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Analytical Method Validation for Anti-Tuberculosis Drugs: A Review of Rifampicin Isoniazid and Pyridoxine

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Abstract

TB is still a major worldwide health concern, especially in low- and middle-income nations. Accurate dosing, pharmacokinetic profile, and quality control of vital medications including pyridoxine, isoniazid, and rifampicin are crucial to the effectiveness of anti-tuberculosis treatment. For methods used for drug quantification in bulk materials, pharmaceutical formulations, and biological matrices to be reliable, specific, and reproducible, analytical method validation is essential. This study offers a thorough summary of the approved analytical techniques used to determine pyridoxine, isoniazid, and rifampicin, namely High-Performance Liquid Chromatography (HPLC), UV-Visible spectrophotometry, and LC-MS/MS. In compliance with ICH and USP criteria, important validation factors are examined, including accuracy, precision, linearity, sensitivity, specificity, robustness, and system adaptability. Rifampicin needs method development techniques that address matrix interference and degradation because of its instability and low solubility. While pyridoxine, which is used prophylactically to avoid neurotoxicity from isoniazid, requires precise monitoring, particularly in fixed-dose combination therapy, isoniazid, a hydrophilic molecule, presents difficulties in derivatization and UV detection. Common problems include matrix effects in biological materials and drug-drug interactions during co-formulation are also highlighted in the paper. Additionally assessed are recent developments in chromatographic methods, such as UPLC and bioanalytical validations for monitoring therapeutic drugs (TDM). In order to promote strong quality assurance and regulatory compliance in anti-TB pharmacotherapy and enhance treatment results and medication safety, this paper attempts to compile important insights into method development and validation.

Keywords: Tuberculosis, TB, Rifampicin, Isoniazid, Pyridoxine, andAnalytical Method Validation

1. INTRODUCTION:

1.1.Overview of Tuberculosis (TB) and Global Burden

One of the biggest risks to world health in the twenty-first century is tuberculosis (TB), an old illness. Mycobacterium tuberculosis is the causative agent of tuberculosis (TB), a communicable and possibly fatal infectious illness that predominantly impacts the lungs (pulmonary TB) but can also spread to other

organs with the value the brain, kidneys, lymph nodes, and bones (extrapulmonary TB). TB is a highly contagious disease, especially in densely populated regions, since it is spread by airborne particles released when an infected person coughs, sneezes, talks, or sings [1].

TB is a long-term illness that develops gradually. A competent immune response may prevent the majority of people exposed to M. tuberculosis from experiencing symptoms right away, but 5–10% of infected people go on to experience active illness at some time in their life. A far higher percentage has latent TB infection (LTBI), which can resurface in immune-suppressive situations including HIV/AIDS, diabetes, or malnourishment [2].

TB is still one of the top 10 causes of mortality globally and the major infectious agent cause of death, exceeding even HIV/AIDS in recent years, despite decades of control attempts and the availability of powerful treatment [3].

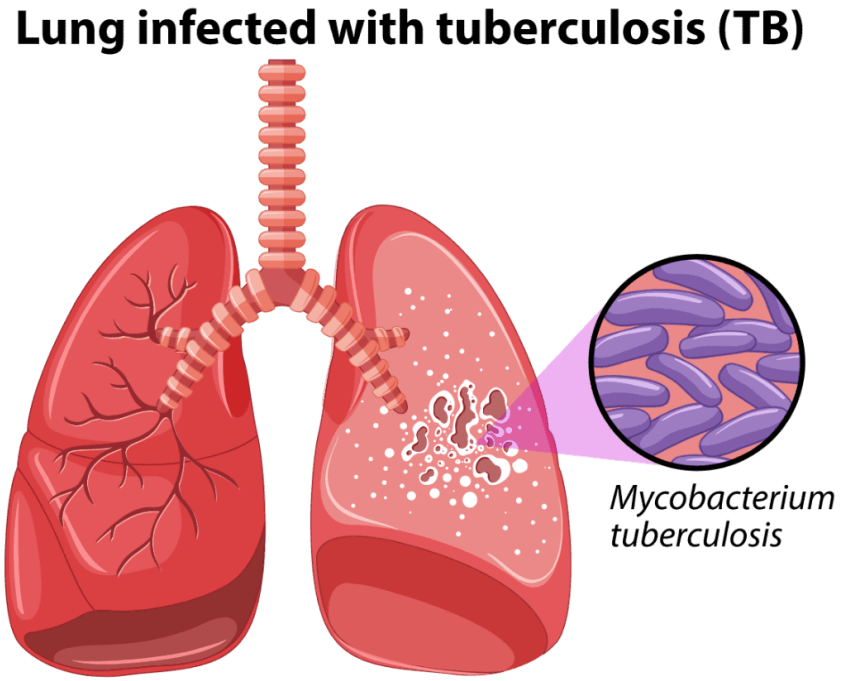

Fig 1: Diagrammatic Representation of Location of T.B [4]

1.2.Epidemiological Burden: Global Perspective

The World Health Organization's (WHO) 2023 Global Tuberculosis Report states that TB still has a significant impact:

- In 2022, approximately 10.6 million persons contracted tuberculosis (TB), of whom 56% were males, 33% were women, and 11% were children.
- There were 187,000 deaths among HIV-positive persons and almost 1.3 million deaths among HIV-negative people [5].

- More than two-thirds of the world's TB infections are in countries like Bangladesh, China, India, Indonesia, the Philippines, Pakistan, Nigeria, and the Democratic Republic of the Congo.
- Moreover, one in three TB cases go unreported or untreated, which makes it extremely difficult to effectively manage the illness.
- In low- and middle-income nations, where access to anti-TB drugs, healthcare, nutrition, and diagnostics is frequently restricted, the burden is disproportionately greater [6].

1.3.Drug-Resistant TB: An Emerging Crisis

The increasing risk of drug-resistant TB (DR-TB) contributes to the worldwide TB burden. A serious threat to public health is multidrug-resistant TB (MDR-TB), which is characterized as TB that is resistant to at least isoniazid and rifampicin, the two most potent anti-TB medications:

- Approximately 410,000 cases of MDR-TB or rifampicin-resistant TB (RR-TB) were reported in 2022 [7].
- MDR-TB treatment is more hazardous, expensive, and time-consuming, and its success rate is lower (about 60%) than that of drug-sensitive TB (about 86%).
- Treatment is made more difficult and fatality rates are raised by extensively drug-resistant TB (XDR-TB), which exhibits resistance to both first-line and second-line medications [8].

1.4.TB and HIV Co-Infection

One of the biggest risk factors for getting active TB is HIV. Persons with HIV have an 18-fold higher risk of developing active tuberculosis than persons without HIV because of immunosuppression. Particularly in sub-Saharan Africa, tuberculosis is the primary cause of mortality for those living with HIV. For these populations to get appropriate disease management, comprehensive TB and HIV care are essential [9].

1.5.Social Determinants and Risk Factors

The socioeconomic issues are closely linked to the TB pandemic. Poverty and overcrowding, which promote transmission, are major indicators of risk for TB infection and disease development.

- Malnutrition, which impairs immunity.
- Abuse of substances, such as alcohol and tobacco.
- Co-morbid conditions such chronic renal disease and diabetes.
- Displacement and migration, which frequently result in poor treatment adherence and interrupted care [10].

These factors demonstrate that tuberculosis is complicated social illnesses that need a multimodal strategy for efficient control, rather than only being a biological problem.

1.6.Global Strategies and the End TB Initiative

The WHO created the End TB Strategy in order to address the persistent global TB burden. The strategy seeks to reduce new cases by 80% and TB fatalities by 90% between 2015 and 2030 when compared to 2015 levels [11].

- One of the main tenets of this approach is integrated, patient-centred prevention and treatment.
- Brave laws and helpful frameworks, such as social security and universal health care.
- More research and development, such as vaccinations, shorter treatment durations, and improved diagnostics [12].

However, the global disruption of TB services caused by the COVID-19 pandemic led to a decrease in case discovery, a delay in treatment, and a setback in reaching TB eradication objectives. Even though TB is preventable and treatable, it nonetheless poses a serious threat to public health, especially in underdeveloped nations.The need for ongoing international commitment, cutting-edge treatments, and comprehensive interventions is highlighted by the rising prevalence of medication resistance, HIV co-infection, and socioeconomic factors including poverty and undernutrition [13].

In order to enhance case detection, guarantee adherence, lessen stigma, and expedite research toward novel vaccines and treatments, it is critical that future TB control initiatives incorporate biological, behavioural, and structural methods. The worldwide burden of tuberculosis may be considerably decreased with concerted efforts and fair access to healthcare [14].

2. OVERVIEW OF ANTI-TUBERCULOSIS DRUGS

The World Health Organization (WHO) estimates that 10.6 million new cases and 1.3 million deaths from tuberculosis (TB) will occur in 2022, making it a persistent worldwide health concern. A multi-drug regimen is the cornerstone of TB therapy in order to guarantee total bacterial eradication and stop medication resistance from developing. Ethambutol, pyrazinamide, isoniazid, and rifampicin are the usual first-line medications. In order to lower the risk of neurotoxicity, pyridoxine (Vitamin B6) is also co-administered, especially in regimens incorporating isoniazid. A thorough pharmacological review of three important medications—pyridoxine, isoniazid, and rifampicin—is provided here [15].

A. Rifampicin

Mechanism of Action

Rifampicin is a strong, all-purpose antibiotic that kills bacteria by attaching itself to the β-subunit of Mycobacterium tuberculosis's DNA-dependent RNA polymerase enzyme. By stopping the RNA chain from elongating, this binding restricts transcription, which in turn suppresses protein production and causes cell death. Rifampicin works well against intracellular organisms that live inside macrophages as well as quickly proliferating external bacilli [16].

B. Metabolism and Pharmacokinetics

- 90–95% bioavailability when taken without food.
- Cmax, or peak plasma concentration, was attained in two to four hours.
- Vd, or volume of distribution, is around 0.9–1.6 L/kg [17].
- Half-Life: 2–5 hours; extended usage may cause auto-induction to shorten it.
- Mainly through hepatic deacetylation, metabolism occurs.
- Excretion: 60–65% in bile, 30% in urine (mostly as metabolites) [18].

Additionally, rifampicin is a strong inducer of cytochrome P450 (CYP3A4) enzymes, which has a major effect on the pharmacokinetics of several other medications, such as oral contraceptives, antiretrovirals, and anticoagulants [19].

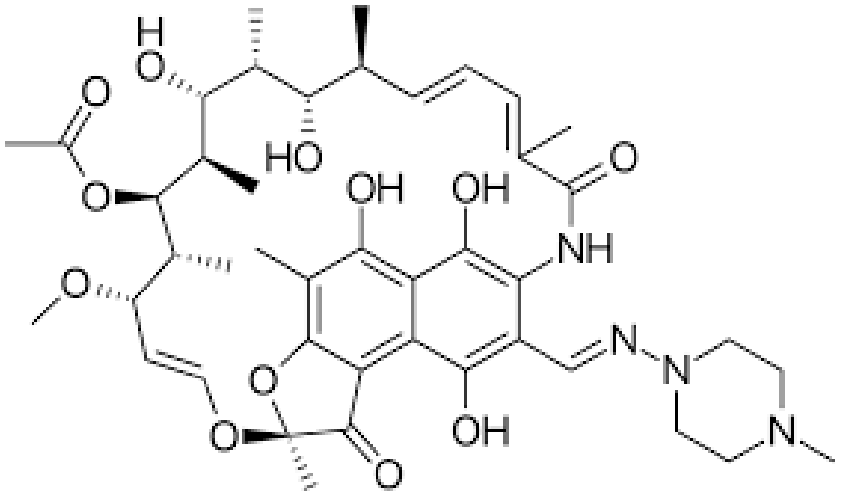

Fig 2: Chemical Structure of Rifampicin [20]

2.1.Isoniazid

A. Mechanism of Action

The antibacterial prodrug isoniazid (INH) has to be activated by the catalase-peroxidase enzyme KatG from M. tuberculosis. When active, it combines with NAD+ to produce a complex that prevents the formation of mycolic acids by InhA, an enoyl-acyl carrier protein reductase. The integrity and impermeability of the mycobacterial cell wall depend on mycolic acids. Bacterial mortality and lysis result from this disturbance [21].

B. Metabolism and Pharmacokinetics

- Bioavailability: around 100% absorption orally.

- Cmax: after 1–2 hours, 3–5 µg/mL was attained.
- Half-Life: Slow acetylators take around three hours, whereas fast acetylators take about one hour [22].
- Distribution: Broad, including the CNS and CSF (attained therapeutic levels).
- N-acetyltransferase 2 (NAT2) facilitates hepatic metabolism. Acetylation rate is influenced by genetic variations.
- Excretion: mostly renal (up to 75 percent in a 24-hour period) [23].

Side Effects: The biggest risk is hepatotoxicity, which is particularly dangerous for slow acetylators, alcohol users, and elderly people. Up to 20% of people without vitamin B6 supplements develop peripheral neuropathy [24].

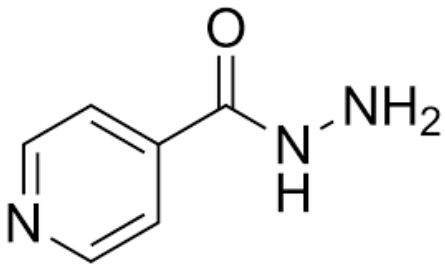

Fig 3: Chemical Structure ofIsoniazid [25]

2.2.Pyridoxine (Vitamin B6)

A. Role in TB Therapy

Pyridoxine is necessary to lessen isoniazid's neurotoxic effects. INH and pyridoxal phosphate, the active form of vitamin B6, combine to produce hydrazone complexes, which results in a functional deficit that hinders the production of neurotransmitters (such as GABA). Peripheral neuropathy is one way this shows up, especially in pregnant women, diabetics, alcoholics, and malnourished people [26].

- Dosage for prevention: 10–50 mg daily.
- Therapeutic Dosage: 100–200 mg daily (should neuropathy develop) [27].

B. Profile of Pharmacological

- Absorption: Effective absorption through the mouth.
- Dispersed widely; penetrates the blood-brain barrier and placenta.
- Metabolism: Mainly to pyridoxal phosphate and pyridoxamine phosphate in the liver.
- Renal excretion [28].

Pyridoxine promotes hemoglobin production, immunological function, and neurological health in addition to avoiding neuropathy. Its anti-inflammatory and neuroprotective properties in TB patients receiving long-term treatment are also being studied [29].

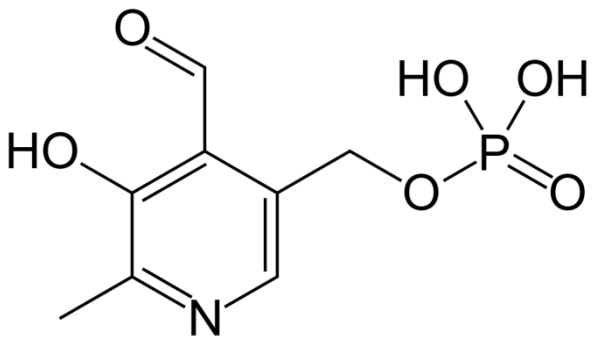

Fig 4:Chemical Structure ofPyridoxine (Vitamin B6) [30]

3. REGULATORY PERSPECTIVE ON ANALYTICAL METHOD VALIDATION

Validation of analytical methods is an essential regulatory need to guarantee the consistency, accuracy, and dependability of test findings, particularly in the quality control of anti-tuberculosis medications like pyridoxine, isoniazid, and rifampicin. To standardize validation procedures, regulatory organizations worldwide have released detailed recommendations that emphasize factors including specificity, linearity, accuracy, precision, detection limit, quantitation limit, robustness, and system appropriateness [31].

Consistency across national boundaries has been guaranteed by the harmonization of analytical technique validation requirements. The USFDA and EMA provide practical details that consider clinical relevance and data integrity, whereas ICH Q2(R1) provides the fundamental framework [32]. In the meanwhile, WHO and Indian Pharmacopoeia are essential in helping low-to-middle-income nations ensure the quality of their anti-TB medications. In addition to guaranteeing patient safety, compliance with these regulatory frameworks raises international confidence in pharmaceutical quality systems [33].

3.1.ICH Guidelines (Q2(R1))

For analytical validation, the International Council for Harmonization's (ICH) Q2(R1) guideline "Validation of Analytical Procedures: Text and Methodology" is regarded as the gold standard worldwide. Four types of analytical techniques are defined [34]:

- Tests for identification
- Quantitative impurity testing
- Limit impurity testing
- Quantitative analysis of active components

Important Validation Criteria for Q2(R1):

- **Specificity:** The capacity to evaluate the analyte when contaminants, degradants, and matrix are present [35].

- **Range and Linearity:** Need to be established across an appropriate concentration range, usually 80–120% of predicted.
- **Accuracy:** For assay procedures, a recovery of 98–102% is usually appropriate.
- Accuracy: Reliability and moderate accuracy (usually %RSD < 2%).
- **LOD & LOQ:** Determined using the slope technique or the standard deviation and signal-to-noise ratio [36].
- **Robustness:** The results shouldn't be impacted by small adjustments to the method's parameters.

The purpose of the Q14 and Q2(R2) drafts (2023) is to supplement these by providing further flexibility in the construction of analytical procedures through the use of Quality by Design (QbD) concepts [37].

3.2.USFDA and EMA Considerations

The European Medicines Agency (EMA) and the United States Food and Drug Administration (USFDA) both adhere to ICH Q2(R1) with the following extra region-specific requirements:

USFDA Recommendations

- Places a strong emphasis on compliance with 21 CFR Part 211 and Good Laboratory Practices (GLP) [38].
- Promotes the use of USP techniques, with complete validation for modified compendial or non-compendial techniques.
- The FDA advises the following for bioanalytical techniques (such as plasma drug levels): Matrix impact assessment, Biological matrix stability research and Reanalysis of the incident sample (ISR) [39].

EMA Regulations

- In accordance with EMA Guideline on Bioanalytical Method Validation (2011), but with a particular emphasis on bioanalytical validation in accordance with ICH Q2(R1) [40].
- Demands proof of the method's functionality in real-world research circumstances (e.g., freeze-thaw stability, re-injection repeatability).
- Both agencies require that New Drug Applications (NDAs) or Abbreviated NDAs (ANDAs) be accompanied by comprehensive validation reports that include statistical explanations and system appropriateness requirements [41].

3.3. WHO and Indian Pharmacopoeia Norms

WHO Guidelines

- In its Technical Report Series (TRS) No. 996, Annex 3, the World Health Organization (WHO) offers worldwide guidelines for the validation of analytical methods [42].
- Validation is emphasized by WHO in both product development and post-marketing monitoring.

- WHO-GMP principles are especially applicable in environments with limited resources when large quantities of TB medications are purchased and dispensed [43].

Indian Pharmacopoeia (IP)

- The General Chapters and particular monographs published by the Indian Pharmacopoeia Commission (IPC) have validation requirements.
- IP incorporates regional adaptation for infrastructure restrictions while adhering to ICH standards [44].
- The most often used validated techniques for isoniazid and rifampicin are TLC, UV-Vis, and HPLC.
- IP mandates: Use of IPC reference standards and the method transfer protocol's incorporation of validation parameters (LOD, LOQ, accuracy, and precision) [45].

Table 1: Comparative Overview of Regulatory Guidelines for Analytical Method Validation [46]

Regulatory Body	Guideline Title / Reference	Key Validation Parameters	Notable Requirements / Remarks
ICH	Q2(R1): Validation of Analytical Procedures	Range, Robustness, Accuracy, Precision, LOD, LOQ, Specificity, and Linearity	worldwide norm that is applicable to both new and pre-existing pharmacological substances and goods [47]
USFDA	Industry-Specific Guidelines for Validating Analytical Procedures and Methods (Chemistry, Manufacturing, and Controls)	identical to ICH + Bioanalytical technique validation for plasma drug levels, etc. [48]	needs matrix stability, ISR (Incurred Sample Reanalysis), and system appropriateness.
EMA	Guideline on Bioanalytical Method Validation (2011)	Every ICH parameter plus performance validation unique to the study [49]	Real-time research settings are emphasized, and biologics and generics are validated under GLP [50].

WHO	Annex 3 (Guidelines on Method Validation) of TRS No. 996	Like ICH: accuracy, linearity, specificity, etc.	Prioritize quality control in public health contexts, particularly for necessary medications such as anti-TB treatments [51].
Indian Pharmacopoeia (IPC)	Generic Method Validation Chapter + Monographs (e.g., Isoniazid, Rifampicin)	Specificity, LOD, LOQ, Accuracy, Precision, Range, Ruggedness [52]	Regionally flexible; prioritizes the application of verified HPLC/UV techniques and IPC reference standards [53].

4. KEY PARAMETERS OF METHOD VALIDATION

4.1.Accuracy

The degree of agreement between the experimental value derived from the analytical approach and the genuine value, or actual known value, is known as accuracy. It guarantees that the technique is accurately determining the concentration of an analyte in a sample. Accurate quantification is crucial for pharmaceutical applications, especially in fixed-dose combinations like isoniazid and rifampicin, to guarantee that patients receive the appropriate therapeutic dosage [54].

Recovery experiments, in which known amounts of the drug (spiked at various levels, such as 80%, 100%, and 120%) have been added to the formulation or matrix, are commonly used to evaluate accuracy. For instance, the accuracy of the Rifampicin tablet assay is 98% if the recovered quantity is 98 mg and the real drug concentration is 100 mg. In accordance with ICH and pharmacopeial criteria, the appropriate recovery range is often 98–102%. High precision reduces batch rejects and regulatory non-compliance in quality control systems [55].

4.2.Precision (Repeatability and Intermediate Precision)

The degree of concordance between a set of measurements derived from numerous samplings of the same homogenous sample is measured by precision. It assesses the method's repeatability in a range of scenarios [56].

- By testing the same sample several times in a single day under the same circumstances (same analyst, same equipment, same laboratory), repeatability (also known as intra-day precision) is assessed. It evaluates variability in the short term.
- Within the same lab, intermediate precision, also known as inter-day or analyst-to-analyst precision, is measured across several days, instruments, or analysts. It evaluates the consistency of the approach in more practical operating environments [57].

A relative standard deviation (RSD) of less than 2% is typically regarded as outstanding in TB medication validation, such as when Rifampicin and Isoniazid are validated using HPLC. Both ordinary batch release in the manufacturing process and drug development depend on accuracy [58].

4.3.Specificity

The capability of an analytical technique to detect and measure the analyte in the presence of other elements like as contaminants, degradation products, excipients, or additional active ingredients is known as specificity [59].

For combo medications, this becomes particularly crucial. For instance, even in the event of degradation or interaction (Rifampicin is known to breakdown in acidic medium or when in contact with Isoniazid), a technique must be able to differentiate and quantify Rifampicin without being influenced by Isoniazid or Pyridoxine [60].

- Studies of forced degradation (acid/base hydrolysis, oxidation, and photolysis) are carried out to assess specificity.
- Photodiode array (PDA) detectors are used to check for peak purity in chromatographic techniques (such as HPLC or LC-MS/MS).

For medications used in long-term TB treatment regimens, a particular technique guarantees that false positives or negatives are prevented during quality control testing [61].

4.4.Linearity and Range

The capacity of an analytical process to produce test findings that are exactly proportionate to the analyte concentration in the sample across a specified range is known as linearity. For quantitative tests to guarantee precise dosage representation, this is essential. To confirm:

- Create calibration curves that span 80–120% of the anticipated drug concentration using a minimum of 5–7 concentrations [62].
- For medicines, a correlation coefficient (R2) of ≥0.999 is generally considered satisfactory. The range is the range between the greatest and lowest analyte concentrations at which the method's linearity, accuracy, and precision are deemed acceptable. For instance: Assay range for rifampicin: 10–100 µg/mL, 5–50 µg/mL of isoniazid and 1–25 µg/mL of pyridoxine [63].

In regulatory submissions, establishing linearity guarantees accurate quantification throughout both low-dose and high-dose studies, which is essential for bioavailability and dissolution testing [64].

4.5.Limit of Detection (LOD) and Limit of Quantitation (LOQ)

These metrics serve as markers of the analytical method's sensitivity.

- LOD is the lowest analyte concentration that is detectable but not always quantifiable. The signal-to-noise (S/N) ratio of 3:1 is frequently used to calculate it.
- LOQ, which is usually at an S/N ratio of 10:1, is the lowest concentration that can be accurately measured [65].

In stability testing, residual solvent analysis, and impurity profiling, these factors are very crucial. For instance, low LOD/LOQ guarantees reliable analytical performance when identifying trace amounts of degraded Rifampicin or looking for residual Isoniazid in plasma [66].

They are computed as follows:

- **LOD = 3.3 × (SD/Slope)**
- **LOQ = 10 × (SD/Slope)**

Where Slope comes from the calibration curve and SD is the response's standard deviation.

4.6.Robustness

Robustness assesses an analytical technique's dependability when minor, intentional changes are made to its parameters, offering information on the stability of the approach under typical circumstances [67].

The following typical characteristics are tested:

- Mobile phase pH fluctuation (±0.2 units)
- Variations in temperature (±5°C)
- Variation in flow rate (±0.1 mL/min)
- A shift in the wavelength of detection (±2 nm)

For instance, even in cases when the mobile phase's pH fluctuates significantly, a validated HPLC technique for isoniazid should reliably produce peak retention and resolution. Throughout large-scale production and quality assurance testing, robustness testing makes sure that small changes in the laboratory or the surrounding environment don't impact the accuracy and precision of the results [68].

4.7. System Suitability Testing (SST)

System Adequacy Prior to sample analysis, tests are a series of parameters carried out to ensure that the system operates as intended. They attest that the tool and technique are appropriate for the planned analysis. Important SST parameters consist of:

- For every analyte, the retention time (tR) can be replicated [69].
- For optimal efficiency, theoretical plates (N) should be greater than 2000.
- A symmetrical peak is indicated by a tailing factor (T) of less than 2.
- Resolution (Rs): There should be more than two peaks between two neighboring peaks.

- To guarantee repeatability, the percentage RSD of duplicate injections should be less than 2% [70].

For instance, before evaluating the bulk samples in HPLC of a fixed-dose combination of pyridoxine, isoniazid, and rifampicin, SST verifies that all peaks are properly resolved and system performance is constant.

5. ANALYTICAL TECHNIQUES FOR DRUG ESTIMATION

5.1.UV-Visible Spectrophotometry

A well-established and popular analytical method for estimating pharmaceutical substances, particularly in bulk and dose forms, is UV-visible spectrophotometry. This approach is predicated on the idea that molecules absorb light at certain visible or ultraviolet wavelengths in proportion to their concentration. The absorbance maxima for anti-tubercular medications are around 337 nm and 475 nm for Rifampicin, 263 nm for Isoniazid, and 290 nm for Pyridoxine. Beer-Lambert's law is usually used for quantification [71].

Despite being an inexpensive and simple technique, UV spectrophotometry has drawbacks. Low specificity is the main disadvantage since accuracy might be hampered by overlapping absorbance spectra and interference from excipients or degradation products. Furthermore, matrix interference makes it unsuitable for biological analysis of specimens.

Key Points:

- Fast and affordable for routine analysis
- Appropriate for straightforward formulations
- Less sensitive for detection at the trace level
- Limited selectivity in complicated matrices [72]

5.2.High-Performance Liquid Chromatography (HPLC)

Because of its great sensitivity, precision, and specificity, HPLC is regarded as the gold standard for estimating anti-TB medications. Under high pressure, it separates compounds according to how they interact with the stationary and mobile phases. For single-component analysis, isocratic elution—constant mobile phase composition—is usually utilized, whereas gradient elution—varying solvent ratio—is used for multi-drug combinations to improve peak form and resolution [73].

HPLC techniques frequently employ reverse-phase C18 columns with mobile phases such as acetonitrile-water, phosphate buffers, or methanol for rifampicin, isoniazid, and pyridoxine. Depending on the substance, UV detectors are used for detection; they are typically set between 254 and 475 nm.

HPLC is very appropriate for stability studies, pharmacokinetic analyses, and regulatory filings, despite the expense of the equipment and upkeep.

Key Points:

- Efficient in time with automated injection and data processing.
- Multifunctional for multi-drug analysis and biological matrices.
- High equipment and solvent expenses.
- Outstanding precision, accuracy, and linearity [74]

5.3.High-Performance Thin Layer Chromatography (HPTLC)

HPTLC is a more sophisticated kind of TLC that provides more automation, repeatability, and resolution. It is especially useful when estimating many medications at once in a single run. On a silica gel plate, drug spots are placed, developed with the proper mobile phases, and then densitometrically scanned.

HPTLC is helpful for stability-indicating tests because it can distinguish between the active ingredients and degradation products in anti-TB medications. Additionally, it is utilized to ensure the quality of herbal extracts used as adjuvants in tuberculosis treatment [75].

Nevertheless, HPTLC is not the best method for trace-level measurement in biological materials and could not be equally sensitive as HPLC or LC-MS/MS.

Key Points:

- Good for quality control and screening
- Permits simultaneous multi-drug quantification
- Has a lower sensitivity than HPLC
- Limited application in clinical pharmacokinetics [76].

5.4.LC-MS/MS and Other Advanced Techniques

The most advanced analytical method for measuring anti-TB medications is liquid chromatography combined with tandem mass spectrometry (LC-MS/MS), particularly in cellular environments like plasma or urine. It combines mass spectrometry's high sensitivity and selectivity with HPLC's capacity for separation.

Pharmacokinetic/pharmacodynamic (PK/PD) modeling, bioequivalence investigations, and therapeutic medication monitoring all depend on this method. For example, Rifampicin, Isoniazid, and Pyridoxine may all be quantified simultaneously in picogram levels using LC-MS/MS with a small sample size [77].

Despite being extremely exact and precise, the main obstacles include complicated data interpretation, high operating costs, and the requirement for expert operators.

Key Points:

- Ultra-sensitive and extremely specific
- Perfect for clinical and research applications
- Costly and technically complex
- Favoured in sophisticated analytical and regulatory laboratories [78].

Table 2: Comparison of Analytical Techniques for Anti-TB Drug Estimation [79]

Parameter	**UV-Visible Spectrophotometry**	**HPLC**	**HPTLC**	**LC-MS/MS**
Sensitivity	Low to Moderate	High	Moderate	Very High
Specificity	Low (interference from excipients)	High	Moderate to High	Very High
Sample Matrix Compatibility	Limited to pure/bulk forms	Suitable for plasma, urine, etc.	Mainly formulation-based	Excellent for biological samples
Detection Limit	µg/mL	ng/mL to µg/mL	µg/mL	pg/mL to ng/mL
Accuracy & Precision	Moderate	High	Moderate	Very High
Throughput	High (simple analysis)	Moderate (depends on method)	High (multiple samples per plate)	Low to Moderate
Cost of Equipment	Low	High	Moderate	Very High
Complexity of Operation	Low	Moderate	Moderate	High
Automation	Limited	Fully automatable	Semi-automated	Fully automated
Regulatory Acceptability	Limited	Widely accepted by ICH, FDA, WHO	Accepted for QC, not PK studies	Gold standard for PK/PD & BE studies

Applications	Routine quality control, dosage form	Stability, QC, PK/PD, formulations	Herbal/complex matrix, screening	Clinical studies, bioanalysis, research

6. METHOD VALIDATION STUDIES

6.1. Rifampicin: Reported Validation Studies

A key component of TB treatment, rifampicin (RIF) requires exact analytical techniques for measurement. A quick and accurate RP-HPLC technique with UV detection at 219 nm for rifampicin in pharmaceutical formulations was created by Gadi Vijaya Lakshmi et al. With a correlation value of 0.9957 and a retention time of less than three minutes, the approach showed linearity. High accuracy was indicated by the relative standard deviation (RSD) value of 0.20% [80].

Rifampicin and other associated chemicals were analysed using a C18 monolithic column in another investigation. With limits of detection (LOD) and quantification (LOQ) of 0.2 µg/mL and 1 µg/mL, respectively, the method's overall run time was less than 11 minutes. The recoveries varied from 99.7% to 100.5%, and the intra- and inter-day RSDs were all less than 2.5 percent.

6.2. Isoniazid: Reported Validation Studies

Another first-line anti-TB medication that needs precise measurement is isoniazid (INH). In order to quantify Rifampicin, Isoniazid, and their metabolites in human plasma simultaneously, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach was created and validated. Excellent linearity ($r2 \geq 0.995$), accuracy (90-115%), and precision (CV% <14) were all displayed by the approach. The technique worked well for a clinical research with thirty-three healthy participants [81].

Furthermore, an HPLC technique was created for the simultaneous measurement of isoniazid and rifampicin in tablet and bulk dose forms. With detection at 239 nm, the technique used a mobile phase consisting of methanol, water, and pH 3.5 phosphate buffer in a 45:30:25 ratio. Rifampicin and isoniazid had retention durations of 2.8 and 3.7 minutes, respectively. The technique demonstrated good accuracy with RSD values within allowable bounds and significant recovery rates within ICH recommendations [82].

6.3. Pyridoxine: Reported Validation Studies

Isoniazid and pyridoxine (Vitamin B6) are frequently used together to avoid peripheral neuropathy. An RP-HPLC technique for the simultaneous measurement of pyridoxine hydrochloride and doxylamine succinate in bulk and pharmaceutical dose forms was created and validated in a research. With a phosphate buffer (pH 5): methanol (40:60) mobile phase and a flow rate of 1 mL/min, the technique employed a Kromosil-C18 column with detection at 263 nm. Pyridoxine showed linearity with a

correlation value of 0.9992 in the concentration range of 5–25 µg/mL. The technique showed recovery values between 99.8% and 100.4% and good accuracy (RSD <2%) [83].

6.4. Simultaneous Estimation Methods for Combination Formulations

For formulations of fixed-dose combinations (FDCs), simultaneous estimate of anti-TB medications is essential. An HPLC technique for the simultaneous measurement of rifampicin, isoniazid, pyrazinamide, and ethambutol hydrochloride in 4-FDC tablets was created and validated in a research. A Waters Symmetry C8 column was used to accomplish the separation. A gradient elution method was used to supply a mobile phase consisting of acetonitrile and 20 mM phosphate buffer solution (pH 6.8) incorporating triethylamine at a rate of 1.5 mL/min. At 210 nm, detection was carried out. According to ICH criteria, the method's robustness, detection limit, quantification limit, accuracy, precision, linearity, and selectivity were all verified. The Indonesian Pharmacopoeia was followed by the drug levels in the 4-FDC tablets [84].

A reverse-phase HPLC technique for the simultaneous measurement of rifampicin, isoniazid, pyrazinamide, and ethambutol hydrochloride in pharmaceutical formulations was developed and validated, according to another study. Using a gradient elution technique and a mobile phase consisting of 20 mM monobasic sodium phosphate buffer with 0.2% triethylamine (pH 7.0) and acetonitrile at a flow rate of 1.5 mL/min, chromatographic separation was performed on a Purospher STAR RP18e column. A diode array detector was used to detect rifampicin, isoniazid, and pyrazinamide at 238 nm and ethambutol at 210 nm. The approach demonstrated robustness, accuracy, precision (RSD <2%), linearity (r2 > 0.99), and specificity [85].

7. CHALLENGES IN ANALYTICAL METHOD DEVELOPMENT FOR ANTI-TB DRUGS

7.1. Stability Concerns

One significant issue that affects the precision, dependability, and repeatability of analytical techniques is the stability of anti-TB medications throughout analysis. Throughout sample collection, storage, or processing, environmental conditions including temperature, light, humidity, and pH may cause these medications to degrade [86].

Key Challenges:

- **Chemical Instability**: Under specific circumstances, a number of anti-TB medications, such as isoniazid and rifampicin, are prone to deterioration or change into related contaminants due to their chemical instability. Rifampicin, for example, is sensitive to both basic and acidic pH, which might cause oxidation or hydrolysis and compromise the accuracy of the test.
- **Photo degradation**: Certain anti-TB medications may undergo photo degradation when exposed to light, which might reduce their effectiveness and produce breakdown products that could obstruct analytical tests.
- **Storage and Handling**: In certain lab or field settings, it may be difficult to strictly regulate storage conditions, which include refrigeration or moisture protection, which is necessary to

maintain medication stability. Drug breakdown prior to analysis due to inadequate storage might result in inaccurate quantification [87].

- **Stability-Indicating Techniques:** It's crucial yet difficult to create techniques that can distinguish and measure the intact medication from its breakdown products. To show that stability-indicating assays can reliably identify and measure all pertinent degradation products, they must undergo thorough validation.
- **Sample Preparation:** Stability may also be impacted by the selection of solvents, buffers, and extraction techniques. Method developers must cautiously modify these parameters since some solvents may promote drug breakdown.

7.2. Drug Interactions in Fixed-Dose Combinations

Numerous active pharmaceutical ingredients (APIs) are combined into a single formulation in fixed-dose combos (FDCs) of anti-TB medications to increase patient compliance and lessen pill burden. These combinations, however, provide difficult analytical problems [88].

Key Challenges:

- **Chemical Interactions:** During formulation or storage, APIs in FDCs may interact chemically, resulting in degradation or the creation of new contaminants. For instance, isoniazid and rifampicin might combine to produce hydrazone derivatives, which make analysis more difficult [89].
- **Differential Stability:** Because the stability profiles of the individual medications in a combination may differ, the analytical technique must be able to identify and quantify each component simultaneously without interfering with one another.
- **Complex Chromatographic Separation:** In chromatographic techniques (such as HPLC or LC-MS), co-eluting peaks or overlapping signals make it more difficult to separate each drug and its breakdown products. Developing a technique with optimum mobile phases, gradients, and detecting wavelengths is necessary for this.
- **Matrix Effects in Formulations:** To guarantee specificity, a comprehensive technique validation is required since excipients and other inactive substances in the FDC may obstruct drug detection or extraction.
- **Dosage Ratio Differences:** The analytical technique must be sensitive and linear enough to precisely measure both high- and low-dosage components since medicines in FDCs may be present at widely variable dose ratios.
- **Regulatory Expectations:** The development and validation of methods are made more difficult by regulatory bodies' requirements for thorough impurity profile for every component in an FDC [90].

7.3. Matrix Interference in Biological Samples (e.g., Plasma/Serum)

Pharmacokinetic research, therapeutic drug surveillance, and bioequivalence testing all depend on the quantification of anti-TB medications in biological matrices such as serum or plasma. Matrix interference, however, presents formidable analytical challenges.

Key Challenges:

- **Complex of Biological Matrices:** Proteins, lipids, salts, and endogenous metabolites found in plasma and serum might obstruct the extraction and detection of drugs. These elements may result in overlapping peaks in chromatographic techniques or ion suppression or amplification in mass spectrometry [91].
- **Protein Binding:** A lot of anti-TB medications have significant levels of plasma protein binding, which makes extraction more difficult and may have an impact on the observed free drug concentration. It is necessary to create effective and repeatable sample preparation methods such liquid-liquid extraction, solid-phase extraction, and protein precipitation.
- **Low Drug Concentrations:** Some anti-TB medications have therapeutic concentrations that are extremely low, necessitating the use of extremely sensitive testing techniques. The limit of detection may be raised or signals may get obscured by matrix elements.
- **Metabolite Interference:** Without ideal chromatographic conditions, it might be challenging to distinguish parent medications from metabolites of anti-TB medications due to their comparable physicochemical characteristics.
- **Variability among Samples:** Diet, illness, and medication all affect biological matrices, which increase variability to matrix effects and make methods less reliable and repeatable.
- **Sample Stability:** After collection, biological samples are vulnerable to enzymatic activity and destruction, necessitating quick processing or the inclusion of stabilizers to protect analytes [92].
- **Internal Standards:** To account for matrix effects and guarantee quantitative accuracy, it is essential to choose appropriate internal standards that exhibit behavior comparable to the target analytes throughout extraction and analysis.

8. APPLICATIONS OF VALIDATED METHODS

8.1. Pharmaceutical Dosage Form Analysis

The exact and accurate quantification of excipients and active pharmaceutical ingredients (APIs) in dosage forms such tablets, capsules, injectables, and fixed-dose combinations depends on validated analytical techniques.

Applications:

- **Assay of Active Ingredients:** Validated techniques such as HPLC, UV spectrophotometry, or TLC are used to precisely quantify APIs in order to guarantee that the right dosage is present in the formulation. This is crucial for anti-TB medications since incorrect dosage might result in resistance or treatment failure [93].

- **Content Uniformity Testing:** Techniques evaluate how uniformly drugs are distributed in different dose units, guaranteeing constant safety and effectiveness.
- **Analysis of Impurities and Degradation Products:** Verified stability-indicating techniques identify impurities or degradation products produced during production or storage, ensuring formulation safety and adherence to legal requirements.
- **Identification and Confirmation:** To prevent the use of phony or inferior medicines, analytical methods aid in confirming the identification of medications in combination formulations [94].

8.2. Bioanalytical and Pharmacokinetic Studies

In order to measure drug concentrations in biological matrices (blood, plasma, serum, and urine) for pharmacokinetic (PK) profiling, therapeutic drug monitoring, and pharmaceutical interaction research, validated bioanalytical techniques are essential.

Applications:

- **Pharmacokinetic Profiling:** Determining absorption, distribution, metabolism, and excretion (ADME) factors is made easier by precise measurement of anti-TB medications and their metabolites across time. These studies aid in optimizing therapy effectiveness by guiding dosage schedules [95].
- **Therapeutic Drug Monitoring (TDM):** Approved techniques guarantee accurate drug level measurements in patients to keep concentrations inside the therapeutic range window, reducing resistance and toxicity.
- **Bioavailability and Bioequivalence Studies:** Techniques for comparing innovator products with generic formulations in order to determine regulatory approval and equivalent.
- Analytical techniques are used in drug-drug interaction studies to evaluate how concurrent drugs affect drug levels, which is crucial in multi-drug anti-TB regimens [96].

8.3. Quality Control and Assurance

The foundation of pharmaceutical manufacturing's quality assurance (QA) and control (QC) procedures is comprised of validated analytical techniques, which guarantee that goods fulfill predetermined standards.

Applications:

- **Batch Release Testing:** Prior to being released into the market, every production batch is subjected to thorough testing for assay, contaminants, dissolution, and other characteristics using approved techniques.
- **In-Process Controls:** Techniques that keep an eye on important quality characteristics throughout production allow for prompt action to preserve product quality [97].
- **Regulatory Compliance:** To produce trustworthy data for product registration, audits, and inspections by regulatory bodies such as the FDA, EMA, or CDSCO, QA uses proven methodologies.

- **Stability Testing:** To guarantee safety and effectiveness until the product's expiry date, validated techniques are used to track the chemical and physical stability of goods over the course of their shelf life.

8.4. Dissolution and Stability Testing

While stability testing evaluates how medication quality changes over time under different environmental circumstances, dissolution testing gauges the rate and magnitude of drug release from a dosage form. Both are essential for determining the shelf life and performance of the product.

Applications:

- **Dissolution Testing:** Reliable analytical techniques measure the quantity of medication dissolved over time in a given medium, forecasting the behavior of drug release in vivo. To guarantee continuous bioavailability, this is particularly crucial for anti-TB medications [98].
- **Formulation Development:** By comparing various batches or generic goods to innovator references, formulation improvement is guided by dissolution data.
- **Determination of Shelf Life:** Validated assays are used in stability testing conducted under long-term and accelerated circumstances to identify deterioration, guaranteeing that the medication is secure and successful during storage.
- **Evaluation of Packaging:** Analytical techniques provide the evaluation of how packaging materials affect drug stability and dissolution, which is crucial for anti-TB medications that are frequently kept in environments with restricted resources.
- **Regulatory Submissions:** Regulatory dossiers must include stability and dissolution data produced using approved techniques [99].

9. FUTURE PERSPECTIVES AND RECOMMENDATIONS

9.1. Use of Green Analytical Chemistry

By lowering the use of hazardous chemicals, waste, energy, and enhancing safety, Green Analytical Chemistry (GAC) aims to lessen the negative effects of analytical procedures on the environment.

Future Directions:

- **Environmentally friendly Solvents:** Toxic organic solvents like methanol or acetonitrile are frequently used in traditional analytical techniques. To lessen toxicity and environmental risks, future techniques will give preference to greener solvents like water, ethanol, or bio-based solvents [100].
- **Waste Reduction:** Chemical waste will be reduced by creating techniques with small amounts of samples and reagents. Solid-phase microextraction (SPME), solvent-free sample preparation, and microextraction procedures are becoming more and more popular.
- **Energy Efficiency:** Analytical devices will develop to run at room temperature and without the need for high pressures or temperatures, and they will use less electricity.

- **Sustainable Instrumentation:** In line with international sustainability goals, innovations in the creation of instruments will prioritize recyclability and reduced resource use.
- **Regulatory Support:** Promoting the use of greener techniques by regulatory bodies will help them be widely adopted, which is crucial in high-volume analysis for anti-TB medications [101].

9.2. Miniaturized and Automated Methods

Analytical technique automation and miniaturization increase productivity, lower costs, and improve reproducibility—all of which are critical for high-throughput capabilities pharmaceutical analysis.

Future Directions:

- **Microfluidic Channels:** Lab-on-a-chip technologies expedite analysis by combining sample preparation, separation, and detection in small devices, significantly lowering sample and reagent quantities.
- **Automated Sample Preparation:** By streamlining time-consuming sample processing procedures including extraction, filtration, and dilution, robotic equipment and automated liquid handling will lower human error.
- **High-Throughput Screening:** Automated techniques and miniature assays allow for the quick examination of several samples at once, which is advantageous for stability testing and drug formulation screening.
- **Compatibility with Analytical Instruments:** LC-MS or other analytical platforms may be seamlessly integrated with automated sample preparation to improve throughput and technique consistency.
- **Point-of-Care Testing:** By enabling near-patient screening for therapeutic drug monitoring of anti-TB medications, miniature analytical instruments may enhance customized therapy.

9.3. AI/ML Applications in Method Optimization

By anticipating ideal parameters, minimizing trial-and-error tests, and improving data interpretation, artificial intelligence (AI) and machine learning (ML) are becoming more potent tools for optimizing analytical techniques.

Future Directions:

- **Automation of Methodology Development:** AI systems may examine historical data on method development to recommend the best chromatographic parameters (such as gradient profiles or mobile phase makeup) or sample preparation methods.
- **Predictive Stability Modelling:** Without requiring extensive experimental effort, ML models may forecast drug degradation routes and stability under a range of circumstances, assisting in the development of stability-indicating techniques.
- **Pattern Recognition in Complex Data:** Artificial intelligence (AI)-driven software can decipher intricate chromatograms or spectra and spot contaminants or co-eluting peaks that human analysis could overlook.

- **Adaptive Quality Control:** During production, AI systems may track real-time analytical data, quickly identifying irregularities and recommending remedial measures.
- **Applications in Personalized Medicine:** By combining patient characteristics and pharmacokinetic data, machine learning may be able to customize analytical techniques for therapeutic medication monitoring, improving TB treatment results [102].

10. CONCLUSION

To guarantee the safety, effectiveness, and caliber of tuberculosis therapies, it is essential to establish strong, dependable, and verified analytical techniques for anti-TB medications. Important issues that make precise drug analysis more difficult have been brought to light by this study, including stability issues, drug interactions in fixed-dose combinations, and interference from matrix molecules in biological materials. Validated techniques continue to be the mainstay of pharmaceutical dosage form analysis, bioanalytical research, quality assurance, and stability testing in spite of these difficulties. Throughout the course of the product lifecycle, their use ensures that the active pharmaceutical components are administered at the appropriate dosages, contaminants are managed, and medicine performance is continuously tracked. These analytical methods are crucial for improving treatment results, reducing toxicity, and fighting drug resistance globally since TB therapy frequently entails intricate multi-drug regimens.

In the future, there are encouraging opportunities to advance TB drug analytics through the combination of downsizing and automation technologies, AI/ML-driven technique improvement, and green analytical chemistry principles. These developments have the potential to revolutionize the analysis and monitoring of anti-TB medications by improving accuracy, increasing throughput, and improving environmental sustainability. To overcome current analytical challenges and stay up with changing treatment approaches and regulatory requirements, research and development expenditures must be sustained. More effective, economical, and patient-focused analytical solutions will be made possible by promoting interdisciplinary cooperation and implementing state-of-the-art technology. All things considered, persistent attempts to improve analytical techniques will be crucial to the worldwide battle against TB by promoting safer, more efficient treatment plans and guaranteeing that those in need receive high-quality pharmaceutical supplies.

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