Jurnal Farmasi Indonesia: Volume 17 Nomor 2 (2025)
https://doi.org/10.35617/jfionline.v17i2.498

Validated RP-HPLC-PDA with Buffer-Free Gradient Elution for Simultaneous Plasma Analysis of Trimethoprim and Sulfamethoxazole

Nicholas Edmund[1], Untung Gunawan[1], Dion Notario[1]

Artikel Penelitian

Abstract: *A simple, cost-effective, and validated reversed-phase high-performance liquid chromatography (RP-HPLC) method is crucial for accurately monitoring trimethoprim and sulfamethoxazole therapy. However, conventional HPLC methods often rely on buffered mobile phases and additives, which can lead to column blockage and shorten column lifespan. To address this issue, we developed and validated a reversed-phase HPLC method with a photodiode array (PDA) detector, using a buffer-free and additive-free mobile phase with gradient elution. Sample preparation was performed using organic solvent-based deproteinization to minimize matrix effects. Chromatographic separation was performed on a ZORBAX Eclipse Plus C18 column (250 × 4.6 mm, 5 µm) using a mobile phase of acetic acid (pH 2.3), methanol, and acetonitrile at a flow rate of 1 mL/min. Detection was carried out at 270 nm with a total run time of 17 minutes. The analytical method exhibited linearity, precision, and accuracy, with adequate sensitivity for the analytes of interest. Stability testing yielded favorable results under various storage conditions, including the autosampler, freeze-thaw cycles, and long-term storage. However, the trimethoprim stock solution was only stable for two days and must be freshly prepared. Full validation was conducted in accordance with Food and Drug Administration (FDA) and International Conference on Harmonization (ICH) bioanalytical guidelines, confirming its suitability for clinical application.*

Keywords: *Bioanalysis, HPLC, Sulfamethoxazole, Trimethoprim, Validation*

Abstrak: Sebuah metode kromatografi cair kinerja tinggi fase terbalik (KCKT-FT) yang sederhana, hemat biaya, dan tervalidasi sangat penting untuk memantau terapi trimetoprim dan sulfametoksazol secara akurat. Namun, metode HPLC konvensional sering kali bergantung pada fase gerak yang menggunakan buffer dan aditif, yang dapat menyebabkan penyumbatan kolom dan memperpendek umur kolom. Untuk mengatasi masalah ini, kami mengembangkan dan memvalidasi metode HPLC fase terbalik dengan detektor *photodiode array* (PDA), menggunakan fase gerak tanpa buffer dan tanpa aditif dengan elusi gradien. Preparasi sampel dilakukan dengan metode deproteinasi berbasis pelarut organik untuk meminimalkan efek matriks. Pemisahan kromatografik dilakukan menggunakan kolom ZORBAX Eclipse Plus C18 (250 × 4,6 mm, 5 µm) dengan fase gerak berupa asam asetat (pH 2,3), metanol, dan asetonitril pada laju alir 1 mL/menit. Deteksi dilakukan pada panjang gelombang 270 nm dengan waktu analisis total selama 17 menit. Metode analisis ini menunjukkan linearitas, presisi, dan akurasi yang baik, serta sensitivitas yang memadai untuk analit yang dituju. Pengujian stabilitas menunjukkan hasil yang baik pada berbagai kondisi penyimpanan, termasuk dalam *autosampler*, siklus pembekuan-pencairan, dan penyimpanan jangka panjang. Namun, larutan stok trimetoprim hanya stabil selama dua hari sehingga harus dibuat segar. Validasi penuh dilakukan sesuai dengan pedoman bioanalitik FDA dan ICH, yang mengonfirmasi kesesuaiannya untuk aplikasi klinis.

Kata kunci: Bioanalisis, KCKT, Sulfametoksazol, Trimetoprim, Validasi

[1] Department of Pharmacy, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, 14440, Indonesia

Korespondensi:

Dion Notario
dion.notario@atmajaya.ac.id

https://doi.org/10.35617/jfionline.v17i2.498

Introduction

A urinary tract infection (UTI) is a condition characterized by the presence of an infection in any part of the urinary system, including the kidneys, ureters, bladder, and urethra (1). Urinary tract infections (UTIs) are among the most common infections worldwide, placing considerable pressure on public health systems. In the United States, the incidence of urinary tract infections is markedly high, with over 250,000 new cases recorded annually. The prevalence of UTI is considerably higher in women than in men, mostly due to anatomical and physiological traits that increase vulnerability (2). Besides their commonality in Western nations, UTIs pose a significant health concern in other areas, including Southeast Asia. According to data from the Indonesian Ministry of Health in 2014, the prevalence of UTI in Indonesia varies from 5% to 15%. The yearly incidence rate in the country is anticipated to range from 90 to 100 cases per 100,000 inhabitants, leading to around 180,000 new cases annually. These statistics emphasize the worldwide and regional public health importance of UTIs, underscoring the necessity for effective preventive and treatment initiatives (3).

A broad range of antibiotic medications exists for the treatment of UTI, with trimethoprim-sulfamethoxazole being one of the most frequently prescribed combinations due to its efficacy against many bacterial pathogens responsible for these infections (4). This combination treatment is extensively used in several countries, emphasising its significance in therapeutic practice. A study by Andrajati et al. revealed that the prescription frequency of trimethoprim-sulfamethoxazole in Depok, Indonesia, was 17.4%, signifying its considerable application in the treatment of UTIs in that region (5). In Norway, a study by Skow et al. revealed a notable prescription rate for trimethoprim-sulfamethoxazole, especially among individuals aged 16 and older. From 2008 to 2018, the prescription rate in this group attained 32.7%, highlighting its significance as a therapy option. The study saw a constant upward trend in the prescription of trimethoprim-sulfamethoxazole over this decade, indicating a growing dependence on this antibiotic over time (6). The prolonged utilization of antibiotics raises important questions regarding the prospective implications for antibiotic resistance, in the context of increasing global concerns about their overuse and misuse. The persistently increased prescription rates of trimethoprim-sulfamethoxazole in both Indonesia and Norway underscore its enduring significance in UTIs treatment, in addition to highlighting the necessity for continual monitoring in its prescription to avoid adverse public health consequences. Moreover, from 2008 to 2018, there was a rising trend in the prescription of trimethoprim-sulfamethoxazole.

The accurate determination of trimethoprim-sulfamethoxazole concentrations is essential not only for patients with UTI but also for numerous other medical problems. This includes its application in preventative therapy and the refinement of dose regimens, which are crucial for achieving optimal therapeutic outcomes. Trimethoprim-sulfamethoxazole has been widely utilised in preventative treatments, especially for illnesses like HIV/AIDS. This combination therapy's efficacy in preventing infections among patients with HIV/AIDS was assessed in a study by Gebresillassie et al. at Gondar University Referral Hospital in Northwestern Ethiopia (7). While this was going on, a study was conducted to determine the amounts of trimethoprim-sulfamethoxazole in the bronchial sputum of patients with cystic fibrosis. The purpose of this investigation was to determine how the medicine was distributed throughout the lungs (7). Both studies highlight the broader clinical relevance of trimethoprim-sulfamethoxazole beyond its use in UTIs, underscoring the importance of monitoring its concentration in different biological matrices for careful monitoring and adjustment based on specific patient conditions.

Various analytical techniques have been established to determine the concentration of trimethoprim-sulfamethoxazole, with High Performance Liquid Chromatography (HPLC) being one of the most frequently employed methods. Lopez et al. conducted a study using HPLC for analysing trimethoprim-sulfamethoxazole in plasma samples, implementing protein precipitation with perchloric acid and isocratic elution for 10

https://doi.org/10.35617/jfionline.v17i2.498

minutes. The mobile phase for this procedure comprised a mixture of 0.1 M phosphate buffer, acetonitrile, and methanol in a volume ratio of 65:20:15 (v/v/v) (8). Amini et al. performed a different study employing a comparable methodology, utilising perchloric acid for sample preparation, succeeded by isocratic elution for a duration of 10 minutes. The mobile phase consisted of a solution comprising 50 mM sodium dihydrogen phosphate, acetonitrile, and triethylamine in a volumetric ratio of 100:25:0.5 (v/v/v) (9). However, certain drawbacks have been associated with methods that utilize phosphate buffer or triethylamine solutions. Specifically, these methods have been reported to cause column blockage and a reduction in column longevity, which can significantly impact the reliability of results over time (10).

In order to solve these concerns, integrating deproteinizing agent from the organic solvent category alongside additive-free and buffer-free mobile phases has been proposed as an alternate strategy. Moreover, gradient elution has demonstrated efficacy in enhancing the separation of analytes in a reduced analysis duration (11). Considering these factors, the current study aimed to establish a streamlined and dependable HPLC method for quantifying trimethoprim-sulfamethoxazole in human plasma. This technique employs acetonitrile for deproteinization and a buffer-free mobile phase, seeking to address the limitations of conventional methods while ensuring precise and efficient quantification of trimethoprim-sulfamethoxazole in biological samples.

Material and Methods

Chemicals

This study employed blood plasma, which was generously provided by the Indonesian Red Cross. Moreover, several types of analytical standards and solvents were utilized, all sourced from Merck. The study specifically included trimethoprim and sulfamethoxazole with a purity of at least 99%. Additionally, high-purity solvents including methanol (HPLC grade), acetonitrile (HPLC grade), and glacial acetic acid were employed in the experimental protocols. The selected reagents ensured the precision, dependability, and uniformity of the study's findings, as they meet the stringent quality standards for analytical and chromatographic techniques.

Methods

Instrumentation and Conditions

The chromatographic equipment utilized in this work comprised a Shimadzu LC-20AD HPLC, equipped with a ZORBAX Eclipse Plus C18 column (250 mm × 4.6 mm, 5 µm particle size). This configuration includes a SIL-20ACHT autosampler, a CTO-20AC column oven, and an SPD-M20A photodiode array (PDA) detector functioning at 230 V. The mobile phase consisted of a carefully prepared mixture of acetic acid solution (pH 2.3), methanol, and acetonitrile. A constant flow rate of 1 mL/min was maintained to ensure accurate and reproducible chromatographic conditions. Analytes were detected at 270 nm, with separation carried out at 35 °C. Each run involved a 20 µL injection and lasted 17 minutes.

Gradient elution method optimization

The mobile phase system was optimized through experiments using various solvent mixtures and a gradient elution strategy, as detailed in Table 1. The study was conducted at the lower limit of quantification (LLOQ) by spiking blank plasma samples with known concentrations of trimethoprim and sulfamethoxazole. The chromatographic run time was carefully optimized to ensure clear peak separation of both analytes, eliminate interference from plasma matrix components, and achieve symmetrical peak shapes. The optimization aimed to balance efficient analyte separation, reduced analysis time, and well-shaped, symmetrical peaks.

https://doi.org/10.35617/jfionline.v17i2.498

Table 1. Gradient elution method development for Trimethoprim and Sulfamethoxazole analysis

Method	Time of Analysis (min)	Mobile Phase
1	0 – 6	C = 10% ; D = 90%
	6 – 8	B = 25% ; C = 5% ; D = 70%
	8 – 12	B = 25% ; C = 5% ; D = 70%
	12 – 15	C = 10% ; D = 90%
	15 – 18	C = 10% ; D = 90%
2	0 – 8	C = 8% ; D = 92%
	8 – 10	B = 25% ; C = 5% ; D = 70%
	10 – 12	B = 25% ; C = 5% ; D = 70%
	12 – 15	C = 8% ; D = 92%
	15 – 18	C = 8% ; D = 92%
3	0 – 10	C = 5% ; D = 95%
	10 – 11	B = 25% ; C = 5% ; D = 70%
	11 – 14	B = 25% ; C = 5% ; D = 70%
	14 – 15	C = 5% ; D = 95%
	15 – 23	C = 5% ; D = 95%

B = Methanol; C = Acetonitrile; D = Acetic Acid pH 2.3. Method development was done in order from method 1 to method 3.

Preparation of standard stock solutions, calibration standards, and quality control (QC) samples

A primary stock solution of trimethoprim (20 µg/mL) and sulfamethoxazole (500 µg/mL) was prepared by dissolving accurately quantified volumes of each analyte standard in methanol. This solvent was selected for its efficacy in dissolving the chemicals, thereby ensuring precise concentrations for subsequent analyses. Working standard solutions were produced by precisely diluting the primary stock solutions in methanol, resulting in trimethoprim concentrations of 14, 12, 10, 5, and 3 µg/mL, and sulfamethoxazole concentrations of 100, 50, 20, 10, and 5 µg/mL.

Calibration standards were established by incorporating a specific volume of 25 µL from each working standard solution into 200 µL of human plasma. This procedure produced six distinct levels of concentration for both trimethoprim and sulfamethoxazole. The concentrations for trimethoprim were established at 1.4, 1.2, 1.0, 0.5, and 0.3 µg/mL, whereas sulfamethoxazole were set at 10, 5, 2, 1, and 0.5 µg/mL. The selected concentration levels encompass the range regularly observed in biological samples, ensuring an extensive dynamic range for excellent calibration.

Alongside the calibration standards, QC samples were independently established to confirm the method's accuracy and precision across various concentration ranges. These QC samples were formulated in human plasma and incorporated into both inter and intra-run experiments. Three distinct QC levels were established: LLOQ (trimethoprim 0.3 µg/mL, sulfamethoxazole 0.5 µg/mL); low QC (trimethoprim 0.9 µg/mL, sulfamethoxazole 1.5 µg/mL); mid QC (trimethoprim 1.1 µg/mL, sulfamethoxazole 4 µg/mL); and high QC (trimethoprim 1.5 µg/mL, sulfamethoxazole 35 µg/mL).

Sample Preparation

A 250 µL plasma sample was treated with 500 µL of acetonitrile in an Eppendorf tube for protein precipitation. The mixture was vortexed for 30 seconds to ensure complete mixing and effective protein removal. It was then centrifuged at 10,000 rpm for 10 minutes to separate the supernatant from the precipitated proteins. The resulting supernatant was carefully transferred and evaporated in a water bath at 80°C. The dried residue was reconstituted with 300 µL of a solution of acetonitrile and acetic acid (pH 2.3, 5:95, v/v), filtered through a 0.22 µm nylon filter, and stored in an HPLC vial at 4°C until injection into the HPLC system.

System Suitability Test

A system suitability test was conducted to ensure that key chromatographic parameters met the specifications set by regulatory standards, particularly those established by the FDA (12). The test verified that parameters such as retention factor, injection repeatability, resolution, tailing factor, and the number of theoretical plates complied with FDA guidelines. Each of these parameters plays a critical role in assessing the effectiveness and reliability of the chromatographic system. To ensure the accuracy and consistency of the method at the LLOQ, the test was performed using five replicate samples at this concentration level, guaranteeing the reliability of the results.

Bioanalytical Method Validation

The method was validated in accordance with the U.S. FDA's *Bioanalytical Method Validation Guidance for Industry* and the ICH M10 Guideline on Bioanalytical Method Validation, ensuring compliance with internationally recognized regulatory standards (13,14). The investigated parameters were linearity, selectivity, carryover, sensitivity, accuracy, precision, and stability.

Linearity

The linearity of the analytical method was assessed by examining calibration curve samples in triplicate to guarantee reliable and consistent outcomes. The peak areas corresponding to the analytes, as shown in the chromatograms, were plotted against their respective concentrations in the samples and analyzed using weighted linear regression in R version 4.4.1. (15). The method's linearity was determined to be acceptable if the concentrations of the calibrator samples ranged within ±15% of their nominal values for a minimum of 50% of the tested samples. This criterion was implemented for all calibrator concentrations, except the LLOQ, where a deviation of up to ±20% from the nominal concentration was considered acceptable.

Selectivity

Selectivity was evaluated by examining six blank samples, generated without the target analytes. The main aim of this evaluation was to ascertain whether any signals or interferences from components in the blank samples aligned with the retention time of the analytes. The method was accepted as selective if no detectable analyte peaks or interferences appeared at the retention intervals of the analytes in the blank samples.

Carryover

Carryover was assessed to ascertain if residual analyte from a prior sample would contaminate subsequent analyses. A blank sample was injected immediately following a sample at the upper limit of quantification (ULOQ), denoting the highest concentration on the calibration curve. The extent of carryover was assessed by comparing the analyte signal in the blank sample with the signal measured at the LLOQ. The criterion for carryover acceptance specifies that the analyte signal in the blank sample must not exceed 20% of the LLOQ.

Sensitivity

Sensitivity was assessed through the examination of five replicates of samples prepared at the LLOQ, denoting the lowest concentration of the analyte that can be consistently detected and quantified by the analytical method. The main aim of this evaluation was to ascertain the method's capability to reliably and precisely quantify the analyte at extremely low concentrations. The acceptance requirements for sensitivity were twofold: first, the accuracy of the measured concentrations must be within ±20% of the nominal concentration, as to guarantee that the results approximate the actual amount. The precision of the measurements was assessed by determining the relative standard deviation (RSD), with an acceptable limit established at ±20%.

Accuracy and Precision

The accuracy and precision were systematically evaluated using the examination of QC samples, specifically intended to examine both within-run and between-run variability. This methodology facilitated a thorough evaluation of the technique's performance in different measurement periods. The method's accuracy was assessed by comparing the measured concentrations of the QC samples to their established nominal concentrations. The accuracy

https://doi.org/10.35617/jfionline.v17i2.498

acceptance criterion defined that the measured concentrations must fall within ±15% of the nominal value for both intra and inter-run studies. At the LLOQ, a wider tolerance of ±20% was considered acceptable, recognizing challenges associated with measuring small quantities. Accompanying accuracy, precision was assessed by determining the RSD for each batch of QC samples, indicating the extent of variation in repeated measurements of the similar sample. In both within-run and between-run analyses, precision considered acceptable if the RSD did not surpass ±15%, except for the LLOQ, when a 20% deviation is tolerable.

Stability

Stability testing was performed to evaluate the integrity and reliability of the analytical method under various conditions. The tests were conducted at low and high QC levels to assess performance across different concentration ranges. Several stability conditions were investigated, including autosampler storage, freeze-thaw cycles, short-term storage of stock solutions at 4°C, and long-term storage at -20°C. Freeze-thaw stability was assessed by subjecting samples to three cycles between -20°C and 25°C, simulating standard handling. Stock solution stability at 4°C was tested for short-term storage, while long-term stability was evaluated by storing samples at -20°C for extended periods. Each stability test was performed in triplicate to ensure reliability, with precision required to stay within ±15% of the nominal concentration.

Results and Discussion

Gradient elution method optimization

The primary objective of chromatography is to achieve efficient separation within a short time, producing peaks that meet analytical quality standards. In the gradient elution profile of Method 1, both trimethoprim and sulfamethoxazole were well separated, and no interference from the plasma matrix was observed, as shown in Figure 1A. However, the trimethoprim peak exhibited significant tailing, making this method suboptimal. To resolve this issue, the gradient profile was modified in Method 2. Although tailing was reduced, the trimethoprim peak overlapped with a matrix-related peak, as illustrated in Figure 1B. Further optimization in Method 3 resulted in well-resolved, symmetric peaks for both analytes without matrix interference, as seen in Figure 1C. Based on these results, Method 3 was selected as the most suitable elution strategy for subsequent sample analysis.

Optimizing the mobile phase composition was critical for effectively separating trimethoprim and sulfamethoxazole, two analytes with markedly different polarities. An acidic mobile phase at pH 2.3 was selected to enhance separation efficiency. Under these conditions, trimethoprim, a basic compound, becomes positively ionized and is less retained on the C18 stationary phase. In contrast, sulfamethoxazole remains largely in its non-ionized, resulting in longer retention in the column. Adjusting the pH to 2.3 also helped suppress the ionization of residual silanol groups on the column surface (16), thereby reducing peak tailing for basic compounds. However, the pH was not set below 2.0 to avoid potential hydrolysis of the C18 bonded phase. This pH adjustment contributed to improved peak symmetry and more reliable quantification, essential for robust bioanalytical performance.

We successfully optimized the mobile phase using an approach based on solvent selectivity and strength. Solvent selectivity was improved by combining a proton donor solvent, methanol, with a proton acceptor solvent, acetonitrile (17). Solvent strength optimization was achieved through gradient elution. To ensure early elution of polar compounds like metabolites and ionized trimethoprim, a weak mobile phase with a low concentration of organic solvent was employed at the start of the run. Later in the run, the concentration of organic solvent was slowly raised to make the elution stronger. This helped elute more nonpolar compounds, such as unionized sulfamethoxazole. The gradient profile was guided by prior laboratory studies (18,19) that produced several elution models—Methods 1, 2, and 3 (Table 1)—with Method 3 ultimately demonstrating the most effective separation and thus selected for the final method and further validated.

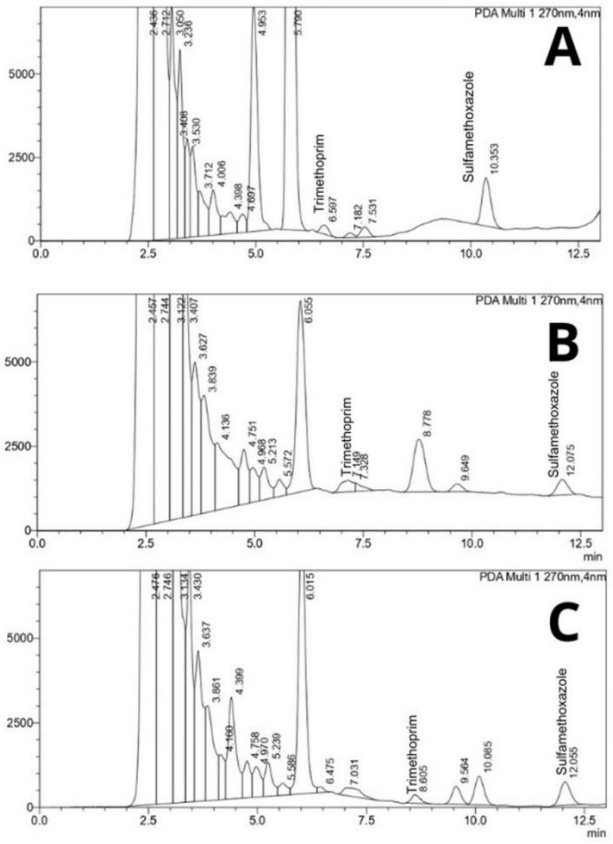

Figure 1. Chromatogram of spiked plasma samples with different mobile phase composition. (A) method 1, (B) method 2, (C) method 3. Mobile phase consists of methanol, acetonitrile, and acetic acid (pH 2.3) with gradient composition as shown in Table 1.

System Suitability

System suitability results are summarized in Table 2. The retention times were 8.6 minutes for trimethoprim and 12.1 minutes for sulfamethoxazole, reflecting their distinct interactions with the stationary phase. The method demonstrated high precision, with RSD values of 0.3% and 0.04%, respectively. Column efficiency, measured by theoretical plates, was 7,800 for trimethoprim and 11,136.2 for sulfamethoxazole, indicating excellent separation performance, particularly for the latter. Capacity factors were 2.5 and 3.9, suggesting stronger column interaction for sulfamethoxazole. Resolutions of 4.1 and 4.5 confirmed effective peak separation, while tailing factors of 1.3 and 1.1 showed good peak symmetry, with sulfamethoxazole exhibiting a slightly more ideal shape. The results support the general appropriateness and dependability of the chromatographic method for detecting trimethoprim and sulfamethoxazole (12,20,21).

Table 2. System suitability test

Parameter	Requirement	Trimethoprim (n = 5)		Sulfamethoxazole (n = 5)	
		$\bar{x}$	RSD (%)	$\bar{x}$	RSD (%)
Retention Time (min)	RSD ≤ 1%	8.63	0.26	12.06	0.04
Theoretical Plate	> 2000	7800	6.58	11136	2.03
Capacity Factor	> 2	2.49	0.48	3.88	0.39
Resolution	> 2	4.11	45.25	4.48	1.93
Tailing Factor	≤ 2	1.26	13.04	1.11	1.09

https://doi.org/10.35617/jfionline.v17i2.498

Method Validation

Linearity

The calibration curve equations for trimethoprim and sulfamethoxazole were derived from linear regression using a weighting factor of 1/x to address heteroscedasticity (22). For trimethoprim, the equation was y = 15,137.6x – 1,186.9 (R^2 = 0.95), and for sulfamethoxazole, it was y = 23,571.6x + 3,203.3 (R^2 = 0.98). The percentage error for all points on the standard curves of trimethoprim and sulfamethoxazole remained within the acceptable range of ±15%. The RSD values for sulfamethoxazole were also within ±15% across all concentrations. For trimethoprim, RSD values were within limits for most concentrations, except at 2 µg/mL, where it slightly exceeded the threshold at 16.53% (Table 3). Nonetheless, five out of six trimethoprim calibration points, or 83%, met the FDA criteria, which exceeds the minimum requirement that at least 50% of the calibration points must comply, indicating that the calibration curve remains acceptable (13,14).

Table 3. Evaluation of calibration curves for Trimethoprim and Sulfamethoxazole (n = 5)

Analyte	Concentration (µg/mL)	Error (%)	RSD (%)
Trimethoprim	0.3	8.67	9.76
	0.5	-4.78	6.21
	1	-12.61	9.05
	1.2	-2.86	14.67
	1.4	4.57	15.13
	2	1.29	16.53
Sulfamethoxazole	0.5	13.94	9.44
	1	-8.26	14.14
	2	1.67	3.75
	5	-11.06	5.95
	10	3.31	13.55
	50	0.40	14.10

Selectivity

Selectivity was evaluated by comparing chromatograms of a blank sample with a QC sample. As shown in Figure 2, no interfering peaks were observed at the retention times of trimethoprim and sulfamethoxazole, confirming the method's ability to distinguish these analytes from matrix components. These results demonstrate that the method meets selectivity requirements and ensures accurate identification and quantification in plasma.

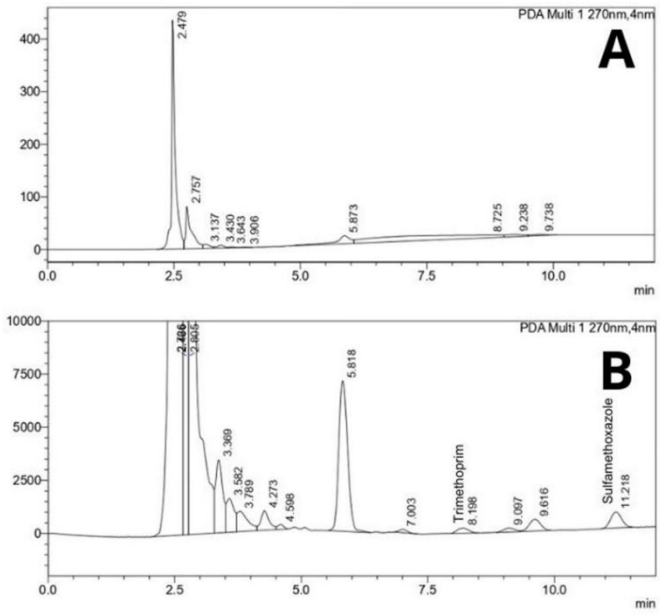

Figure 2. Comparison between blank sample chromatogram (A) and spiked sample chromatogram at LLOQ (B)

https://doi.org/10.35617/jfionline.v17i2.498

The method was designed for single-drug quantification under controlled conditions. However, a limitation of the current method is the absence of selectivity testing against potential metabolites and co-administered medications that may be encountered in clinical practice. To address this, further studies are planned to evaluate potential interferences from commonly used drugs and known metabolites using both incurred and spiked samples. These additional investigations will strengthen the method's robustness and support its future application in more complex clinical scenarios involving multiple drugs.

Carryover

Carryover in bioanalysis implies the inadvertent transfer of analytes or interfering chemicals from one sample to another during an analytical process, resulting in contamination and possible inaccuracies in the results. This is a significant concern in bioanalytical methodologies, particularly in assays such as HPLC, LC-MS, or other chromatographic procedures, where precise identification of trace analytes is essential. Carryover may provide erroneous results by transferring residual quantities of the analyte from prior injections into the subsequent assessments, so misrepresenting their actual concentrations (23,24).

Figure 3 demonstrates the chromatogram outcome derived from the blank sample after an injection at the ULOQ. Analysis revealed no carryover from the preceding injection in the blank sample, demonstrating that the system successfully removed any residual analyte from the prior injection. The lack of carryover contamination in the chromatogram of the blank sample following ULOQ injection verifies that the analytical equipment operates with sufficient precision and sensitivity. The results indicate that the procedure adheres to the specified standards for carryover, ensuring that the measurements of following samples remain unaffected by residual analyte from prior injections. Thus, the analytical process is considered sufficiently robust to comply with the requisite requirements for carryover assessment (25).

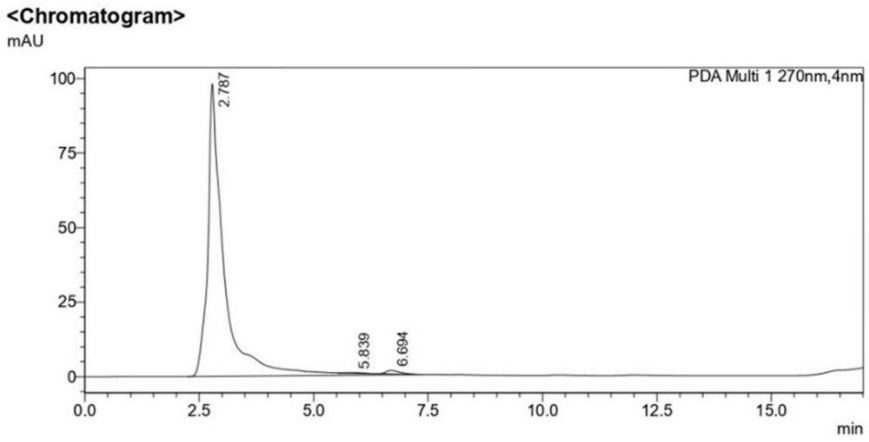

Figure 3. Chromatogram of blank after QC sample injection at ULOQ

Sensitivity

Method sensitivity was evaluated at the LLOQ using five replicates for each analyte. The LLOQ was 0.3 µg/mL for trimethoprim and 0.5 µg/mL for sulfamethoxazole. Accuracy and precision assessments yielded percent errors of 15.9% and -16.4%, and RSDs of 6.03% and 13% for trimethoprim and sulfamethoxazole, respectively. Although the percentage errors slightly deviated from zero, all values remained within acceptable ranges. These findings confirm the method's adequate sensitivity and reliability in quantifying low analyte concentrations in plasma.

https://doi.org/10.35617/jfionline.v17i2.498

Accuracy and Precision

Accuracy and precision of the method were evaluated using QC samples for both intra- and inter-assay performance. As shown in Table 4, the mean percent error and RSD for trimethoprim and sulfamethoxazole were within acceptable limits, confirming that the method provides accurate and reproducible results suitable for routine quantitative analysis.

Table 4. Accuracy and precision of Trimethoprim and Sulfamethoxazole

Analyte	Concentration (µg/mL)	Day of Analysis	Intra-Run (n = 5)		Inter-Run (n = 15)	
			Error (%)	RSD (%)	Error (%)	RSD (%)
Trimethoprim	0.3	Day 1	9.46	4.61	15.50	8.23
		Day 2	19.25	10.80		
		Day 3	17.77	6.34		
	0.9	Day 1	4.10	1.96	-1.36	9.44
		Day 2	2.87	8.70		
		Day 3	-11.04	7.30		
	1.1	Day 1	5.20	2.80	2.97	4.45
		Day 2	1.35	5.70		
		Day 3	2.36	4.52		
	1.5	Day 1	0.52	2.89	4.49	5.50
		Day 2	6.94	7.35		
		Day 3	6.02	3.71		
Sulfamethoxazole	0.5	Day 1	-10.89	3.55	-10.24	9.05
		Day 2	-15.40	12.06		
		Day 3	-4.44	6.71		
	1.5	Day 1	-8.74	3.85	-10.82	7.96
		Day 2	-10.29	9.74		
		Day 3	-13.43	9.91		
	4	Day 1	-5.90	4.40	-3.96	7.94
		Day 2	-9.24	7.26		
		Day 3	3.27	5.98		
	35	Day 1	4.71	3.60	6.35	7.34
		Day 2	5.68	12.09		
		Day 3	8.67	4.61		

Stability

The stability of trimethoprim and sulfamethoxazole was assessed under various conditions, including autosampler, freeze-thaw, long-term storage, and stock solution stability. As summarized in Table 5, most stability results met the required criteria, with sulfamethoxazole showing satisfactory long-term stability.

However, trimethoprim did not meet stability criteria for long-term storage and stock solutions, indicating potential degradation over time (Table 5 and Figure 4). Therefore, we recommend that trimethoprim stock solutions be freshly prepared prior to use. These findings highlight the importance of considering analyte stability for reliable results.

https://doi.org/10.35617/jfionline.v17i2.498

Table 5. Stability test of plasma spiked with Trimethoprim and Sulfamethoxazole standards

Analyte	Stability Test	Error Low QC (%)	RSD (%)	Error High QC (%)	RSD (%)
Trimethoprim	Autosampler (24 hours)	0.82	6.93	-3.84	16.24
	Freeze-thaw (3 cycle)	-12.67	2.30	3.17	20.80
	Long Term	-17.60	6.75	-17.90	3.24
Sulfamethoxazole	Autosampler (24 hours)	3.91	6.80	4.03	9.12
	Freeze-thaw (3 cycle)	-9.45	10.70	-13.54	12.57
	Long Term	-9.23	7.35	-4.62	3.37

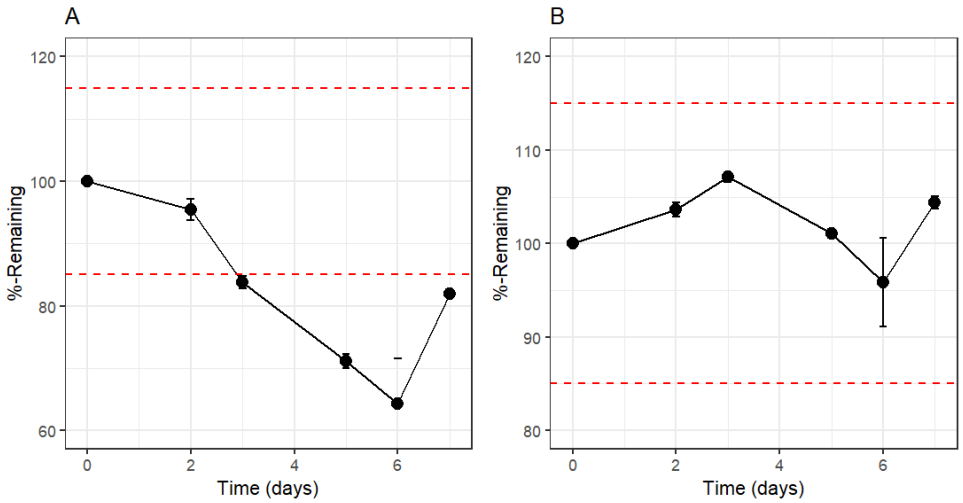

Figure 4. Stock solution stability of Trimethoprim (A) and Sulfamethoxazole (B). The red dashed line represents the acceptance limit.

Compared with previously published methods, our RP-HPLC approach offers both advantages and limitations. While it has a slightly longer separation time than the method reported by Amini et al and López et al., (8,9) it avoids the use of buffer components in the mobile phase, which reduces the risk of column clogging. Additionally, our method has been fully validated in accordance with both FDA and ICH guidelines, ensuring its reliability and regulatory compliance, whereas the method developed by Lopez et al. was not validated according to either of these guidelines (8,13,14). The method developed by Nong et al. offers better sample clean-up efficiency; however, it is more costly and time-consuming due to the use of two-dimensional chromatography involving both an extraction and an analytical column (26). In contrast, the method presented in this study is more practical and economical for routine analysis.

The goal of developing this method was to cover the expected pharmacokinetic ranges of trimethoprim and sulfamethoxazole, even though it hasn't been applied to clinical samples yet. Following a single oral dose of 160 mg trimethoprim and 800 mg sulfamethoxazole in adults, the typical Cmax values are approximately 1.4 µg/mL for trimethoprim and 40 µg/mL for sulfamethoxazole, while the Cmin values are around 0.4 µg/mL and 10 µg/mL, respectively (27). The method demonstrated acceptable linearity, accuracy, and precision, meeting established validation criteria across the relevant pharmacokinetic range. These findings support the method's potential for future application in pharmacokinetic studies or routine therapeutic drug monitoring.

Conclusions

The development of a simplified and efficient HPLC method for quantifying trimethoprim-sulfamethoxazole in human plasma signifies a considerable improvement for pharmaceutical analysis. This method minimizes the drawbacks of conventional analytical techniques, especially those related to phosphate buffer and triethylamine solutions, which may result in column obstruction and diminished lifetime. The approach enhances efficiency and accuracy of analysis by employing acetonitrile for deproteinization and a buffer-free mobile phase. The method has undergone thorough validation for essential analytical performance metrics, encompassing selectivity, linearity, sensitivity, precision, and accuracy. The linearity of the calibration curves demonstrates a robust link between analyte concentration and peak area, hence enabling dependable quantification across a broad concentration spectrum. The sensitivity tests indicated that the approach can identify low amounts of both drugs, demonstrating its suitability for bioanalysis at low concentration ranges. Moreover, evaluations of accuracy and precision demonstrated that the method yields consistent results, both intra-run and inter-run, according to regulatory norms. Stability studies indicated that sulfamethoxazole demonstrates excellent long-term stability, whereas trimethoprim exhibited degradation during prolonged storage durations. This discovery underscores the necessity of evaluating the stability of pharmaceutical substances during bioanalytical assessments, especially in long-term investigations or clinical environments where accurate drug monitoring is essential. This study presents a reliable, precise, and efficient approach for measuring trimethoprim-sulfamethoxazole concentrations in human plasma.

Acknowledgements

We would like to express our gratitude to Atma Jaya Catholic University of Indonesia for providing support for this study.

Disclosure Statement

All authors declare that there are no conflicts of interest in this research.

Reference

1. Kr infections in adults. Singapore Med J. India; 2016;57(9):485–90. https://doi.org/10.11622/smedj.2016153
2. Medina M, Castillo-Pino E. An introduction to the epidemiology and burden of urinary tract infections. Ther Adv Urol. England; 2019;11:1756287219832172. https://doi.org/10.1177/1756287219832172
3. Teguh Firdaus, Rina Yunita. Urinary Tract Infection Bacterial at RSUP H. Adam Malik Medan in 2019: an Overview Study. Sumatera Medical Journal. 2021;4(1 SE-). https://doi.org/10.32734/sumej.v4i1.5849
4. Zeind CS, Carvalho MG, Cheng JWM, Zaiken K. Urinary Tract Infection. In: Applied Therapeutics: The Clinical Use of Drugs. 12th ed. LWW; 2023. p. 1594–618.
5. Andrajati R, Tilaqza A, Supardi S. Factors related to rational antibiotic prescriptions in community health centers in Depok City, Indonesia. J Infect Public Health. 2017;10(1):41–8. https://doi.org/10.1016/j.jiph.2016.01.012
6. Skow MAH, Vik I, Høye S. Antibiotic switch after treatment with UTI antibiotics in male patients. Infect Dis. Taylor & Francis; 2020;52(6):405–12. https://doi.org/10.1080/23744235.2020.1736329
7. Tron C, Belleguic C, Piau C, Brinchault G, Deneuville E, Ricordel C, et al. Determination of amoxicillin and cotrimoxazole concentrations in sputum of patients with cystic fibrosis. Biomedical Chromatography. 2021;35(12). https://doi.org/10.1002/bmc.5208
8. López AT, Olguín HJ, Lares-Asseff I, González FR, Medina RA, Guillé GP, et al. A Reliable Method to Quantify Trimethoprim and Sulfamethoxazole in Plasma of Children by HPLC with Application in Pharmacokinetic Studies. Am J Analyt Chem. 2019;10(07):247–53. https://doi.org/10.4236/ajac.2019.107019
9. Amini H, Ahmadiani A. Rapid and

simultaneous determination of sulfamethoxazole and trimethoprim in human plasma by high-performance liquid chromatography. J Pharm Biomed Anal. J Pharm Biomed Anal; 2007;43(3):1146–50. https://doi.org/10.1016/j.jpba.2006.09.004
10. Garg NK, Sharma G, Singh B, Nirbhavane P, Katare OP. Quality by Design (QbD)-Based Development and Optimization of a Simple, Robust RP-HPLC Method for the Estimation of Methotrexate. J Liq Chromatogr Relat Technol. Taylor & Francis; 2015;38(17):1629–37. https://doi.org/10.1080/10826076.2015.1087409
11. Tuzimski T, Petruczynik A. Review of Chromatographic Methods Coupled with Modern Detection Techniques Applied in the Therapeutic Drugs Monitoring (TDM). Vol. 25, Molecules MDPI AG; 2020. https://doi.org/10.3390/molecules25174026
12. Swartz M. Method Validation. Introduction to Modern Liquid Chromatography. John Wiley and Sons; 2010;531–67. https://doi.org/10.1002/9780470508183.CH12
13. FDA. Bioanalytical Method Validation Guidance for Industry [Internet]. 2018 [cited 2021 Apr 2].
14. ICH. [Internet]. 2023. ICH M10 on bioanalytical method validation - Scientific guideline | European Medicines Agency (EMA) [cited 2024 Sep 3]. https://www.ema.europa.eu/en/ich-m10-bioanalytical-method-validation-scientific-guideline#current-version-9785
15. Team RC. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2023. https://www.r-project.org/
16. Zhang J, Wang Q, Kleintop B, Raglione T. Suppression of peak tailing of phosphate prodrugs in reversed-phase liquid chromatography. J Pharm Biomed Anal. 2014;98:247–52. https://doi.org/10.1016/j.jpba.2014.05.027
17. Johnson AR, Vitha MF. Chromatographic selectivity triangles. J Chromatogr A. 2011;1218(4):556–86. https://doi.org/10.1016/j.chroma.2010.09.046
18. Notario D, Amelia J, Della G. Pengembangan dan Validasi Metode Bioanalisis Trimetoprim dalam Sampel Plasma dan Urin Manusia Simulasi Menggunakan KCKT-PDA. Jurnal Kefarmasian Indonesia. 2023;41–9. https://doi.org/10.22435/JKI.V13I1.6189
19. Notario D, Suwita A, Kelviyana K. Analytical Method Validation of Sulfamethoxazole in Human Plasma and Urine by HPLC-PDA. Jurnal Sains dan Kesehatan. Faculty of Pharmacy, Mulawarman University; 2022;4(SE-1):31–8. https://doi.org/10.25026/JSK.V4ISE-1.1687
20. Epshtein NA. System Suitability Requirements for Liquid Chromatography Methods: Controlled Parameters and their Recommended Values (Review). Pharm Chem J. 2020;54(5):518–25. https://doi.org/10.1007/s11094-020-02231-w
21. Briscoe CJ, Stiles MR, Hage DS. System suitability in bioanalytical LC/MS/MS. J Pharm Biomed Anal. 2007;44(2):484–91. https://doi.org/10.1016/j.jpba.2007.03.003
22. Sonawane SS, Chhajed SS, Attar SS, Kshirsagar SJ. An approach to select linear regression model in bioanalytical method validation. J Anal Sci Technol. 2019;10(1):1. https://doi.org/10.1186/s40543-018-0160-2
23. Zeng W, Musson DG, Fisher AL, Wang AQ. A new approach for evaluating carryover and its influence on quantitation in high-performance liquid chromatography and tandem mass spectrometry assay. Rapid Communications in Mass Spectrometry. John Wiley & Sons, Ltd; 2006;20(4):635–40. https://doi.org/https://doi.org/10.1002/rcm.2353
24. Abdessadek M, Kharbach M, Ben-Saghroune H, El Amraoui B, Khabbal Y. Validation steps and parameters of bioanalytical methods

using in clinical studies: A narrative review. Vol. 13, Journal of Applied Pharmaceutical Science Open Science Publishers LLP Inc.; 2023. p. 68–83. https://doi.org/10.7324/JAPS.2023.109956

25. Jogpethe A, Jadav T, Rajput N, Kumar Sahu A, Tekade RK, Sengupta P. Critical strategies to pinpoint carryover problems in liquid chromatography-mass spectrometry: A systematic direction for their origin identification and mitigation. Microchemical Journal. 2022;179:107464. https://doi.org/https://doi.org/10.1016/j.microc.2022.107464
26. Nong L, Tang Y, Qin X, Zhou Z, Lai Z, Wei W, et al. Simultaneous determination of concentration of sulfamethoxazole and trimethoprim in human plasma by dual-quaternary HPLC and its clinical application. Precision Medication. 2024;1(2):100017. https://doi.org/10.1016/j.prmedi.2025.100017
27. Thompson EJ, Wu H, Maharaj A, Edginton AN, Balevic SJ, Cobbaert M, et al. Physiologically Based Pharmacokinetic Modeling for Trimethoprim and Sulfamethoxazole in Children. Clin Pharmacokinet. 2019;58(7):887–98. https://doi.org/10.1007/s40262-018-00733-1istiani SNM, Kapantouw MG, Pandaleke TA. Hubungan Indeks Massa Tubuh dan Angka Kejadian Akne Vulgaris pada Siswa-Siswi di SMA Frater Don Bosco Manado. E-Clinic. 2017;5(2).