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Identification and Assay of Isoniazid and Pyrazinamide in Fixed-Dose Dispersible Tablets Containing Rifampicin Using High-Performance Liquid Chromatography

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Abstract

Tuberculosis (TB) remains a major global health challenge, particularly in Indonesia, which ranks among the countries with the highest TB burden. Fixed-dose dispersible anti-tuberculosis formulations containing rifampicin, isoniazid, and pyrazinamide are widely recommended to enhance patient adherence and minimize the risk of drug resistance. Ensuring the quality and appropriate dosage of active pharmaceutical ingredients is essential for achieving therapeutic effectiveness and maintaining TB control. This study aimed to perform routine quality assessment and regulatory compliance assays on commercial anti-TB fixed-dose combination (FDC) dispersible tablets using a reversed-phase high-performance liquid chromatography (HPLC) method as specified in the International Pharmacopoeia 2025. The HPLC system was equipped with a C18 column and used a mobile phase consisting of acetate buffer (pH 5) and methanol (94:6 v/v). System suitability testing was conducted before analysis, with %RSD values below 2%, resolution greater than 2.0, and tailing factors ≤ 2.0 , indicating acceptable precision, selectivity, and chromatographic performance. The retention times of isoniazid and pyrazinamide were consistent with those of the reference standards, confirming identity. The assay results showed that pyrazinamide (100.3%) and isoniazid (99.9%) were within the specification limits set by the International Pharmacopoeia. These findings demonstrate that the official monograph method is suitable for regulatory-grade quality control testing of dispersible anti-tuberculosis fixed-dose combination tablets, and that the tested product met pharmacopeial quality requirements.

Keywords: fixed-dose combination, international pharmacopoeia, isoniazid, pyrazinamide, reversed-phase HPLC.

Introduction

Tuberculosis (TB) remains one of the most persistent infectious diseases worldwide, caused by *Mycobacterium tuberculosis* (Chai et al., 2018). According to the WHO Global TB report in 2023, an estimated 10.6 million people fell ill with TB and 1.3 million died globally in 2022 (WHO, 2023). In Indonesia, TB remains a significant public health problem, as the country ranks second globally after India, with over one million estimated cases and about 125,000 deaths every year (around 14 deaths per hour), and with about 885,000 cases detected in 2024 across men, women, and children, highlighting the urgent need to strengthen TB prevention and treatment effort (Kemenkes, 2025). Therefore, quality control of anti-TB pharmaceutical products remains a critical component in ensuring the success of TB control programs.

Fixed-dose combination (FDC) anti-TB pharmaceutical products, such as rifampicin, isoniazid, and pyrazinamide, are recommended, as they reduce the number of pills a patient must take, improve adherence, and reduce the risk of functional monotherapy (Gallardo et al., 2016). Accurate quantification of active pharmaceutical ingredients in FDC anti-TB products is essential to verify compliance with labeled content, assure therapeutic equivalence, and prevent sub-optimal dosing that can drive the emergence of drug resistance development (Singh et al., 2020). Evidence shows that ingredients in FDC can directly affect plasma drug concentrations and lead to poorer clinical outcomes, especially in high-burden TB settings (Bargaje et al., 2022). Several analytical studies have demonstrated that some marketed FDC anti-TB tablets failed to meet pharmacopeial specifications for content uniformity and dissolution, indicating a real risk of underdosing and reduced clinical effectiveness (Cáceres-Pérez et al., 2023). Therefore, continuous quality surveillance and routine post-market evaluation of FDC products are critical to ensure optimal therapeutic performance and to protect antimicrobial stewardship efforts (Tabernero & Newton, 2023).

Measurement of rifampicin, isoniazid, and pyrazinamide levels is generally performed using UV-vis spectroscopy. Because rifampicin, isoniazid, and pyrazinamide produce UV absorbance profile that overlaps each other, UV-vis spectroscopy is not reliable to measure them at the same time (Goicoechea & Olivieri, 1999). When UV-vis spectroscopy cannot provide adequate selectivity, chromatographic analysis becomes necessary. High-Performance Liquid Chromatography (HPLC) is widely recognized as the gold standard analytical technique for multi-component pharmaceutical analysis due to its selectivity, high resolving power, and suitability for direct regulatory confirmation (Gupta et al., 2022). For rifampicin, isoniazid, and pyrazinamide combination, the International Pharmacopoeia established official monographic procedures by HPLC for identification and assay (WHO, 2025). However, published research applying this official monographic method to real-market dispersible FDC tablets remains limited. Most reports on triple-ingredient anti-TB analysis either modify chromatographic conditions or apply multivariate spectrophotometric solutions that are not routinely used for regulatory confirmation (Staden et al., 2023). Consequently, there remains a translational evidence gap between the monograph's existence and its actual analytical performance when directly applied to commercial FDC dispersible products circulating in Indonesia.

Accordingly, this research aims to apply the official International Pharmacopoeia HPLC monograph for the identification and assay determination of isoniazid and pyrazinamide in dispersible tablets containing rifampicin commercially available in Indonesia. This research will provide empirical performance evidence of the official method and evaluate whether the obtained assay results comply with the pharmacopeial acceptance criteria. This research is expected to support harmonized laboratory implementations of validated reference methods in Indonesian pharmaceutical quality control settings.

Method

Instruments

The instrument used in this research consisted of analytical balance (Precisa), analytical microbalance (Sartorius), volumetric flasks (50 mL and 1000 mL), pH-meter (Mettler Toledo), mortar and pestle, an HPLC Shimadzu LC-20AD equipped with SPD-20A Prominence UV-vis detector,

reversed-phase C-18 column (Phenomenex) 150 × 4.6 nm, 5 μm, sample vials, ultrasonic bath (Branson), and shaker (Torta).

Materials

The materials used in this research were HPLC-grade methanol (Supelco), ammonium acetate (Merck), glacial acetic acid (Merck), certified reference standards of isoniazid (BPFI) and pyrazinamide (BPFI), and dispersible tablets containing the mixture of rifampicin 75 mg, isoniazid 50 mg, and pyrazinamide 150 mg.

Procedure

Preparation of Buffer and Mobile Phase Liquid

25 mg of ammonium acetate was weighed and transferred into 50 mL volumetric flasks. Ammonium acetate was dissolved in HPLC-grade water, and the volume was adjusted to the mark. The solution was homogenized, and the pH was adjusted to 5 using glacial acetic acid. For the mobile phase, 30 mL of methanol and 470 mL of acetate buffer (pH 5) were mixed (ratio 6:94 v/v), homogenized, and filtered through 0.45 μm membrane filter, in accordance with the International Pharmacopoeia monograph (WHO, 2025).

Preparation of Working Standard Solution

5 mg of isoniazid and 15 mg of pyrazinamide (Indonesian Pharmacopoeia reference material /BPFI) were weighed and transferred into 50 mL volumetric flasks. Approximately 35 mL of purified water was added, the mixture was mechanically agitated for 15 minutes, and subsequently sonicated for 2 minutes. The solution was then diluted to volume with purified water and filtered through a 0,45 μm membrane filter.

Preparation of Sample Solution

Twenty sample tablets were weighed, finely powdered, and homogenized. A portion of powder equivalent to 10 mg of isoniazid was transferred into 100 mL volumetric flasks. 80 mL of purified water was added, and the mixture was mechanically agitated for 15 minutes. The dispersion was then sonicated for 2 minutes. When foaming was observed, 3 mL of methanol, equivalent to 4% of 80 mL of water, was added. The solution was diluted to volume with the same solvent, then filtered through a 0.45 μm membrane filter (WHO, 2025)

Preparation of Spiked Solution

5 mL of the working standard solution and 5 mL of the sample solution were transferred into 10 mL volumetric flasks and mixed. The mixture was then filtered through a 0,45 μm membrane filter.

The Calculation of Chromatogram Resolution and Tailing Factor

Resolution is the degree of separation measured quantitatively between two chromatographic peaks, A and B. A value ≥ 1.5 generally indicates good baseline separation. If $R < 1.0$, the peaks are considered to overlap. The resolution (R) is calculated using **Equation 1**.

$$R = \frac{2(t_{R2} - t_{R1})}{w_1 + w_2} \quad (1)$$

Where:

R = resolution between two peaks

t_{R2} and t_{R1} = retention time of the second and first peaks

w_1 and w_2 = baseline width for the first and second peak

Tailing factor indicates a tail at the end of a chromatographic peak, usually due to the presence of a very active site in the stationary phase. The calculation is shown in **Equation 2**.

$$R = \frac{2(t_{R2} - t_{R1})}{w_1 + w_2} \quad (2) \quad (1)$$

Where:

T = tailing factor

W_{0.05} = peak width at 5% of peak height

F = distance from the center line of the peak to the front side of the peak (front half width) at a height of 5%.

Chromatographic Condition

The chromatographic analysis was carried out using an HPLC system equipped with a UV-Vis detector. Separation was achieved on a C18 reversed-phase column (150 4,6 mm) with an isocratic mobile phase consisting of acetate buffer at pH five and methanol in a 94:6 ratio. The mobile phase was filtered through a 0,45 µm membrane and degassed prior to use. The flow rate was maintained at 1.0 mL/min, and detection was performed at 240 nm.

Validation Parameters

Method validation included system suitability testing to ensure that the chromatographic system provided adequate precision, resolution, and peak performance. Parameters evaluated were retention time reproducibility, peak area repeatability (%RSD), resolution between isoniazid and pyrazinamide, tailing factor, and theoretical plate number. The method met all required criteria, demonstrating an acceptable %RSD (<2%), good resolution, and appropriate peak symmetry, confirming its reliability for routine analysis.

Result and Discussion

This research employed the official monograph-based reversed-phase HPLC method from The Twelfth Edition of The International Pharmacopeia (2025) for the determination of isoniazid and pyrazinamide in dispersible fixed-dose combination tablets containing rifampicin (WHO, 2025). Because this protocol was already an official compendial method, the analytical design used here was directly aligned with internationally recognized reference procedures. However, because the reference standards used during this research were Indonesian Pharmacopeia reference material (BPFI), not International Chemical Reference Substance (ICRS) from World Health Organization (WHO), the laboratory first performed method verification before use the sample which is in line with WHO guidance that laboratory may use local certified standards as long as the laboratory demonstrates suitability (WHO, 2025). The tablet used in this research is an anti-TB fixed-dose combination. In principle, High-Performance Liquid Chromatography (HPLC) separates analytes based on differential interactions between each molecule and the stationary phase surface as the mobile phase flows through the column. Compounds that interact more strongly with the stationary phase are retained longer and elute at longer retention times, whereas those with weaker interactions elute sooner (Hussein, 2025). This separation principle is well established in pharmaceutical analysis and has been widely used to separate multiple anti-TB drugs in combination formulations with high selectivity (Staden *et al.*, 2023). For dispersible dosage forms, this mechanism is even more relevant, as excipient composition can vary between manufacturers and influence chromatographic selectivity.

Selection of Mobile Phase and Buffer

Regarding the mobile phase, a hydro-organic mixture was used in accordance with the International Pharmacopoeia monograph, specifically a 94:6 (v/v) acetate buffer pH five mixed with methanol. The

mobile phase not only serves as the solvent to dissolve the analytes but also as the transport medium that governs elution strength and ultimately determines resolution. The mobile phase consists of a mixture of miscible solvents, which collectively determine the elution strength and separation resolution (Wulandari *et al.*, 2024). In addition to dissolving the analytes, the mobile phase transports the sample components through the stationary phase, providing retention times within the required analytical range and ensuring adequate selectivity for the compounds to be separated. In reverse-phase HPLC, methanol was chosen as the organic modifier due to its excellent miscibility and capability to solubilize both analytes (Fitri and Pratiwi, 2023). The selection of a glacial acetic acid-methanol composition was justified based on polarity considerations, as isoniazid and pyrazinamide are polar molecules; hence, a polar mobile phase is required to allow sufficient interaction, elution, and selectivity along the chromatographic pathway. Achieving a good separation in HPLC is strongly influenced by the composition and pH of the mobile phase (Heering *et al.*, 2024). Selecting an appropriate mobile phase is crucial for achieving efficient, effective separation under the specific reversed-phase HPLC conditions.

According to the International Pharmacopoeia, Tenth Edition (2025), the monograph recommends a 5% acetate buffer and a methanol mixture in a 94:6 (v/v) ratio (WHO, 2025). In the present research, the mobile phase consisted of an acetate buffer at pH five and a methanol mixture at a 94:6 (v/v) ratio. Methanol was selected as the organic modifier due to its good solubility for the analytes. Polarity considerations justify the use of glacial acetic acid and methanol, as both isoniazid and pyrazinamide are polar molecular compounds, and a polar hydro-organic mobile phase is required to dissolve and transport them effectively through the chromatographic system. These considerations are consistent with recent research in chromatography, which reported that the balanced polarity of the aqueous buffer and organic modifier critically governs retention and selectivity for simultaneous multi-drug TB analysis (Xing *et al.*, 2021). Thus, maintaining the correct mobile phase composition and chemical environment is essential to ensure reproducible separations.

Based on polarity, the mobile and stationary phases of the chromatographic system used in this research are reversed-phase, where the mobile phase is more polar than the stationary phase (C18 octadecylsilane column) (Klimek-Turek *et al.*, 2020). Because isoniazid and pyrazinamide are polar, a polar mobile phase allows them to be eluted efficiently. The nonpolar C18 stationary phase provides separation due to the varying strengths of interaction between each analyte and the hydrophobic surface of the stationary phase (Bibire *et al.*, 2018). In reversed-phase HPLC, the polarity contrast between a polar aqueous mobile phase and a nonpolar C18 stationary phase is the fundamental mechanism driving retention differences among analytes (Xing *et al.*, 2021). Therefore, controlling the pH of the mobile phase is critical, as ionizable analytes change charge state with pH.

The ionization behavior of analytes is strongly pH-dependent. Thus, the pH of the mobile phase must be controlled to maintain ionizable analytes in a stable, dominant ionization state. Buffer plays a central role in stabilizing the protonation state of isoniazid and pyrazinamide (Safavi *et al.*, 2004). This condition improves the peak shape, prevents excessive tailing, and maintains predictable retention under reserved-phased HPLC (Zhang *et al.*, 2014). The importance of adequate pH and buffering is well documented, as evidenced by the chromatographic literature, which shows that sufficient buffer capacity increases selectivity, improves resolution, and ensures method robustness in multi-drug RP-HPLC analysis systems. Pyrazinamide is an acidic compound with pKa values of 0.5 and 1.8, while isoniazid has three pKa values: 1.8, 3.5, and 10.8, making them highly sensitive to pH shifts (Keating *et al.*, 2020). In reversed-phase HPLC, the pH is ideally adjusted at least two units above or below the analyte pKa to maintain the analyte in a single ionization state (Studzińska & Buszewski, 2012). At pH 5, both analytes predominantly remain in their deprotonated forms, resulting in stable, predictable retention times. In contrast, if the mobile phase pH is set closer to any of the analytes' pKa values, the analytes undergo partial deprotonation or protonation in the column, leading to fluctuating ionization states. This condition would produce peak tailing or fronting and reduced quantitative accuracy. **Figure 1** shows the chemical structures of isoniazid and pyrazinamide, along with their retention times of 3.482 min and 4.206 min, respectively, obtained from HPLC measurements (**Table 1**).

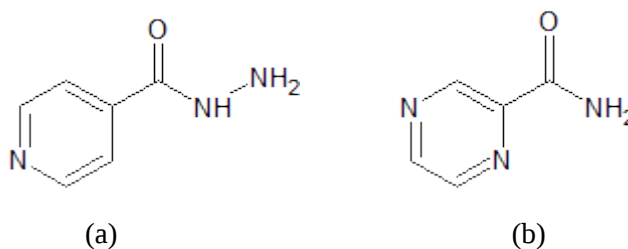


Figure 1. The chemical structure of isoniazid (a) and (b) pyrazinamide

This difference in retention time indicates that the two analytes were effectively separated by the reversed-phase HPLC system using a C18 column. The relatively short retention times indicate that both compounds have high polarity, allowing them to interact weakly with the nonpolar stationary phase and be rapidly eluted by the polar mobile phase.

Table 1. The retention time of isoniazid and pyrazinamide

Time of retention	
Isoniazid	3.482 min
Pyrazinamide	4.206 min

These results align with previous research reports by Jongrungruangchok and Songsak in 2015, who reported that in the reversed-phase HPLC system with a C18 column and an acetate-methanol buffer-based mobile phase, isoniazid and pyrazinamide eluted with retention times of approximately 3.4 and 4.2 min, respectively, which shows a similar elution pattern to this research, as shown in **Table 1**. Xing *et al.* in 2021 also demonstrated that isoniazid and pyrazinamide, due to their highly polar nature, exhibited retention times below 5 minutes on a C18 column with an aqueous mobile phase, confirming that their separation could be achieved without a long gradient. Chellini *et al.* in 2015 reported a similar trend in 2015, where the elution order (isoniazid first, followed by pyrazinamide) was consistent across a range of mobile-phase compositions, indicating high reproducibility of the International Pharmacopoeia monograph-based HPLC system. Thus, the retention times obtained in this research agree with the literature, indicating that the HPLC system used exhibits good reproducibility and selectivity for both main analytes. This condition also confirms that the pharmacopoeia monograph method can be directly applied to dispersible FDC tablets without requiring significant modifications to the chromatographic parameters.

System Suitability Test

A system suitability test is performed to verify that the chromatographic system demonstrates sufficient resolution, sensitivity, and reproducibility for the intended analysis. The principle is that the equipment, electronic components, analytical operation, and the sample should be analyzed as a single integrated system that must be evaluated before and during analysis. In this research, system suitability testing was conducted to ensure that the HPLC system provided accurate results throughout the analysis. The purpose of this step is to confirm that the analytical system maintains adequate detection sensitivity, maintains resolution between adjacent peaks, and preserves acceptable reproducibility of chromatographic performance during the analytical run. System suitability testing parameters typically assessed include retention time reproducibility, resolution, tailing factor, and theoretical plates (Webster & Kott, 2019). A system is considered acceptable when the relative standard deviation (RSD) of replicate injections does not exceed 2% and the number of theoretical plates is not less than 1,000, indicating good method reproducibility and acceptable column efficiency (Epshtein, 2020). System suitability was evaluated by injecting the mixed working standard solution of isoniazid and pyrazinamide five times consecutively, followed by calculation of the relative standard deviation (RSD) of the peak responses. The results of the system suitability test are presented in **Tables 2 and 3**.

Table 2. The Suitability test of isoniazid and pyrazinamide

Parameters	Isoniazid 1		Isoniazid 2		Pyrazinamide 1		Pyrazinamide 2	
Concentration (mg/mL)	0.106		0.102		0.300		0.305	
Corrected standard weight (mg)	5.312		5.102		15.022		15.240	
Sequencing	RT	Area	RT	Area	RT	Area	RT	Area
1	3.487	927478	3.478	888108	4.213	6242133	4.199	6354307
2	3.483	935855	3.472	889729	4.208	6248566	4.193	6357621
3	3.483	930459	3.472	884550	4.207	6246632	4.192	6356476
4	3.479	931158			4.202	6247597		
5	3.478	929499			4.201	6246660		
Mean	3.482	930890	3.474	8887462	4.206	6246318	4.195	6356135
SD	0.004	3101,832	0.003	2649.184	0.005	2470.786	0.004	1683.161
RSD (%)	0.104	0.333	0.100	0.299	0.116	0.040	0.090	0.026
Response Factor	8762537		8696787		20790577		20853232	
%Difference			0.75				-0.30	
%Difference requirement			≤ 2%				≤ 2%	
Conclusion	The system suitability test meets the acceptance criteria.							

Table 3. Summary of System Suitability Analysis

Parameter	Result	Specification
% RSD of The Working Standard Solution		
1) Isoniazid	0.33 %	$\leq 2\%$
2) Pyrazinamide	0.29 %	
% Difference between isoniazid control standard solution and the working standard solution		
1) Isoniazid	0.75 %	$\leq 2\%$
2) Pyrazinamide	0.30 %	
Resolution	3.860	$\geq 2\%$
Tailing Factor (Asymmetry)	1.370	$\leq 2\%$

The system suitability test report, presented in **Tables 2 and 3**, demonstrated that the chromatographic system performed within the acceptable limits of the USP requirements. The RSDs of the working standard solutions for isoniazid and pyrazinamide were both below the 2% limit (isoniazid: 0.33%; pyrazinamide: 0.0%), indicating excellent short-term precision of the detector response. In addition, the % difference between the isoniazid control standard solution and the working standard solution was also below 2% for both analytes (isoniazid, 0.75%, and pyrazinamide, 0.30%), indicating that the system provided consistent quantitation between reference levels. According to USP, an $RSD \leq 2\%$ is considered evidence that the method is precise and suitable for quantitative analysis in HPLC (Epshtein, 2020).

Moreover, the tailing factor (asymmetry) value of 1.370 complied with the acceptance criteria of $\leq 2\%$, indicating symmetric peak shapes and minimal secondary interactions with residual silanol groups in the stationary phase. Proper peak symmetry is crucial because poor tailing can bias the integration and quantification. This finding aligns with previous research demonstrating that adequate buffering and controlled ionization conditions significantly reduce tailing in reversed-phase HPLC (McCalley, 2004). Overall, these results confirm that the system possesses sufficient precision, resolution, and peak symmetry to produce reliable quantitative data for isoniazid and pyrazinamide using the current reversed-phase HPLC method. In other words, the analytical system met the regulatory criteria.

The Quantitative Analysis of Isoniazid and Pyrazinamide

The retention times obtained for isoniazid and pyrazinamide in the sample solution were in close agreement with those of the reference standard solutions (**Table 4**). This similarity in retention time

indicated that the peaks detected in the sample chromatogram corresponded to the analyte identities and that no co-eluting interfering peaks were observed in the retention window. Retention time matching between sample and standard is a routinely accepted identity confirmation parameter in pharmacopeial chromatography.

Table 4. Retention Time

Analyte	Retention Time of The Reference Standard Solution	Retention Time of the Analytes
Isoniazid	3.482	3.469
Pyrazinamide	4.206	4.189

Moreover, based on Table 3, the assay results showed that pyrazinamide concentration in the sample was 100.3% (150.40 mg per tablet), while isoniazid content was 99.9% (49.97 mg per tablet). These values in line with the specification criteria stated in the International Pharmacopoeia monograph for anti-TB fixed dose combination dispersible tablets, which states that rifampicin and isoniazid should not be less than 90.00% and not more than 130.00% of the label claim and pyrazinamide should not be less than 90.00% and not more than 110.00% of the label claim (WHO, 2025). The results obtained here are consistent with previous chromatographic studies on multi-drug TB tablet formulations, in which properly optimized reversed-phase HPLC methods produced assay values typically between 95 and 105% of the labeled claim (Panda *et al.*, 2020). Overall, both the identity confirmation through retention time alignment and the quantitative assay values demonstrated that the product tested met the acceptable quality range according to international pharmacopeial standards.

Conclusion

The reversed-phase HPLC method used in this study successfully confirmed the identity and quantified isoniazid and pyrazinamide in fixed-dose dispersible anti-tuberculosis tablets. The system suitability parameters met the pharmacopeial acceptance criteria, indicating that the analytical system was precise, selective, and capable of producing reliable results. The assay results obtained for both active ingredients complied with the specification limits stated in the International Pharmacopoeia 12th edition, confirming that the tested product met the required quality standard. Therefore, the applied reversed-phase HPLC method is considered suitable for routine quality control analysis of isoniazid and pyrazinamide within multi-drug anti-TB formulations.

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