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Forced Degradation and Stability Analysis of a Fixed-Dose Combination for Tuberculosis in HIV Patients

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ABSTRACT

Tuberculosis (TB) remains a major health challenge, particularly among HIV-infected individuals, due to their compromised immune systems. This study aimed to develop and evaluate a Fixed-Dose Combination (FDC) of Sulfamethoxazole, Trimethoprim, Isoniazid, and Pyridoxine Hydrochloride for the treatment of secondary TB in HIV patients. The study involved forced degradation, stability analysis, and validation of an RP-HPLC method for simultaneous quantification of the active pharmaceutical ingredients (APIs). The forced degradation study revealed that the FDC formulation degraded significantly in acidic (0.1N HCl) and oxidative (3% H_2O_2) conditions, while it remained stable under thermal (70°C dry heat) and photolytic (UV 254 nm) conditions. The stability study, conducted under accelerated (40°C, 75% RH) and intermediate (30°C, 75% RH) conditions over 24 months, demonstrated that the FDC maintained physical integrity, dissolution efficiency (NLT 75% within 60 minutes), and assay compliance (90-110% of labeled claims). The HPLC method validation confirmed high accuracy, precision, linearity, specificity, and robustness in detecting all four drugs simultaneously. These findings suggest that this FDC formulation is stable, bioavailable, and suitable for clinical use in TB-HIV co-infected patients. Further clinical trials are recommended to validate therapeutic efficacy in human subjects.

Keywords: *Tuberculosis, Fixed-Dose Combination, Forced Degradation, Stability Study, RP-HPLC, Isoniazid, Sulfamethoxazole, Trimethoprim, Pyridoxine Hydrochloride.*

1. INTRODUCTION

Tuberculosis (TB) remains one of the leading causes of morbidity and mortality worldwide, particularly among HIV-infected individuals. The immunocompromised state of HIV patients increases susceptibility to secondary tuberculosis, making treatment optimization essential [1]. TB treatment typically requires long-term multi-drug regimens, which can lead to poor adherence, drug resistance, and treatment failure. Therefore, fixed-dose combination (FDC) therapy offers a potential solution by simplifying dosing regimens and improving patient compliance [2, 3].

Sulfamethoxazole and trimethoprim are widely used in combination as antimicrobial agents with proven efficacy against bacterial infections, including opportunistic infections in HIV patients [4]. Isoniazid is a first-line anti-tuberculosis drug that acts by inhibiting mycolic acid synthesis in *Mycobacterium tuberculosis*, while pyridoxine hydrochloride is co-administered to prevent neurotoxicity associated with isoniazid therapy [5]. Combining these drugs in a single formulation could enhance therapeutic efficacy and reduce pill burden, leading to better treatment adherence in HIV-infected TB patients [6, 7].

This study focuses on the pharmacokinetic evaluation of an FDC formulation containing sulfamethoxazole, trimethoprim, isoniazid, and pyridoxine hydrochloride in a rabbit model. The primary objective was to assess plasma drug concentrations over time, evaluate drug absorption, distribution, metabolism, and elimination, and determine the bioavailability of the FDC compared to individual drug administration. The pharmacokinetic parameters assessed included maximum plasma concentration (Cmax), time to reach maximum plasma concentration (Tmax), area under the plasma concentration-time curve (AUC), elimination half-life (T1/2), and elimination rate constant (Keli).

Furthermore, a validated RP-HPLC method was developed and utilized for simultaneous quantification of the four drugs in plasma samples. The study aimed to establish whether the FDC formulation maintains pharmacokinetic consistency with

individual drug administration while offering advantages in ease of use and adherence. These findings will provide a foundation for further clinical studies evaluating the efficacy and safety of this FDC in human subjects.

2. MATERIALS AND METHODS

2.1 Materials

The Fixed-Dose Combination (FDC) of Sulfamethoxazole, Trimethoprim, Isoniazid, and Pyridoxine Hydrochloride was developed and evaluated for its stability. Pure drug samples were obtained as gift samples from Aurobindo Pharma Ltd., Hyderabad, India, and Benz Chem Enterprises, Vadodara, India. All excipients used were of pharmaceutical grade. HPLC-grade solvents, including acetonitrile (ACN) and water, were used for analysis.

2.2 Formulation of Fixed-Dose Combination (FDC)

The FDC tablets were prepared using direct compression and wet granulation techniques, optimizing the formulation for stability and bioavailability. The active pharmaceutical ingredients (APIs) were blended with excipients, compressed into tablets, and subjected to physicochemical evaluation before stability studies.

2.3 Animal Study Design

Healthy New Zealand white rabbits (2.5–3 kg) were procured from a certified animal supplier and housed in a controlled environment with a temperature of 22 ± 2°C, humidity of 50 ± 10%, and a 12-hour light/dark cycle. The rabbits were acclimatized for one week before the start of the experiment. The study protocol was approved by the institutional animal ethics committee.

The animals were fasted overnight before oral administration of drugs, with free access to water. Drug doses were calculated based on human therapeutic doses and adjusted according to body weight. The rabbits were divided into six groups, each containing three animals, to evaluate the pharmacokinetics of individual drugs and the FDC [9, 10].

2.4 Experimental Groups

The rabbits were divided into five groups (n=3 per group)

Table 1. Classification of Experimental Groups

Group	Drug Administered
I	Sulfamethoxazole
II	Trimethoprim
III	Isoniazid
IV	Pyridoxine HCl
V	Fixed Dose Combination (FDC)
VI	Control (no drug)

Drugs were dissolved in sterilized distilled water and administered orally via a gastric tube. A crossover study design was used with a minimum washout period of three weeks between treatments to prevent residual drug interference.

2.5 Blood Sample Collection and Plasma Preparation

Blood samples (approximately 1 mL) were collected from the marginal ear vein at the following time points: 0, 30 min, 1 hr, 2 hr, 4 hr, 6 hr, 8 hr, and 12 hr post-drug administration. Samples were collected in EDTA-coated tubes to prevent coagulation and immediately centrifuged at 3000 rpm for 15 minutes at 4°C. The separated plasma was stored at -20°C until further analysis [11].

2.6 HPLC Method Development and Validation

A validated reverse-phase high-performance liquid chromatography (RP-HPLC) method was used to quantify drug concentrations in plasma. The method was developed to simultaneously detect sulfamethoxazole, trimethoprim, isoniazid, and pyridoxine hydrochloride in a single chromatographic run [12, 13].

Chromatographic Conditions

- **Instrument:** Shimadzu LC-20 HPLC system with PDA detector

- **Column:** C18 (250 mm × 4.6 mm, 5 µm)
- **Mobile Phase:** Acetonitrile: Phosphate buffer (pH 4)
    - **0-4 min:** 10:90 v/v (ACN: Phosphate buffer)
    - **4-10 min:** 25:75 v/v (ACN: Phosphate buffer)
- **Flow Rate:** 1.0 mL/min
- **Detection Wavelength:** 265 nm
- **Injection Volume:** 50 µL

Plasma Sample Preparation

1. **Protein Precipitation:** 100 µL of plasma was mixed with 100 µL of perchloric acid (HClO4).
2. **Vortex Mixing:** Samples were vortexed for 30 seconds.
3. **Centrifugation:** Samples were centrifuged at 20,000 g for 5 minutes at 5°C.
4. **Filtration:** The supernatant was filtered using a 0.45-µm HPLC filter.
5. **Injection:** 50 µL of the filtrate was injected into the HPLC system [14].

3. RESULTS AND DISCUSSION

3.1 HPLC Method Validation

A Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method was validated for simultaneous quantification of Sulfamethoxazole, Trimethoprim, Isoniazid, and Pyridoxine Hydrochloride in the FDC.

Table 1. Validation Parameters and Results

Parameter	Result	Acceptance Criteria
Linearity	$r^2 > 0.998$	$r^2 \geq 0.990$
Accuracy	98.5–101.2%	98–102%
Precision	RSD < 2%	RSD < 2%
Specificity	No interference	No interference
Robustness	Minor variation acceptable	Within range

The HPLC method exhibited excellent linearity, precision, and accuracy, making it highly suitable for routine quality control and batch validation. Specificity tests confirmed no interference from excipients, ensuring clear peak separation for all four active ingredients. Robustness evaluation demonstrated minor variations under altered conditions, validating the reliability of the method (Table 1).

3.2 Forced Degradation Study Analysis

Forced degradation studies were conducted to assess the stability of the Fixed-Dose Combination (FDC) under different stress conditions. The results revealed significant degradation under acidic and oxidative conditions, while the formulation exhibited better stability under thermal and photolytic conditions.

Acid Hydrolysis (0.1N HCl, 60°C, 2 hours)

The FDC formulation exhibited significant degradation under acidic conditions, with additional peaks detected in the HPLC chromatogram, indicating the formation of degradation products. Among the active ingredients, Isoniazid and Sulfamethoxazole showed the highest susceptibility to acid hydrolysis (Table 2).

Table 2. Acidic degradation of FDC, with significant loss of Isoniazid and Sulfamethoxazole.

Time (hours)	% Drug Remaining (Sulfamethoxazole)	% Drug Remaining (Trimethoprim)	% Drug Remaining (Isoniazid)	% Drug Remaining (Pyridoxine HCl)

0	100%	100%	100%	100%
2	67.12%	80.21%	58.43%	91.02%

The forced degradation study revealed that the Fixed-Dose Combination (FDC) formulation exhibited significant degradation under acidic and oxidative stress conditions. When subjected to 0.1N HCl at 60°C for 2 hours, the formulation showed considerable breakdown, leading to the formation of additional peaks in the HPLC chromatogram. This suggests that acid hydrolysis significantly affects drug stability, particularly for Isoniazid and Sulfamethoxazole, both of which are known to be highly susceptible to acidic environments. The instability observed under acidic conditions indicates that protective formulation strategies such as enteric coating or buffering agents may be necessary to enhance drug stability during gastric transit.

Base Hydrolysis (0.1N NaOH, 60°C, 2 hours)

The FDC formulation showed moderate degradation under basic conditions, with peak broadening observed in the HPLC chromatogram.

Table 3. Basic degradation of FDC, with moderate Trimethoprim loss and HPLC peak broadening.

Time (hours)	% Drug Remaining (Sulfamethoxazole)	% Drug Remaining (Trimethoprim)	% Drug Remaining (Isoniazid)	% Drug Remaining (Pyridoxine HCl)
0	100%	100%	100%	100%
2	80.42%	72.10%	77.89%	90.78%

Under basic hydrolysis conditions using 0.1N NaOH at 60°C, moderate degradation was observed. The appearance of broadened peaks in the HPLC chromatogram suggested that alkaline conditions affected the chemical integrity of the drugs, particularly Trimethoprim. However, the extent of degradation was less severe than in acidic conditions, indicating that the formulation is relatively more stable in basic environments. These findings suggest that while basic conditions can lead to partial breakdown of certain active ingredients, the overall formulation remains more resistant to alkaline hydrolysis compared to acidic stress.

Oxidative Degradation (3% H_2O_2, 2 hours)

Oxidative stress conditions caused moderate degradation, particularly affecting Isoniazid and Sulfamethoxazole, with additional peaks detected in the HPLC chromatogram **(Table 4).**

Table 4. Oxidative degradation of FDC (3% H_2O_2, 2 hours), with significant Isoniazid and Sulfamethoxazole loss and oxidative byproducts in HPLC.

Time (hours)	% Drug Remaining (Sulfamethoxazole)	% Drug Remaining (Trimethoprim)	% Drug Remaining (Isoniazid)	% Drug Remaining (Pyridoxine HCl)
0	100%	100%	100%	100%
2	76.35%	81.25%	63.90%	89.34%

Oxidative degradation studies conducted with 3% H_2O_2 for 2 hours resulted in moderate degradation, with the formation of oxidative degradation products detected through additional peaks in the chromatogram. The oxidative susceptibility of Isoniazid and Sulfamethoxazole was evident, as these compounds are known to undergo oxidation-related degradation. The presence of degradation products under oxidative stress suggests the necessity of incorporating antioxidants into the formulation or employing oxygen-resistant packaging such as blister packs with desiccants to minimize oxidative exposure and ensure product stability over its shelf life.

Thermal Degradation (70°C Dry Heat, 24 hours)

Thermal stability studies showed minimal degradation, with no major peak shifts or additional peaks in the HPLC chromatogram (Table 5).

Table 5. Thermal degradation of FDC (70°C, 24 hours), showing minimal drug loss and maintained chemical integrity.

Time (hours)	% Drug Remaining (Sulfamethoxazole)	% Drug Remaining (Trimethoprim)	% Drug Remaining (Isoniazid)	% Drug Remaining (Pyridoxine HCl)
0	100%	100%	100%	100%
24	94.68%	97.23%	95.89%	98.12%

Thermal degradation studies at 70°C for 24 hours demonstrated minimal degradation, indicating that the FDC formulation has good thermal stability. No significant changes were observed in the HPLC chromatograms, suggesting that the chemical integrity of the active ingredients remains intact under high-temperature conditions. This result is promising, as it indicates that the formulation can withstand elevated temperatures, making it suitable for distribution and storage in regions with warm climates without compromising drug potency.

Photolytic Degradation (UV Light, 254 nm, 24 hours)

Exposure to UV light for 24 hours caused slight degradation, with minor peak shifts in the HPLC chromatogram (Table 6).

Table 6. Photolytic degradation of FDC (UV 254 nm, 24 hours), showing slight Pyridoxine HCl loss with minor HPLC peak shifts.

Time (hours)	% Drug Remaining (Sulfamethoxazole)	% Drug Remaining (Trimethoprim)	% Drug Remaining (Isoniazid)	% Drug Remaining (Pyridoxine HCl)
0	100%	100%	100%	100%
24	92.15%	94.70%	93.88%	89.75%

Photolytic degradation studies under UV light exposure at 254 nm for 24 hours resulted in slight degradation, with minor peak shifts detected in the HPLC analysis. Pyridoxine Hydrochloride, known to be light-sensitive, exhibited slight degradation, whereas the other active ingredients remained relatively stable. This suggests that while the formulation is not highly susceptible to photodegradation, the use of appropriate light-resistant packaging, such as amber-colored bottles or aluminum foil wrapping, may be beneficial in further enhancing its stability.

3.3 Forced Degradation Profile of Fixed-Dose Combination (FDC) Under Various Stress Conditions

The forced degradation study was conducted to evaluate the stability of the Fixed-Dose Combination (FDC) under different stress conditions, including acidic, basic, oxidative, thermal, and photolytic environments, as per ICH Q1A(R2) guidelines. The study aimed to assess the extent of degradation and the formation of any degradation products (Table 7).

Table 7. Degradation profile of FDC, showing significant acid and oxidative degradation, with stability under thermal and photolytic conditions.

Stress Condition	Reagent/Exposure	Time	Degradation Observed
Acid Hydrolysis	0.1N HCl, 60°C	2 hours	Significant degradation, additional peaks in HPLC.
Base Hydrolysis	0.1N NaOH, 60°C	2 hours	Moderate degradation, peak broadening observed.
Oxidative Stress	3% H_2O_2	2 hours	Moderate degradation, oxidative degradation peaks detected.
Thermal Degradation	70°C Dry Heat	24 hours	Minimal degradation, formulation remained stable.
Photolytic Stability	UV Light (254 nm)	24 hours	Slight degradation, minor peak shifts detected.

3.4 Stability Studies

The stability study was performed under accelerated (40°C, 75% RH) and intermediate (30°C, 75% RH) storage conditions over 24 months. The aim was to evaluate changes in physical appearance, assay values, dissolution efficiency, and disintegration time.

Table 8. Stability study of FDC, showing maintained integrity and compliance under stress conditions.

Parameter	Initial Value	Accelerated (6M, 40°C, 75% RH)	Intermediate (12M, 30°C, 75% RH)	Specification Limit
Physical Appearance	No change	No change	No change	Complies
Disintegration Time	5 min 04 sec	4 min 19 sec	3 min 42 sec	NMT 30 min
Dissolution (NLT 75% in 60 min)	Pass	Pass	Pass	NLT 75%
Sulfamethoxazole Assay	99.23%	97.69%	98.93%	90-110%
Trimethoprim Assay	99.52%	100.87%	98.40%	90-110%
Isoniazid Assay	102.01%	101.75%	101.20%	90-110%
Pyridoxine Assay	112.13%	112.24%	110.44%	90-115%

The stability study conducted over 24 months under both accelerated (40°C, 75% RH) and intermediate (30°C, 75% RH) storage conditions confirmed that the formulation maintains its physical and chemical stability over time. No significant changes were observed in the physical appearance of the tablets, indicating that the formulation remains structurally intact throughout the study period. Disintegration time showed a slight reduction over time, likely due to moisture absorption, but remained well within the acceptable range, ensuring rapid breakdown of the tablet upon administration. Dissolution studies demonstrated that all active ingredients met the required NLT 75% dissolution criteria within 60 minutes, ensuring that the drugs are bioavailable and can be effectively absorbed (Table 8).

Assay values for Sulfamethoxazole, Trimethoprim, Isoniazid, and Pyridoxine Hydrochloride remained within the 90-110% acceptable limit, indicating that the active pharmaceutical ingredients retained their potency over the entire storage period. While slight variations in assay values were observed, particularly for Isoniazid and Pyridoxine Hydrochloride, they remained within the specified range, confirming that the formulation maintains its therapeutic efficacy over time. These findings suggest that the formulation is highly stable and suitable for long-term storage under standard conditions.

The forced degradation and stability study results indicate that while the FDC formulation demonstrates excellent thermal and photolytic stability, it is highly susceptible to acidic and oxidative degradation. This necessitates protective formulation strategies such as enteric coating to enhance acid resistance and antioxidant incorporation or moisture-resistant packaging to mitigate oxidative damage. The stability study findings further reinforce that the formulation maintains its integrity and efficacy over time, making it a suitable candidate for the treatment of secondary tuberculosis in HIV patients.

3.5 Discussion

The forced degradation study assessed the stability of the Fixed-Dose Combination (FDC) of Sulfamethoxazole, Trimethoprim, Isoniazid, and Pyridoxine Hydrochloride under different stress conditions [15]. The results showed significant degradation under acidic and oxidative stress, whereas the formulation exhibited good stability under thermal and photolytic conditions. Acidic hydrolysis resulted in extensive degradation, particularly affecting Isoniazid and Sulfamethoxazole, with additional peaks observed in the HPLC chromatogram. Isoniazid showed the highest susceptibility, with a degradation rate of 41.57%, followed by Sulfamethoxazole at 32.88%, while Trimethoprim and Pyridoxine HCl remained relatively stable, degrading by 19.79% and 8.98%, respectively. The degradation in acidic conditions suggests the necessity of enteric coating or buffering agents to prevent early breakdown during gastric exposure [16].

Base hydrolysis led to moderate degradation, with Trimethoprim showing the highest degradation at 27.90%, indicating that alkaline conditions significantly affect its stability. Sulfamethoxazole and Isoniazid also exhibited moderate degradation of 19.58% and 22.11%, respectively, while Pyridoxine HCl remained stable. The peak broadening in HPLC suggests partial hydrolytic cleavage of the active ingredients. The moderate degradation under basic conditions highlights the need for pH stabilizers in the formulation. Oxidative stress conditions caused substantial degradation, particularly in Isoniazid, which degraded by 36.10%, followed by Sulfamethoxazole at 23.65%. The appearance of oxidative degradation peaks in HPLC suggested structural modifications due to oxidation, necessitating the incorporation of antioxidants and oxygen-resistant packaging [17].

Thermal degradation studies at 70°C for 24 hours showed minimal degradation across all active ingredients, with Sulfamethoxazole retaining 94.68% of its potency, Trimethoprim 97.23%, Isoniazid 95.89%, and Pyridoxine HCl 98.12%. The results confirm the formulation's suitability for storage in high-temperature environments. Photolytic degradation under UV exposure showed minor peak shifts, with Pyridoxine HCl degrading by 10.25%, suggesting some light sensitivity, whereas the other active ingredients remained stable. Protective packaging such as amber-colored bottles is recommended. These findings highlight that while the FDC formulation is stable under normal and high-temperature conditions, it requires acid protection and oxidation-resistant measures to ensure long-term stability and efficacy [18].

3.6 Conclusion

The forced degradation study of the Fixed-Dose Combination (FDC) of Sulfamethoxazole, Trimethoprim, Isoniazid, and Pyridoxine Hydrochloride revealed that the formulation is highly susceptible to acidic and oxidative degradation, while it exhibits good stability under thermal and photolytic conditions. Acidic conditions caused the most significant degradation, particularly in Isoniazid and Sulfamethoxazole, indicating the need for acid-resistant coatings or buffering agents. Oxidative stress also led to substantial degradation, suggesting that antioxidants and oxygen-resistant packaging should be incorporated. Base hydrolysis resulted in moderate degradation, whereas thermal and photolytic conditions had minimal effects, confirming that the formulation is suitable for storage in warm environments. The findings demonstrate that while the FDC maintains stability and efficacy under standard storage conditions, protective formulation strategies such as enteric coating, moisture-resistant packaging, and antioxidant incorporation are recommended to enhance long-term stability. Further clinical evaluation is necessary to validate its therapeutic effectiveness in tuberculosis treatment for HIV patients.

List of Abbreviations

FDC: Fixed-Dose Combination

HIV: Human Immunodeficiency Virus

TB: Tuberculosis

HPLC: High-Performance Liquid Chromatography

RP-HPLC: Reverse-Phase High-Performance Liquid Chromatography

Cmax: Maximum Plasma Concentration

Tmax: Time to Reach Maximum Plasma Concentration

AUC: Area Under the Curve

T½: Elimination Half-Life

Keli: Elimination Rate Constant

PO: Per Oral (Oral Administration)

µg/mL: Micrograms per Milliliter

ACN: Acetonitrile

FTIR: Fourier Transform Infrared Spectroscopy

PDA: Photodiode Array Detector

UV: Ultraviolet

EDTA: Ethylenediaminetetraacetic Acid

RH: Relative Humidity

ICH: International Council for Harmonisation

NLT: Not Less Than

NMT: Not More Than

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