

Development and Optimization of HPLC Conditions for Quantitative Determination of Methadone in Hospital-Prepared Syrup Formulation

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Abstract: Reliable and practical analytical methods are essential for confirming the quality and safety of locally compounded methadone syrup in clinical settings. This study aimed to develop and optimize high-performance liquid chromatography (HPLC) conditions for the quantitative determination of methadone in hospital-compounded formulations. Five distinct mobile phase compositions, varying in buffer systems and organic solvent ratios, were systematically evaluated using a reversed-phase C18 column. Chromatographic consistency was assessed based on retention time, peak area, signal reproducibility, and peak symmetry. Among the evaluated conditions, a mobile phase consisting of 20 mM ammonium formate and acetonitrile (13:87, v/v) demonstrated the most favorable chromatographic performance. The optimized condition yielded an ideal retention time of approximately 6.0 minutes, symmetrical peak profiles ($As_{10} \approx 1.25-1.45$), and very low variability (%RSD < 3%), indicating consistent chromatographic response under the tested conditions. Additionally, the method provided a stable signal intensity with no major observable interfering peaks. This optimized framework was successfully applied to quantify methadone in hospital- prepared syrup samples. The developed methodology is streamlined, cost-effective, and highly suitable for routine implementation into hospital laboratory workflows. Overall, this study establishes a practical analytical framework for the quality control of methadone formulations in clinical environments.

Keywords: Methadone, HPLC, Mobile phase optimization, Hospital-compounded formulation, Quality control, Method optimization

INTRODUCTION

Opioid dependence remains a major global public health issue, contributing significantly to morbidity and mortality rates worldwide [1]. Methadone, a synthetic opioid, is extensively utilized in opioid substitution therapy for the clinical management of opioid dependence [1]. Clinical evidence indicates that sustained retention in methadone maintenance treatment programs substantially lower the risk of all-cause and overdose-related mortality [2].

Given methadone's complex pharmacokinetic profile and essential clinical role, precise quantification within pharmaceutical preparations is critical to ensure therapeutic efficacy and safeguard patient safety [3, 4]. In specialized healthcare and hospital settings, methadone syrup formulations are often compounded on-site [1]. This decentralized compounding necessitates strict quality assurance protocols that depend on practical analytical methods for routine quality control.

While commercially manufactured formulations undergo strict regulatory oversight, hospital-prepared oral solutions require independent, practical validation to prevent dosing irregularities, verify active pharmaceutical ingredient (API) concentrations, and monitor longitudinal physicochemical stability [1, 5, 6]. While numerous analytical methods exist for methadone, many rely on complex instrumentation, making them impractical for routine clinical application. Advanced techniques such as gas chromatography (GC) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) offer high specificity but are limited by substantial operational costs, the need for highly specialized personnel, and extensive sample preparation times [3, 5-8]. In contrast, high-performance liquid chromatography (HPLC) offers a cost-effective, and reproducible solution readily accessible for routine clinical quality control [5, 9, 10]. However, careful optimization of chromatographic conditions is critical for achieving adequate separation, optimal peak geometry, and reliable analytical performance. Variables including the mobile phase buffer system, organic modifier ratio, and ionic strength directly dictate the chromatographic efficiency, retention behavior, and peak symmetry for lipophilic analytes like methadone [11]. The efficacy of HPLC methods relies heavily on these parameters. Alterations in buffer systems, pH, and organic solvent ratios can strongly affect retention behavior and overall analytical reliability [13, 14], particularly for lipophilic compounds such as methadone. Despite existing research, there remains a lack of systematically optimized HPLC protocols specifically tailored for hospital-prepared methadone syrup formulations [1, 14, 15]. Although several chromatographic methods for methadone analysis have been reported, many were developed for biological matrices or comprehensive analytical validation studies and often require complex sample preparation, specialized instrumentation, or extended analytical procedures. Limited information is available regarding chromatographic condition optimization specifically for hospital-compounded methadone syrup formulations intended for routine quality control. Therefore, a practical and accessible HPLC method optimization approach remains necessary to support routine pharmaceutical analysis in

healthcare settings. Consequently, this study aimed to develop and comprehensively optimize HPLC conditions for the quantitative determination of methadone in hospital-compounded syrup formulations through the systematic evaluation of diverse mobile phase compositions. This research aims to provide a streamlined, robust, and highly practical analytical methodology that can be deployed in hospital laboratories to enhance routine quality control and ultimately improve patient safety within opioid substitution programs. The present investigation was designed as a chromatographic optimization study. Comprehensive analytical method validation, including specificity, linearity, accuracy, precision, LOD, and LOQ determination, was beyond the scope of the current work and is recommended for future studies.

MATERIALS AND METHODS

Chemicals and Reagents

Acetonitrile (HPLC grade) was sourced from Merck (Germany). Methanol (HPLC grade) and ammonium acetate (analytical grade) were obtained from RCI Labscan (Thailand). Potassium dihydrogen phosphate (KH_2PO_4 , ACS grade) and glacial acetic acid were acquired from Merck (Germany), formic acid (98%) from Fisher Scientific (UK), and orthophosphoric acid (85%) from Loba Chemie (India). Methadone- D_3 (1.0 mg/mL in methanol) was procured from Merck (Germany). A 25 mM sodium acetate solution (1 L) was formulated by dissolving 2.05 g of sodium acetate in deionized water and adjusting the volume to 1 L. A 20 mM ammonium formate solution (1 L) was prepared by dissolving 1.54 g of ammonium acetate in deionized water, adding 0.76 mL of 98% formic acid, and diluting to 1 L. A 10 mM potassium dihydrogen phosphate solution (KH_2PO_4 , 1 L) was prepared by dissolving 1.36 g of potassium dihydrogen phosphate in deionized water, adjusting the pH to 2.5 with orthophosphoric acid, and making up to volume. A 0.1% (v/v) acetic acid solution was prepared by diluting 1 mL of glacial acetic acid with deionized water to a final volume of 1 L.

Preparation of Standard and Sample Solutions

The Methadone-D₃ stock solution (100 µg/mL) was prepared by diluting 0.1 mL of a 1 mg/mL standard solution with methanol to a final volume of 1 mL. The solution was filtered through a 0.45 µm syringe filter, transferred into an HPLC vial, and stored at 2-8 °C for up to 2-4 weeks. Methadone-D₃ was selected as the internal standard because its isotopically labeled structure closely resembles methadone while remaining chromatographically distinguishable under the tested conditions. Methadone syrup samples (2 mg/mL) were prepared by pipetting 20 µL of the sample and diluting with 80 µL of deionized water to obtain a final working concentration of approximately 40 µg/mL, yielding a working concentration of approximately 40 µg/mL for chromatographic evaluation. The methadone syrup used in this study was a hospital-prepared formulation obtained from Thanyarak Mae Hong Son Hospital by diluting methadone oral concentrate solution (10 mg/mL) with modified syrup to achieve the required concentration. The identical formulation was applied across all batches, and samples were stored at a controlled room temperature (25 °C) without accelerated stability evaluations.

Chromatographic Conditions

Analysis was performed using a SCION LC6000 HPLC system equipped with a diode array detector (DAD) and a COSMOSIL C18 column (4.6 mm I.D. × 250 mm). Chromatographic separation was conducted under isocratic conditions at a flow rate of 1.0 mL/min with an injection volume of 10 µL. The column oven temperature was maintained at 30 °C, and detection was performed at 205 nm. The five mobile phase systems were selected to represent different chromatographic environments commonly reported for methadone analysis, including variations in buffer composition, pH conditions, and organic modifiers. This approach was intended to systematically evaluate their effects on retention behavior, signal response, and peak quality in methadone syrup samples and to identify the most suitable chromatographic condition for routine pharmaceutical analysis (Table 1). Prior to use, all mobile phases were filtered through a 0.45 µm membrane filter and ultrasonically degassed [11].

Table 1. Mobile phase compositions investigated for the optimization of chromatographic conditions in methadone analysis

Condition	Composition
1	25 mM Sodium acetate : ACN (45:55, v/v)
2	20 mM Ammonium formate : ACN (13:87, v/v)
3	10 mM KH ₂ PO ₄ (pH 2.5) : ACN (60:40, v/v)
4	0.1% v/v Acetic acid : ACN (65:35, v/v)
5	0.1% v/v Acetic acid : ACN : MeOH (50:25:25, v/v/v)

*ACN = Acetonitrile

Analytical Procedure

Methadone-D₃ internal standard and methadone syrup sample solutions were filtered through a 0.45 µm syringe filter prior to injection. The HPLC system was equilibrated until a stable baseline was achieved. Samples were injected using an autosampler, and chromatograms were monitored for 5-10 minutes under optimized conditions.

Evaluation of Chromatographic Performance

The chromatographic conditions were systematically evaluated based on key analytical parameters, including retention time, peak area, and peak symmetry, as assessed by the asymmetry factor [16, 17].

RESULTS AND DISCUSSION

Comparison of Mobile Phase Conditions

Table 2 summarizes the chromatographic performance of methadone and the internal standard (Methadone-D₃) under five mobile phase conditions. Condition 1 yielded no detectable peaks for either analyte, rendering it entirely unsuitable for methadone analysis. Although Conditions 2 through 5 produced measurable responses, significant performance disparities were observed. Condition 2 provided optimal retention time of 6.05 ± 0.02 min for the internal standard and 6.13 ± 0.01 min for methadone. Both analytes exhibited consistent chromatographic performance with %RSD values below 1%, satisfying commonly accepted chromatographic criteria [16].

Table 2. Comparison of chromatographic performance under different mobile phase conditions

Condition	Analyte	Rt (min)	%RSD Rt	Area	%RSD Area	Height	%RSD Height
Condition 1	IS	ND	-	ND	-	ND	-
	Sample	ND	-	ND	-	ND	-
Condition 2	IS	6.05 ± 0.02	0.33	89392.6 ± 205.1	0.23	372703.6 ± 2997.9	0.80
	Sample	6.13 ± 0.01	0.16	73190.2 ± 1735.5	2.37	261390.7 ± 8868.7	3.39
Condition 3	IS	7.57 ± 0.27	3.57	53610.8 ± 784.7	1.46	180454.0 ± 5518.0	3.06
	Sample	7.58 ± 0.17	2.27	39836.7 ± 165.5	0.42	135572.9 ± 4466.2	3.29
Condition 4	IS	7.67 ± 0.44	5.79	51998.4 ± 1130.7	2.17	79044.3 ± 2767.6	3.50
	Sample	7.89 ± 0.43	5.44	33990.7 ± 1041.4	3.06	53557.3 ± 2715.3	5.07
Condition 5	IS	4.61 ± 0.10	2.19	44151.1 ± 228.6	0.52	154532.2 ± 1690.5	1.09
	Sample	4.73 ± 0.05	1.12	30660.4 ± 534.1	1.74	124077.3 ± 1864.2	1.50

* IS = Methadone-D₃ internal standard, ND = non-detectable

In contrast, Conditions 3 and 4 resulted in prolonged retention times (≈ 7.5 -7.9 min) coupled with increased variability, whereas Condition 5 yielded abbreviated retention times (≈ 4.6 -4.7 min). These shorter retention times may compromise chromatographic separation efficiency by reducing analyte resolution and peak separation [17]. In terms of chromatographic signal response, Condition 2 generated the largest peak area for the internal standard ($89,392.6 \pm 205.1 \mu\text{AU}\cdot\text{min}$), indicating stronger chromatographic signal intensity. Additionally, peak heights under Condition 2 were robust and highly consistent, with low %RSD values reflecting stable signal intensity. Together, the data in Table 2 demonstrate that Condition 2 offers the most favorable chromatographic performance providing a strong balance between retention time, signal response, and chromatographic consistency.

Evaluation of Peak Shape and Chromatographic Quality

Table 3 details the peak shape parameters, including the area-to-height ratio and peak asymmetry (As_{10}), evaluated under the various mobile phases. Condition 2 demonstrated favorable peak characteristics. The peak asymmetry (As_{10}) values were 1.45 ± 0.02 for the internal standard and 1.25 ± 0.02 for methadone; these values closely approximate

unity, indicating relatively symmetrical peak profiles free from significant fronting or tailing, which is generally acceptable for well-resolved peaks [18-21]. The low %RSD values for asymmetry ($< 2\%$) further highlight the consistent peak shape characteristics. The area-to-height ratios under Condition 2 were consistent, with 0.24 ± 0.00 for the internal standard and 0.28 ± 0.01 for methadone, both with low %RSD values. These results indicate stable peak geometry and acceptable chromatographic performance, consistent with previous HPLC methods for methadone analysis [1, 22]. In contrast, Conditions 3 and 4 exhibited elevated asymmetry values ($As_{10} > 2.5$), indicative of distorted peak geometries and underlying tailing effects. Condition 5 produced severe asymmetry for the internal standard ($As_{10} \approx 4.18$), indicating poor peak symmetry despite acceptable retention time and signal intensity. Overall, the combined data confirm that Condition 2 offers favorable peak shape and chromatographic quality, justifying its selection as the best condition for methadone analysis. Based on the combined assessment of chromatographic performance (Table 2) and peak quality parameters (Table 3), Condition 2 was selected as the most suitable mobile phase condition among the tested systems condition for subsequent analyses.

Table 3. Evaluation of peak shape and chromatographic quality

Condition	Analyte	Area/Height ratio	%RSD	Peak Asymmetry (As_{10})	%RSD
Condition 2	IS	0.24 ± 0.00	1.01	1.45 ± 0.02	1.43
	Sample	0.28 ± 0.01	2.68	1.25 ± 0.02	1.60
Condition 3	IS	0.30 ± 0.01	4.54	2.85 ± 0.09	3.00
	Sample	0.29 ± 0.01	3.05	2.51 ± 0.13	5.23
Condition 4	IS	0.66 ± 0.01	1.86	2.58 ± 0.04	1.57
	Sample	0.64 ± 0.05	8.29	2.25 ± 0.03	1.18
Condition 5	IS	0.29 ± 0.00	1.59	4.18 ± 0.12	2.80
	Sample	0.25 ± 0.01	2.30	2.98 ± 0.48	16.11

* IS = Methadone-D₃ internal standard, As_{10} = peak asymmetry at 10% height

Chromatographic Profiles and Practical Implications

Representative chromatograms obtained under Condition 2 (Figure 1) demonstrate well-resolved peaks for Methadone-D₃ and methadone at retention times of 6.05 ± 0.02 min and 6.13 ± 0.01 min, respectively, with no major observable interfering peaks under the tested condition [16, 23]. Although the retention times of methadone and Methadone-D₃ were relatively close, the chromatographic profiles

showed distinguishable peak positions without observable co-elution under the optimized condition. Therefore, no observable peak overlap was evident under the optimized chromatographic condition. The symmetrical peak profiles were consistent with the quantitative asymmetry data, supporting the consistency of the optimized chromatographic condition. In contrast, analyses conducted under suboptimal conditions revealed less favorable chromatographic characteristics (Figure 2).

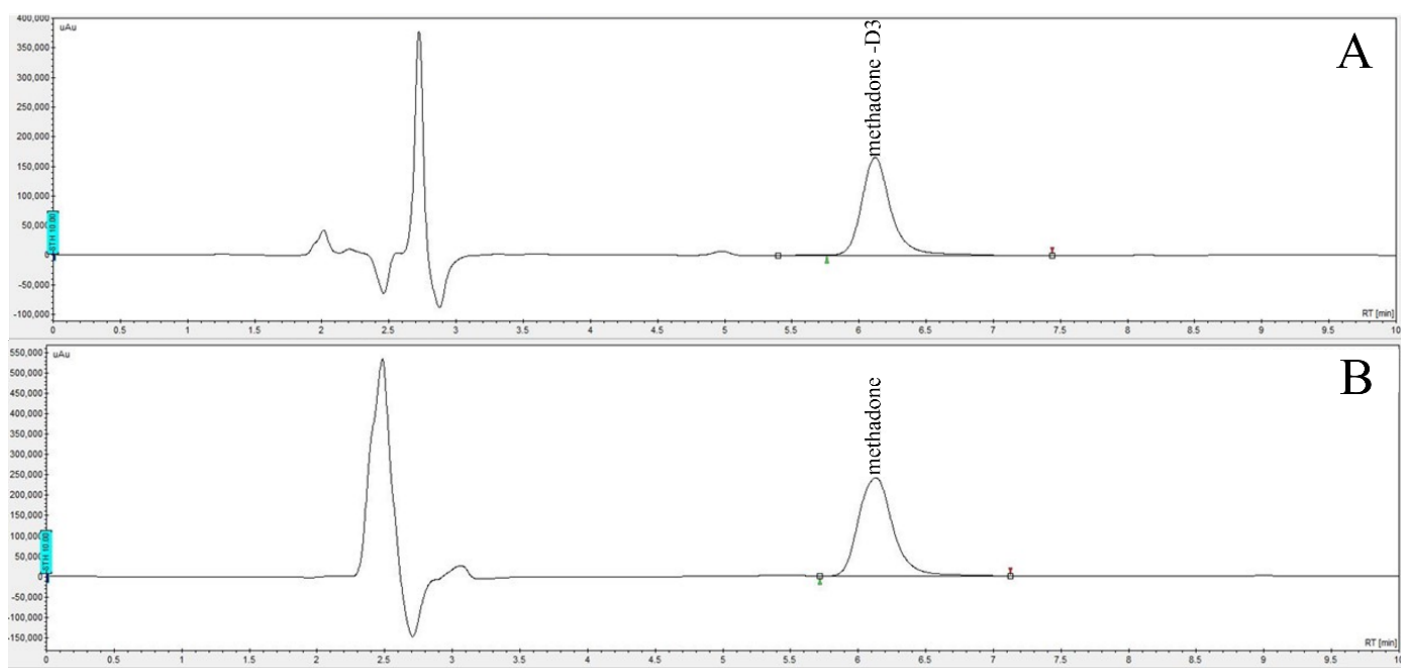


Figure 1. Representative chromatograms of methadone analysis under optimized conditions (Condition 2). A Chromatogram of the Methadone-D₃ internal standard (IS), and B chromatogram of the methadone syrup sample. Both analytes exhibited well-resolved peaks at approximately 6 min with no interfering signals.

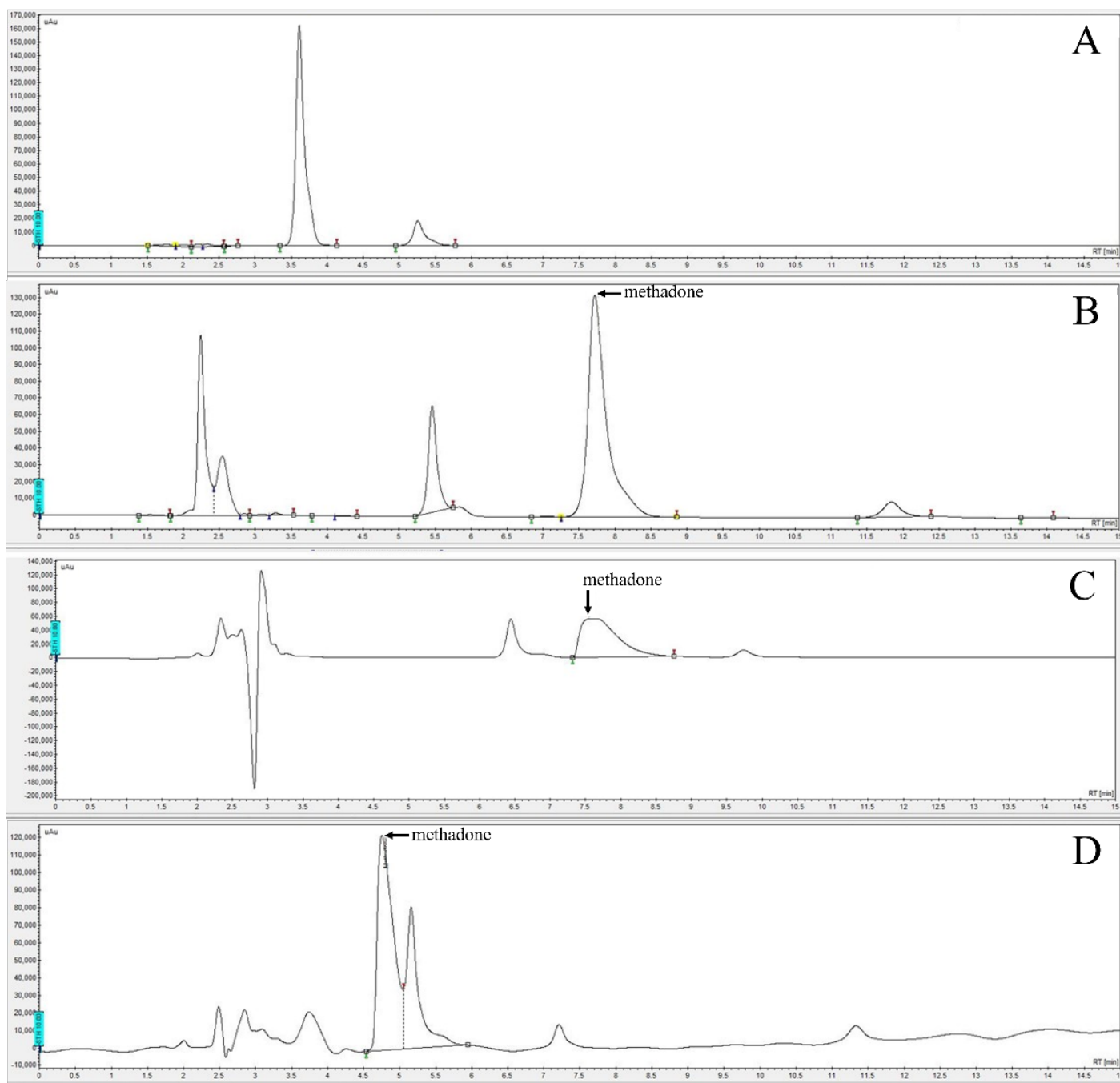


Figure 2. Representative chromatograms of methadone syrup samples under suboptimal mobile phase conditions. (A) Condition 1 showing no detectable methadone peak, (B) Condition 3 showing pronounced peak tailing, (C) Condition 4 showing distorted peak geometry, and (D) Condition 5 showing poor peak symmetry and increased baseline noise.

Figure 2 displays representative chromatograms of methadone samples analyzed under selected suboptimal conditions. Condition 1 failed entirely to elute or detect the analyte. Condition 3 yielded a detectable peak, but pronounced tailing and distortion compromised chromatographic efficiency, a phenomenon commonly associated with suboptimal

analyte-stationary phase interactions. Condition 5 similarly produced a markedly asymmetrical peak with increased baseline noise, which may affect chromatographic consistency. This visual evidence of peak distortion corroborates the quantitative asymmetry data ($As_{10} > 2$) presented in Table 3.

In summary, Condition 2 was confirmed as the optimal chromatographic method, distinguished by a clean retention time with no major observable interfering peaks, highly symmetrical peaks ($As_{10} \approx 1.25 - 1.45$) and low variability in signal response ($\%RSD < 3\%$), indicating a consistent chromatographic response. Practically, this optimized methodology provides a cost-effective and practical chromatographic approach, explicitly tailored for routine integration into hospital laboratory workflows. By employing standard reagents and a simplified sample preparation protocol, the method significantly minimizes operational complexity, supporting efficient workflow in resource-limited settings [1, 25]. The optimized retention time facilitates high sample throughput without compromising analytical integrity. Ultimately, this framework ensures routine quality control of locally compounded methadone, maintaining compliance with pharmaceutical standards and safeguarding patient health [16].

CONCLUSION

Based on our systematic evaluation, Condition 2 was identified as the most suitable mobile phase composition among the evaluated conditions for quantitative methadone determination. This selection is supported by an integrated assessment of chromatographic performance and peak quality parameters. The optimized method produced retention times of approximately 6.0 minutes, demonstrating acceptable chromatographic separation and a complete absence of co-eluting matrix interferences. Additionally, the analytical protocol exhibited low variability in chromatographic response, validated by low relative standard deviations ($\%RSD$) across retention times, peak areas, and peak heights. The system also produced symmetrical chromatographic profiles, with peak asymmetry factors (As_{10}) closely approximating unity (1.45 for the internal standard and 1.25 for methadone), ensuring acceptable peak geometry. Analytically, Condition 2 maximized detector response, providing the strongest chromatographic signal among the evaluated conditions. Consequently, the optimized HPLC framework provides a practical and suitable chromatographic approach for the routine quality assurance of locally compounded

methadone formulations. Nevertheless, this study primarily focused on chromatographic condition optimization and preliminary analytical performance. Full analytical method validation, including specificity evaluation using placebo analysis, accuracy, linearity, robustness, inter-day and intra-day precision, as well as LOD and LOQ determination, should be conducted in future studies prior to routine clinical implementation.

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