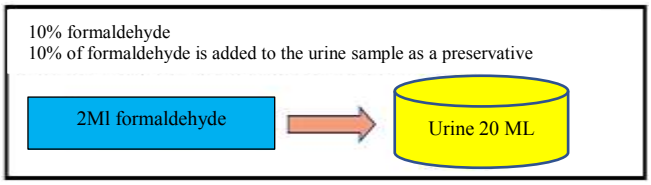


Figure 1. Sample Preparation Using Salt Mixture as Preservative.



Figures 2. Sample Preparation Using Formaldehyde as Preservative.

MATERIALS AND METHODS

Sample

Nine hundred and sixty methadone-positive samples were used to study the effects of storage temperature and duration on methadone stability and analytical reliability. The samples were divided into three groups: without preservatives, with preservatives (salt Mixture, and with formaldehyde as a preservative. Each group included 80 samples.

The study included 240 samples and four subgroups based on storage temperature (room temperature, 4°C, −20°C and 40°C) and addition of a preservative in equal distribution in each subgroup. Samples were analyzed at the time of collection (Day 1), one, three and six months later by using immunoassay screening followed by confirmation by gas chromatography–mass spectrometry (GC–MS) in a research project to mimic the routine work of a forensic laboratory.

-An initial screening employed Atellica (Siemens) Randox Evidence & Evidence MultiSTAT alongside One-Step cup tests.

-Following designated storage periods, samples with positive initial screens underwent confirmatory analysis using GC-MS to evaluate potential analyte degradation over time.

GC-MS instrument conditions

Specific safety protocols were followed for each instrument in accordance with the manufacturer's safety guidelines. Additionally, periodic maintenance was conducted to ensure the safety and proper functioning of each instrument. An Agilent GC-MS system equipped with a DB-5MS Ultra Inert column (30 m × 0.25 mm × 0.25 µm) was utilized in split less mode. The initial oven temperature was set to 70°C, then increased to 280°C at a rate of 12°C/min, where it was held constant for 11 minutes. The total flow rate, purge flow rate, and pressure were set at 24 mL/min, 3 mL/min, and 8.8085 psi, respectively. For detection, the mass spectrometer was set to a scan range of 50–550 m/z, with the ion source temperature maintained at 230°C and the quadrupole temperature set at 150°C.

Toxi-Tubes (A) General Procedure: Add up to 5 mL of sample to the desired Toxi-tube, mix it for 2 minutes, add 50 µl appropriate internal standard, centrifuge for 2 minutes, and remove organic extract for analysis (Figure 3).

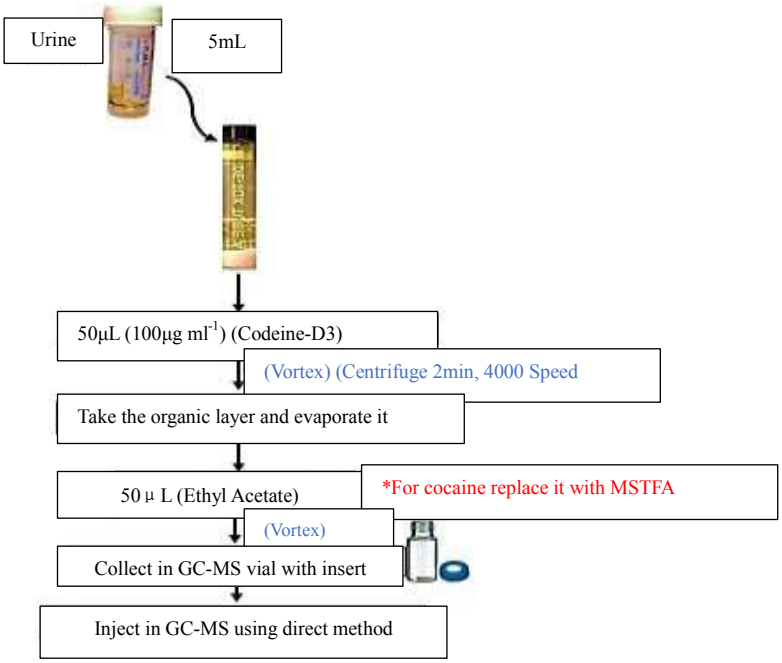


Figure 3. Toxi-Tubes (A) Direct Extraction Method.

Methadone, a long-acting mu-opioid receptor agonist, plays a vital role in Medication-Assisted Treatment (MMT) programs for heroin dependence. However, individual responses to methadone can vary significantly due to a complex interplay of pharmacokinetic and pharmacodynamics factors to optimize treatment efficacy and ensure patient compliance, accurate monitoring of methadone levels in biological samples was essential. One reliable method for such monitoring involves gas chromatography-mass spectrometry (GC-MS). Diong and team successfully developed and validated a GC-MS approach for quantifying methadone and its major metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), in plasma and urine samples collected from MMT patients. This method offers several advantages, including simplicity, accuracy, and reproducibility. It allows for the effective monitoring of both methadone and EDDP concentrations across a wide range, from 15 to 1000 ng/mL for methadone and 50 to 2000 ng/mL for EDDP. Table 1 show the ability to measure both the parent drug and its metabolite provides a more comprehensive picture of methadone absorption and elimination within the body [14].

Table 1. Validation Parameters for Quantitation of Methadone and EDDP in Plasma and Urine Using GC-MS.

Plasma	Parameters	Urine
Methadone		EDDP
	Calibration curve	
15–1,000	Range (ng mL^{-1})	15–1,000
y = 1.493x − 0.001	Equation of line	y = 10.928x − 0.158
0.999	r^2	0.996
5	LOD (ng mL^{-1})	2
15	LOQ (ng mL^{-1})	5
	Selectivity	
9	Accuracy (%bias)	6.3
1.7	Precision (%CV)	10.5
	Intraassay	
1	Accuracy (%bias)	-5.4
1.6	Precision (%CV)	1.2

	Intraassay	
0.7	Accuracy (%bias)	-4
1.5	Precision (%CV)	4.3
100.1	Recovery (%)	95.8
	Stability	
2.5	4 h at room temperature (%bias)	2.6
12.3	One day at 4°C (%bias)	-2.1

Stability of methadone metabolites in urine samples concerning temperature and time

The stability of methadone & its primary metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, in urine was important for the interpretative accuracy of drug testing results, specifically in the context of methadone maintenance treatment. The effects of temperature & time on the stability of these substances can highly influence the reliability of urine drug tests.

Effects of time & temperature on methadone stability

Several factors, including storage temperature and duration, can significantly impact methadone stability in urine samples. The research of Kapur and Aleksa addresses the sensitivity and bias in the results [18].

-At room temperature for 4 hours: Methadone showed a difference of 8.7 percent, which means a certain degree of instability over room temperature and short-term storage.

-Stored for one day at 4°C: Methadone exhibited a bias of 9.9%, which exhibited moderate stability for a short span in a refrigerated condition.

These findings highlight the surprising instability of methadone, even under short-term room temperature or refrigerated conditions. Such rapid degradation highlights the potential for misinterpretations in urine drug testing if samples are not processed promptly or stored under appropriate conditions

Stability of EDDP

Unlike methadone itself, its primary metabolite, 2-ethylidene-1,5-dimethyl-3,3 diphenylpyrrolidine (EDDP), exhibits greater stability in urine. This was particularly advantageous, as EDDP was not affected by fluctuations in urine pH, making it a more reliable marker for monitoring methadone adherence.

-At room temperature for 4 hours: EDDP reveals a bias of 3.0%, which indicates good stability in short-term room temperature conditions.

-Stored for one day at 4°C: EDDP exhibited a difference of -2.6%, which suggests that it remains stable under cold conditions for at least 24 hours [15].

These findings highlight EDDP's superiority as a marker for methadone adherence due to its enhanced stability compared to methadone, particularly under common storage conditions.

Clinical interpretation & practical considerations

Inaccurate interpretations of drug test results can arise from compromised urine sample integrity, a critical concern for clinical laboratories. Sample tampering practices, such as patient resubmission of previously submitted samples or the use of the same sample by multiple individuals, can significantly hinder result validity.

To address these stability concerns, Kapur and Aleksa's proposed a urine fingerprinting algorithm utilizing diet-dependent factors (chromium, urine pH, sodium, and chloride) to identify potential sample manipulation or resubmission. This approach has the potential to enhance the reliability of urine drug tests by ensuring quantitative and precise measurements [18]. Furthermore, monitoring steady-state concentrations of methadone and its metabolites was essential for assessing patient adherence to methadone maintenance treatment (MMT). Deviations from the expected excretion window of the primary metabolite, EDDP, can indicate non-adherence, while

chronic MMT patients typically exhibit consistent EDDP levels (Figure 4). Therefore, due to its stability and minimal influence from urine pH variations compared to methadone itself, urinary EDDP concentration serves as a reliable marker for treatment compliance (Figure 4 adopted from Kapur and Aleksa's, 2020).

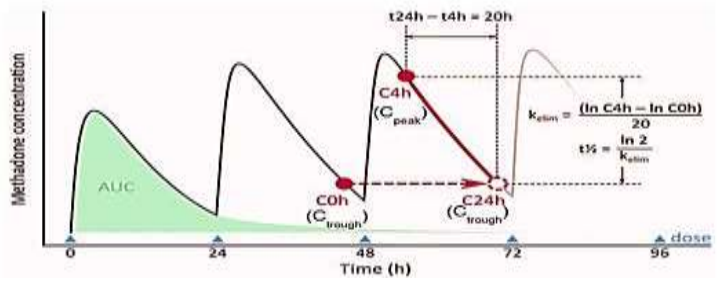


Figure 4. Calculation of methadone half-life. In a once a day (24 h) dosing. C4h (C post peak) must be measured four or more hours after last dose to ensure drug distribution has taken place.

Statistical analysis

The statistical analysis aims to evaluate differences in the mean values of physical parameters across the study groups. Comparisons between groups were performed using One-Way ANOVA, with a 95% confidence interval (CI). The analysis was based on the formulation of two mutually exclusive hypotheses: the null hypothesis and the alternative hypothesis. Equal variances were assumed for the analysis.

Null hypothesis: All means are equal.

Alternative hypothesis: Not all means are equal.

Significance level: $\alpha = 0.05$.

RESULTS AND DISCUSSION

In the present study, methadone was examined in 960 urine samples using four different testing conditions (temperatures: room temperature, 40°c, 4°c, and -20°c). The results of the present study were summarized as follows:

One month analysis

-Room temperature (RT): The stability of methadone decreases slightly by 10 to 20% over time, with minor variations observed across the different categories: initial concentration (IC), with preservatives (WP), with formaldehyde preservatives (WF), and without preservatives (WOP).

-Storage at 4°C: Similar trends were observed, with stability showing a marginal decrease over one month.

-Storage at 40°C: Significant degradation occurred, with more than 50% loss in stability across all categories.

-Storage at -20°C: The most stable condition was observed at -20°C, where methadone retained relatively high stability even after one month (Table 2a & b).

-Figure 5: The data indicate that the concentration of methadone at the start and after one month without preservatives showed negligible change. In contrast, the concentrations remained nearly identical in the samples with preservatives and those with formaldehyde.

Table 2a. Observation of methadone stability over 1 month.

Samples	No.	Mean	StDev	95% CI	
IC	20	0.445	0.1791	(0.3838,	0.5062)
WP_RT	20	0.365	0.1565	(0.3038,	0.4262)
WF_RT	20	0.2715	0.1181	(0.2103,	0.3327)
WOP_RT	20	0.1825	0.0783	(0.1213,	0.2437)
WP_4C	20	0.447	0.174	(0.3858,	0.5082)
WF_4C	20	0.3445	0.143	(0.2833,	0.4057)
WOP_4C	20	0.241	0.0997	(0.1798,	0.3022)
WP_(-)20C	20	0.42	0.1642	(0.3588,	0.4812)
WF_(-)20C	20	0.313	0.1231	(0.2518,	0.3742)
WOP_(-)20C	20	0.2325	0.0963	(0.1713,	0.2937)
WP_40C	20	0.37	0.1658	(0.3088,	0.4312)
WF_40C	20	0.275	0.1245	(0.2138,	0.3362)
WOP_40C	20	0.2525	0.14	(0.1913,	0.3137)

Abbreviations: IC – Initial Concentration; WP_RT – With Preservative at Room Temperature; WF_RT – With Formaldehyde Preservative at Room Temperature; WOF_RT – Without Preservative at Room Temperature; WP_4C – With Preservative at 4oC; WF_4C – With Formaldehyde Preservative at 4oC; WOF_4C – Without Preservative at 4oC; WP_(-)20C – With Preservative at -20oC; WF_(-)20C – With Formaldehyde Preservative at -20oC; WOF_(-)20C – Without Preservative at -20oC; WP_40C – With Preservative at 40oC; WF_40C – With Formaldehyde Preservative at 40oC; WOF_40C – Without Preservative at 40oC.

Table 2b. Analysis of Variance of methadone stability over 1 month

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Factor	12	1.773	27.08%	1.773	0.14778		
Error	247	4.774	72.92%	4.774	0.01933	7.65	0
Total	259	6.547	100.00%				

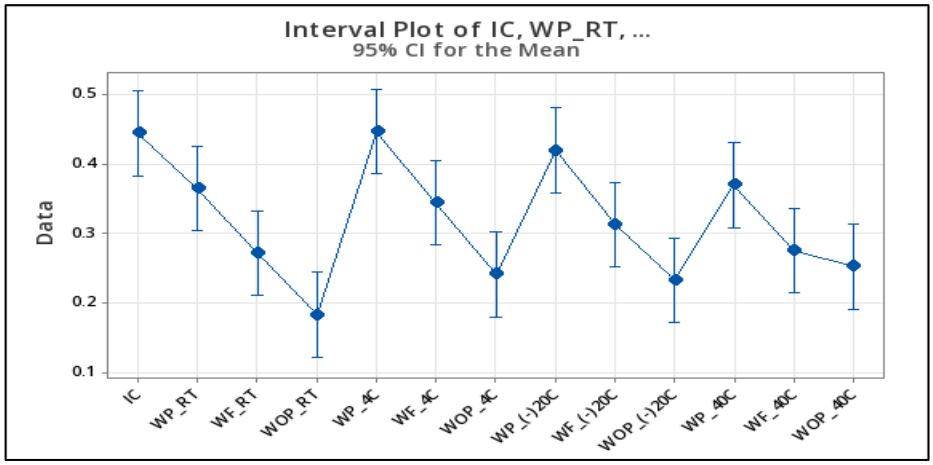


Figure 5. The 95% confidence intervals for each sample group were calculated as mean ± t* × (SD/√n), and displayed using an interval plot to compare group estimates.

Three months analysis

-Room temperature and 4°C: Similar trends were observed at both temperatures, with methadone stability decreasing gradually by 20 to 30% over three months.

-Storage at -20°C: This condition proved to be the most stable, although slight degradation was noted when compared to the one-month results.

-Storage at 40°C: Methadone stability declined significantly, with a decrease of more than 50%, indicating substantial degradation after three months (Table 3a & b).

-With preservatives: After three months, methadone concentration declined slightly by 10 to 20%. Samples with formaldehyde preservatives exhibited more degradation compared to those with other preservatives. Notably, degradation in samples without preservatives was more pronounced, with a loss exceeding 50% (Figure 6).

Table 3a. Observation of methadone stability over 3 months

Samples	No.	Mean	StDev	95% CI	
IC	20	0.445	0.1791	(0.3854,	0.5046)
WP_RT	20	0.2175	0.1042	(0.1579,	0.2771)
WF_RT	20	0.16	0.0791	(0.1004,	0.2196)
WOP_RT	20	0.221	0.1699	(0.1614,	0.2806)
WP_4C	20	0.383	0.1592	(0.3234,	0.4426)
WF_4C	20	0.2835	0.1192	(0.2239,	0.3431)
WOP_4C	20	0.1905	0.081	(0.1309,	0.2501)
WP_(-)20C	20	0.4275	0.169	(0.3679,	0.4871)
WF_(-)20C	20	0.313	0.1245	(0.2534,	0.3726)
WOP_(-)20C	20	0.2055	0.0784	(0.1459,	0.2651)
WP_40C	20	0.224	0.1626	(0.1644,	0.2836)
WF_40C	20	0.168	0.1215	(0.1084,	0.2276)
WOP_40C	20	0.173	0.1496	(0.1134,	0.2326)

Table 3b. Analysis of Variance of methadone stability over 3 months.

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Factor	12	2.383	34.52%	2.383	0.19859		
Error	247	4.521	65.48%	4.521	0.0183	10.85	0
Total	259	6.904	100.00%				

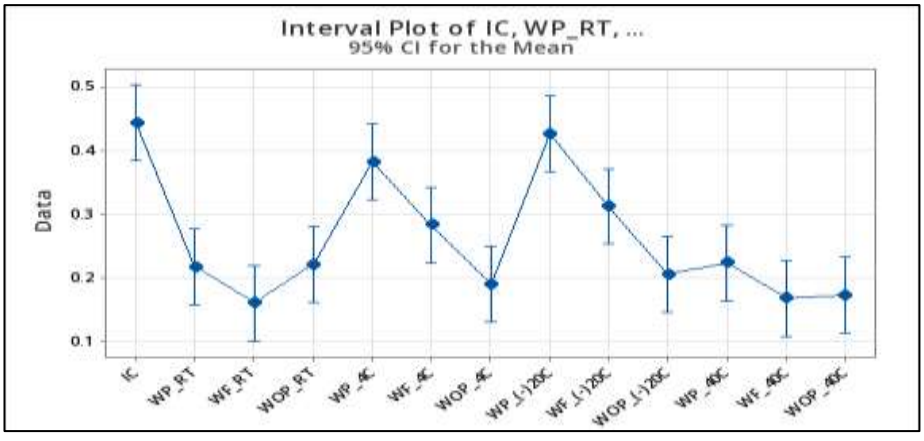


Figure 6. The 95% confidence intervals for each sample group were calculated as mean ± t* × (SD/√n), and displayed using an interval plot to compare group estimates

Six months analysis

-Room temperature and 4°C: Methadone stability continued to decline by 50 to 60%, albeit at a slower rate than observed at earlier time points.

-Storage at -20°C: Methadone retained the highest

stability under these conditions, though some degradation was noted compared to the three-month results.

-Storage at 40°C: Significant degradation occurred, with stability declining by approximately 70%, indicating increased instability over six months.

-Temperature effects: Lower temperatures, particularly -20°C, preserved methadone stability over time, while higher temperatures accelerated degradation. These findings underscore the critical importance of temperature control in maintaining the integrity of urine samples for accurate methadone drug testing (Table 4a & b).

-Figure 7: After six months, degradation of methadone concentration became more noticeable compared to the concentration observed after one month with preservatives. Notably, samples without preservatives showed a decline of more than 50%.

Table 4a. Observation of methadone stability over the period of 6 months.

Samples	No.	Mean	StDev	95% CI	
IC	20	0.445	0.1791	(0.4027,	0.4873)
WP_RT	20	0.1665	0.09	(0.1242,	0.2088)
WF_RT	20	0.1155	0.0675	(0.0732,	0.1578)
WOP_RT	20	0.0835	0.0465	(0.0412,	0.1258)
WP_4C	20	0.2755	0.1174	(0.2332,	0.3178)
WF_4C	20	0.2025	0.0878	(0.1602,	0.2448)
WOP_4C	20	0.1375	0.0581	(0.0952,	0.1798)
WP_(-)20C	20	0.3195	0.1354	(0.2772,	0.3618)
WF_(-)20C	20	0.262	0.1219	(0.2197,	0.3043)
WOP_(-)20C	20	0.1635	0.0695	(0.1212,	0.2058)
WP_40C	20	0.131	0.074	(0.0887,	0.1733)
WF_40C	20	0.0945	0.0551	(0.0522,	0.1368)
WOP_40C	20	0.0655	0.03873	(0.02322,	0.10778)

Table 4b. Analysis of Variance of methadone stability over 6 months.

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Factor	12	2.869	55.75%	2.869	0.239076		
Error	247	2.277	44.25%	2.277	0.009218	25.94	0
Total	259	5.146	100.00%				

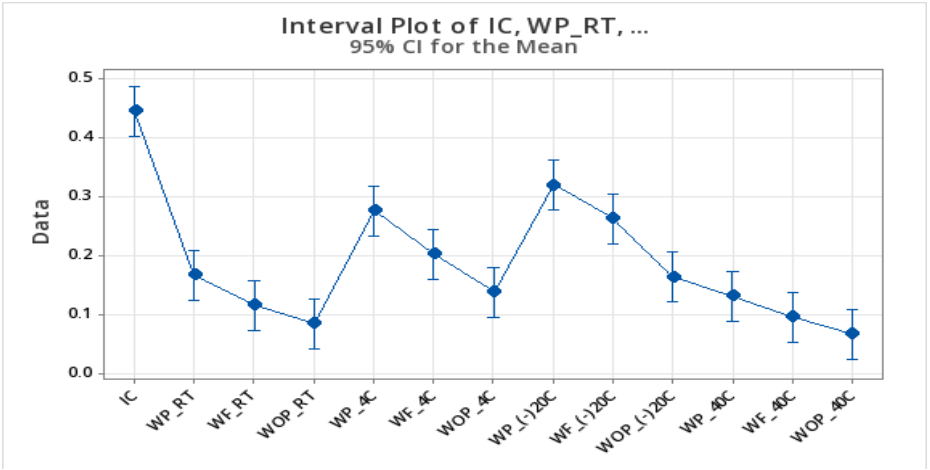


Figure 7. The 95% confidence intervals for each sample group were calculated as mean ± t* × (SD/√n), and displayed using an interval plot to compare group estimates

The present study investigated the influence of temperature and storage duration on methadone stability in urine. Our findings elucidate critical factors affecting the reliability of drug testing in forensic and clinical settings, particularly for methadone, a synthetic opioid used in both addiction treatment and pain management. The analysis aims to detect and quantify methadone to monitor its presence and concentration in urine samples.

By interpreting the chromatogram peaks, mass spectra, and calibration data, analysts can effectively monitor methadone levels and ensure the reliability of the analytical process. Cod-D3is an internal standard used in GC-MS for calibrating and validating the measurement of target analytes, such as methadone. The internal standard helps correct for variations in the analytical process. The GC-MS results for the internal standard Cod-D3 provide crucial data for calibrating and validating the analytical process. By examining the chromatogram and mass spectra of Cod-D3, analysts can ensure accurate quantification of target analytes, such as methadone, and maintain high-quality standards in analytical testing.

A multitude of studies has highlighted the significant impact of storage conditions on the stability of analytes in biological samples. Dahlin and Petrides demonstrated that prolonged storage (exceeding 1.5 months) negatively affects analyte performance due to matrix interactions, likely caused by precipitation dynamics during the freezing process [7]. In another study, the stability of methamphetamine and amphetamine in highly doped urine, both with and without preservatives, was assessed. Notably, while estazolam remained stable under the same conditions, nitrazepam underwent complete degradation within 14 days at 25°C in the absence of preservatives [19]. Cocaine and 6-acetylmorphine also exhibited complete degradation after 1.5 months, even at reduced concentrations. The optimal storage conditions for cocaine in urine samples were identified as temperatures ranging from –16°C to –21°C, along with a controlled pH (~5.0) maintained using ascorbic acid, which proved more effective than hydrochloric acid in preserving sample integrity. However, such conditions require advanced freezing equipment, which may pose challenges for laboratories with spatial limitations. Additionally, it was recommended that dark, sanitized glass containers be used to mitigate degradation during prolonged storage (exceeding 4 months) [8]. Riggio et al. observed that cocaine remained stable for up to 6 days at room temperature, but only 1–2 days at 37°C. Benzoylecgonine exhibited similar stability patterns, while THC remained stable for 7 days at room temperature and 2–6 days at 37°C [11]. Another study confirmed that drug concentrations in biological fluids stored below freezing gradually declined over time, highlighting that suboptimal storage conditions could compromise analyte integrity and the overall quality of specimens [20]. The results of the present study demonstrate degradation in drug concentration over time under different temperature conditions, supporting findings from studies by Aldubayyan et al., Riggio et al., and Aldubayyan et al. [9, 11, and 12].

The emergence of preservative additives has proven valuable in mitigating drug concentration degradation, especially in high-volume testing scenarios. By suppressing bacterial growth and maintaining analyte solubility, these additives play a critical role in safeguarding test integrity and ensuring accurate results. The finding of present study underscores the importance of incorporating effective preservative measures into testing protocol standards. Our investigation focused on the impact of storage conditions, including temperature variations and duration, on methadone stability in urine. Samples were subjected to a range of temperatures (-20°C, 4°C, room temperature, and 40°C) over varying time periods (1 to 6 months) to assess the integrity and reliability of drug concentrations under real-world storage conditions. Employing industry-standard methods (e.g., analyte stability, pharmacokinetics) and incorporating authentic sample submission procedures, our research prioritized sample integrity and ensured the accuracy of the obtained results. The present study contributes to the understanding of methadone's unique pharmacokinetic profile compared to other opioids. While its efficacy in addiction treatment and anesthesia was well-established, methadone presents distinct challenges in terms of dose prediction and risk management. These findings underscore the importance of vigilant monitoring and close clinical oversight for

patients receiving methadone therapy.

The observed variability in methadone stability across different storage conditions further emphasizes the need for standardized protocols for urine sample collection, handling, and analysis. Implementing robust quality control measures throughout the testing process was crucial for ensuring the accuracy and reliability of methadone drug testing results. The complex pharmacokinetic profile of methadone necessitates a personalized approach to treatment planning. Careful monitoring and ongoing clinical evaluation were essential for optimizing therapeutic outcomes and mitigating potential risks associated with methadone use. Future research efforts could explore the development of more precise dosing strategies and tailored treatment regimens to further improve patient care in methadone maintenance programs.

CONCLUSIONS

In conclusion, the present study highlights the complex relationship between storage temperature, time, and methadone stability in urine samples. These findings have significant implications for healthcare professionals, toxicologists, and laboratory analysts involved in urine drug testing. By elucidating the impact of these factors on methadone degradation, this study empowers stakeholders to optimize sample handling protocols, thereby ensuring the consistent quality of methadone metabolite screening in both clinical and forensic settings.

Moreover, the research extends beyond methadone, suggesting the broader applicability of these insights to other analytes in toxicological analysis. By enhancing our understanding of how environmental variables affect drug stability, the study advocates for the development of robust, evidence-based guidelines to improve the accuracy and reliability of toxicological examinations across various contexts. Ultimately, the aim is to contribute to the refinement of clinical and forensic toxicology practices, leading to better patient outcomes and preserving the integrity of legal proceedings.

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Conflict of interests

There are no conflicts of interest to declare by the authors.

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Data availability

The datasets and results are available from the corresponding author on reasonable request.

Supplementary information

Not applicable.

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