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Analytical Method Development and Validation for Thioacetazone and Isoniazide (INH) by using RP-HPLC in Pharmaceutical Dosage forms as per ICH Guidelines

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A B S T R A C T

This research workoutlines the advancement& verification of a HPLC technique for the parallel quantification of Isoniazid & Thiaacetazone. The optimized chromatographic conditions employed a SPURSIL C18-EP column with a MP composed of 66% HCOOH &34% ACN, a flow speed of 1 ml per min, & detection at 260 nm. SST demonstrated that all parameters, including resolution (greater than 2), theoretical plates (above 2000), & tailing factor (below 2), met the required consent. The assay results showed a % assay of 99.55% for Isoniazid & 98.31% for Thiaacetazone. The technique exhibited linearity over the conc. extent of 10–50 µg per ml for both compounds, with R2 of 0.9998 & 0.9994, separately. Precision studies indicated low %RSD values (0.9% for Isoniazid & 1.5% for Thiaacetazone), confirming the method's reliability. LOD were determined at 1.6 ppm for Isoniazid & 1.5 ppm for Thiaacetazone, while LOQ were found to be 5.6 ppm & 5.4 ppm, separately. Robustness testing revealed that variations in flow speed & MP composition didn't notable affect the technique performance. Overall, the verified HPLC technique is suitable for regular QC of Isoniazid &Thiaacetazone in formulations.

Keywords: Isoniazid, Thiaacetazone, %RSD, Detector, Analytical, Spectra.

A R T I C L E I N F O

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C o n t e n t s

1. Introduction:

In the pharmaceutical industry, the development and validation of analytical methods are of paramount importance to ensure the quality, efficacy, and safety of drug products. Among the numerous analytical techniques available, High-Performance Liquid Chromatography (HPLC) stands out due to its high resolution, sensitivity, and specificity. Reverse Phase HPLC (RP-HPLC) is particularly favored for its capability to separate compounds with diverse polarities. This study focuses on the analytical method development and validation for the simultaneous estimation of Thioacetazone and Isoniazide (INH) in pharmaceutical dosage forms, in accordance with the stringent guidelines set forth by the International Council for Harmonization (ICH). Thioacetazone is an antitubercular drug used in the treatment of tuberculosis. It works by inhibiting the synthesis of mycolic acids, essential

components of the mycobacterial cell wall. Isoniazide (INH) is another cornerstone drug in tuberculosis therapy. It is highly effective against Mycobacterium tuberculosis and operates by inhibiting the synthesis of mycolic acids, thereby disrupting the bacterial cell wall and killing the bacterium. The combination of Thioacetazone and Isoniazide in pharmaceutical formulations is used to provide a synergistic effect, enhancing therapeutic outcomes and reducing the risk of drug resistance.

The development of an RP-HPLC method for the simultaneous estimation of Thioacetazone and Isoniazide involves several critical steps, including the optimization of chromatographic conditions. This process requires the careful selection of the mobile phase, column type, flow rate, and detection wavelength. The mobile phase, typically composed of a mixture of water, methanol, and acetonitrile, must be optimized to achieve the best separation and resolution of the drug compounds. The choice of column, often a C18 column, is crucial as it directly affects the efficiency and resolution of the separation process.

Once the optimal chromatographic conditions are determined, the method must undergo rigorous validation according to ICH guidelines. Validation ensures that the analytical method is reliable, reproducible, and suitable for its intended purpose. The key parameters assessed during validation include specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantitation (LOQ), and robustness. Specificity tests the method's ability to accurately identify and quantify Thioacetazone and Isoniazide in the presence of other components, such as excipients and potential degradation products. Linearity evaluates the method’s ability to produce results that are directly proportional to the concentration of the analytes over a specified range.

Accuracy and precision are critical attributes for ensuring the reliability of the analytical method. Accuracy is determined by comparing the test results with those obtained using a reference method or known standards, while precision is assessed by measuring the consistency of results from multiple analyses of the same sample. The LOD and LOQ are essential for determining the method’s sensitivity, indicating the smallest amount of the analyte that can be reliably detected and quantified. Robustness testing evaluates the method's reliability under varying conditions, such as changes in pH, flow rate, and temperature, ensuring that the method remains consistent and accurate under different operational scenarios.

The successful development and validation of an RP-HPLC method for the simultaneous estimation of Thioacetazone, Isoniazide have significant implications for pharmaceutical quality control. It ensures that these critical antitubercular drugs meet the required standards of efficacy, safety, and quality, thereby enhancing patient outcomes and contributing to public health. Furthermore, this validated method can be applied in routine quality control, stability testing, and during the manufacturing process to ensure the consistent quality of pharmaceutical products.

The development and validation of an RP-HPLC method for the simultaneous estimation of Thioacetazone and Isoniazide represent a crucial step in pharmaceutical analysis. By following a systematic approach to method development and adhering to rigorous validation protocols as per ICH guidelines, this study aims to establish a robust, reliable, and accurate analytical method. This method will not only ensure compliance with regulatory standards but also contribute to the overall improvement of pharmaceutical analytical practices.

Drug Profile

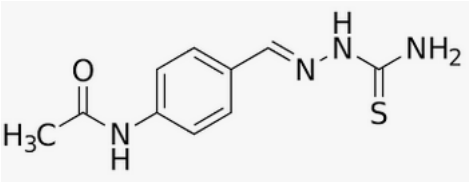

Figure.1. Thioacetazone

Basic Information
IUPAC Name: N-{4-[(Ethanethioamidoimino) methyl] phenyl}acetamide
Molecular Formula: $C_{10}H_{12}N_4OS$
Molecular Weight: 236.29 g/mol
Category: Antituberculosis agent
Physical Properties
Melting Point: Approximately 204-205°C
pKa: 9.38 (acidic), 11.42 (acidic),13.37(acidic), 2.45 (basic)
Solubility: Sparingly soluble in water; soluble in ethanol and acetone.
Description
Thioacetazone is an oral antibiotic used in the treatment of tuberculosis. It has largely fallen out of use due to its toxicity and the availability of more effective drugs like isoniazid12.
Mechanism of Action
Thioacetazone is believed to interfere with mycolic acid synthesis in Mycobacterium tuberculosis, although its exact biological target remains elusive1.
Pharmacodynamics: Thioacetazone has weak activity against Mycobacterium tuberculosis and is primarily used to prevent resistance to more potent drugs.
Pharmacokinetics: Detailed pharmacokinetic data is limited.
Metabolism and Elimination
Metabolism: Not well-documented.
Route of Elimination: Primarily renal.
Protein Binding and Half-Life
Protein Binding: Not well-documented.
Half-Life: Not well-documented.
Uses: Primarily used in combination with other antituberculosis drugs to prevent resistance.
Dosage: Typically administered orally in combination with other drugs; specific dosage should be determined by a healthcare provider.
Side Effects: Severe skin reactions, especially in HIV-positive patients, and other toxic effects such as thyroid

gland weakening and serum accumulation in the brain. Drug Interactions Should not be used with other drugs that cause similar toxic effects. Storage store in a cool, dry place away from direct sunlight.

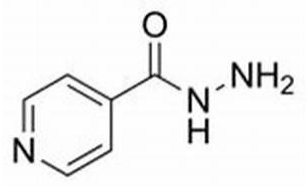

Figure.2. Isoniazide

Basic Information
IUPAC Name: Pyridine-4-carbohydrazide
Molecular Formula: $C_6H_7N_3O$
Molecular Weight: 137.14 g/mol
Category: Antituberculosis agent
Physical Properties
Melting Point: 171-173°C
pKa: 1.8 (acidic), 3.5 (acidic), 10.8 (basic)
Solubility: Soluble in water, ethanol, and methanol.
Description: Isoniazid, also known as isonicotinic acid hydrazide (INH), is an antibiotic used for the treatment and prevention of tuberculosis (TB). It is often used in combination with other TB medications to prevent resistance12.

Mechanism of Action

Isoniazid works by inhibiting the synthesis of mycolic acids, which are essential components of the bacterial cell wall in Mycobacterium tuberculosis.
Pharmacodynamics:
Isoniazid is bactericidal against actively growing intracellular and extracellular Mycobacterium tuberculosis organisms.
Pharmacokinetics: Absorption: Rapidly absorbed from the gastrointestinal tract.
Distribution: Widely distributed in body fluids and tissues.
Metabolism: Metabolized in the liver by acetylation.
Excretion: Primarily excreted in the urine.
Metabolism and Elimination
Metabolism: Metabolized in the liver by acetylation.
Route of Elimination: Primarily renal.
Protein Binding and Half-Life
Protein Binding: Very low (0-10%)
Half-Life: 0.5-1.6 hours (fast acetylators), 2-5 hours (slow acetylators).
Uses: Treatment and prevention of tuberculosis.
Dosage: Typically 5 mg/kg daily (maximum 300 mg daily) for adults; specific dosage should be determined by a healthcare provider1.
Side Effects: Common side effects include peripheral neuropathy, liver toxicity, and gastrointestinal disturbances. Severe side effects can include hepatitis and hypersensitivity reactions12.

Drug Interactions

Isoniazid can interact with various drugs, including phenytoin, carbamazepine, and alcohol, potentially increasing the risk of liver damage. Storage Store in a cool, dry place away from direct sunlight.

2. Methodology

Instruments used

For the analytical procedures, an HPLC system is used, specifically the WATERS model with Empower software, incorporating a 2695 separation module and a 2487 UV detector. A UV/VIS spectrophotometer, model LABINDIA UV 3000+, is also employed. The pH levels are measured using a pH meter, model Adwa – AD 1020. For precise measurements, a weighing machine, model Afcoset ER-200A, is utilized. Additionally, pipettes and burettes, as well as beakers, from Borosil are used for various procedures.

Chemicals used

For the estimation and analysis of Thioacetazone and Isoniazide, various chemicals and reagents are utilized. Thioacetazone and Isoniazide are supplied by MSN LAB. Potassium dihydrogen phosphate (KH2PO4) is sourced from FINAR Chemical LTD. Water and methanol for HPLC are provided by Standard Solutions Ltd, which also supplies acetonitrile for HPLC. Additionally, hydrochloric acid (HCl), hydrogen peroxide (H2O2), and sodium hydroxide (NaOH) are sourced from MERCK.
Methoddevelopment:
Choosing λmax:
spectrum of UV with 10µg/ml Thioacetazone& Isoniazid in MP ratio) was noted by examining in the scale of 200 to 400nm and the isobestic λmax of both the drugs obtained at 258 nm.
Optimization of Column:
INSPIRE (150 x 4.6 mm, 5µm) is find out optimum as it produce excellentshape of peak & RS at 1.0ml per min flow speed.

Optimized Chromatographic Conditions

Instrument used: RP-HPLCWith Auto Sampler and PDA detector
Column: INSPIRE (150X4.6mm 5□m)
Mobile phase: (65: 35) ACN: Trifluoroacetic acid
Flow rate : 1ml per min
λmax: 258 nanometers
Volume Injected: 10 µl
Time duration: 10 min.

Buffer & mobile phase making:

TFA pH 3Preparation:
By adding 1ml TFA in 1L HPLC grade water. Adjust this solution to pH 3 by using acid / base based on the PH of the resulted solution.

Mobile phase making:

Mix a 350 ml TFA (35%) ,ACN 650ml (65%)& remove gases in ultra-sonication water bath for few min. Filter by vacuum filtration instrument using 0.45µ filter paper.
Diluent:
Acetonitrile: TFA (65:35) ratio.

System Suitability:

- Tailing factor for Thioacetazone&Isoniazid in Std solution shouldn ‘t>2.0.
- For Standard solution Theoretical plates for the Thioacetazone&Isoniazid peaks shouldn ‘t<
- 2000.

limitations of System Suitability:
1) Tailing factor should be < 2.

2) Theoretical Plates should be > 2000.

Method validation parameters:

Assay:

Standard Solution Preparation:

Precisely measure & poured 10 mg Isoniazid &5mg Thioacetazone standard into a 10 ml VF add Diluents & to dissolve fully sonicate & fill up till the margin. Additional takeout 0.6mlfrom solutions into a 10ml VF & fill up till the margin using dilutant. (60ppm of isoniazide& 30ppm of thiaacetazone).

Sample Solution Preparation:

Precisely measure & poured equal to 10mg Isoniazid &5mg of Thioacetazoneequal wt of the sample into a 10ml VF add few ml of dilutant& to fully dissolve sonicate fully get volume up till the margin. Additional take out 0.6mlfrom solutions into a 10ml VF & fill up till the margin using diluent. (60ppm of isoniazide& 30ppm of thiaacetazone).

Procedure:

Inject 10 µL of the std, sample into the HPLC system & note down the areas for the Thioacetazone and Isoniazid peaks.

Linearity:

Stock solution Preparation:

Precisely measure & poured mg of 10 mg Isoniazid and 5mg Thioacetazone standard into a 10 ml VF add Diluents & sonication to dissolve fully &fill up till the margin.

Level – I Preparation (Isoniazide20ppm &Thiaacetazone 10ppm):Pipette out 0.2mlfrom solution into10ml of VF fillup till the margin using diluent.

Level – II Preparation (Isoniazide 40ppm &Thiaacetazone 20ppm):Pipette out 0.4ml from solution into10ml of VF fill up till the margin using diluent.

Level – III Preparation (Isoniazide 60ppm &Thiaacetazone 30ppm):Pipette out 0.6mlfrom solution into10ml of VF fillup till the margin using diluent.

Level – IV Preparation (80ppm of Isoniazide and Thiaacetazone 40ppm):

0Pipette out 0.8mlfrom solution into10ml of VF fillup till the margin using diluent.

Level – V Preparation (Isoniazide 100ppm&Thiaacetazone 50ppm):Pipette out 1mlfrom solution into10ml of VF fillup till the margin using diluent.

Procedure: Introduce each concentration level into the chromatographic system and record the corresponding peak area. Construct a graph with concentration on the X-axis and peak area on the Y-axis, then determine the correlation coefficient from the plotted data

Precision:

Stock Solution Preparation:

Precisely measure & poured mg of 10 mg Isoniazid and 5mg Thioacetazone standard into a 10 ml VF add Diluents and to fully dissolve sonicate& get volume up till the margin using the solvent. Additional take out 0.6mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent. (60ppm of isoniazide& 30ppm of thiaacetazone).

Procedure:

Injecte the std solution for 6 times & we get all 6 replicas area's in HPLC. The %RSD for the areas of 6 replicas was calculated to be under the boundaries.

Intermediate precision/ruggedness:

To examine the ID method precision, it wasdone on separate days under the lab.

Stock solution Preparation:

Precisely measure & poured mg of 10 mg Isoniazid and 5mg Thioacetazonestandard into a 10 ml VF add Diluents and to fully dissolve sonicate& get volume up till the margin using the solvent. Additional take out 0.6mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent. (60ppm of isoniazide& 30ppm of thiaacetazone).

Procedure:

Introduce the standard solution into the HPLC system six times and record the peak areas for each injection. The %RSD for the peak areas of these six replicate injections was found to be within acceptable limits

Accuracy:

For identification of accuracy, 3 separate conc. were individually prepared i.e. 50, 100 & 150 percent for the desired analytes & chromatographs are noted for them.

Standard solution Preparation:

Precisely measure & poured mg of 10 mg Isoniazid and 5mg Thioacetazone standard into a 10 ml VF add Diluents and to fully dissolve sonicate& get volume up till the margin using the solvent. Additional take out 0.6mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent.

Sample solutions Preparation:

50% solution preparation:

Precisely measure & poured mg of 5mg Isoniazid and 2.5mg Thioacetazone standard into a 10 ml VF add Diluents and to fully dissolve sonicate& get volume up till the margin using the solvent.

Additional take out 0.6mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent..

100% solution preparation:

Precisely measure & poured mg of 10 mg Isoniazid and 5mg Thioacetazonestandard into a 10 ml VF add Diluents and to fully dissolve sonicate& get volume up till the margin using the solvent.

Additional take out 0.6mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent.

150% solution preparation:

Precisely measure & poured mg of 15 mg Isoniazid and 7.5mg Thioacetazone standard into a 10 ml VF add Diluents and to fully dissolve sonicate& get volume up till the margin using the solvent. Additional take out 0.6mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent. (60ppm of isoniazide& 30ppm of thiaacetazone).

Procedure: Introduce standard solution, Accuracy -50%, 100% & 150% solutions. Find out the amount found & added for Isoniazid and Thioacetazone& find the separate l recovery &Avg recovery values.

Limit of detection:

1.6µg/ml Isoniazide solution Preparation:

Precisely measure & poured 10mg of Isoniazid standard into a 10 ml VF add Diluents and to fully dissolve sonicate& get volume up till the margin using the solvent.

Additional take out 1mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent. Additional take out

1mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent. Additional take out 1.6 ml from stock solutions into a 10ml VF & get up till the margin with Diluent.(1.6ppm).

1.5µg/ml Thiaacetazone solution Preparation:

Precisely measure & poured 10mg of Thioacetazone standard into a 10 ml VF add Diluents and to fully dissolve sonicate& get volume up till the margin using the solvent.
Additional take out 1mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent.
Additional take out 1mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent.
Additional take out 1.5 ml from stock solutions into a 10ml VF & get up till the margin with Diluent. (1.5ppm).
Limit of quantification:

5.6µg/ml Isoniazide solution Preparation:

Precisely measure & poured 10mg of Isoniazid standard into a 10 ml VF add Diluents and to fully dissolve sonicate& get volume up till the margin using the solvent. Additional take out 1mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent. Additional take out 1mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent. Additional take out 5.6mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent.(5.6ppm).

5.4µg/ml Thiaacetazone solution Preparation:

Precisely measure & poured 10mg of Thioacetazone standard into a 10 ml VF add Diluents and to fully dissolve sonicate& get volume up till the margin using the solvent.
Additional take out 1ml of the above stock solution into a 10ml volumetric flask and dilute upto the mark with Diluents.
Additional take out 1mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent.
Additional take out 5.4mlfrom stock solutions into a 10ml VF & get up till the margin with Diluent.(5.4ppm).

Robustness: Robustness, is a deliberate change in the Flow speed, MP ratios, Temperature changes was done to check the affect on this technique.

- The flow speed was differ at 0.8ml per min to1.2ml per min.
- Standard solution 30µg/ml of Thiaacetazone and Isoniazide made & analyzed using the different flow speed across with actual flow.
- The ratios of Organic in the MP was differ from 40% to 60% Standard solution 30µg/ml of Thiaacetazone, Isoniazide was made &analyzed using the different MP ratios across with the real MP rato in the method.

3. Results and Discussion

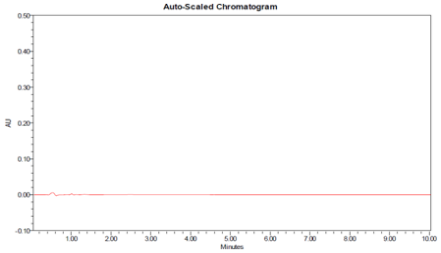

Figure 3:Chromatogram for system suitability

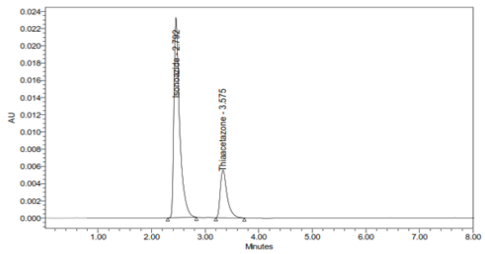

Figure 4: Standard Chromatogram for system suitability

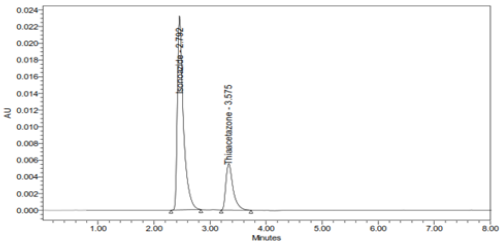

Figure 5: Standard Chromatogram

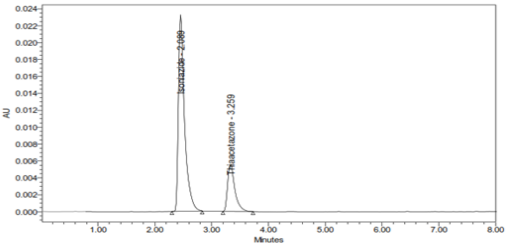

Figure 6: Sample Chromatogram

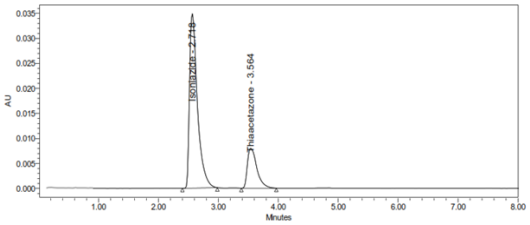

Figure 7: Linearity Chromatogram

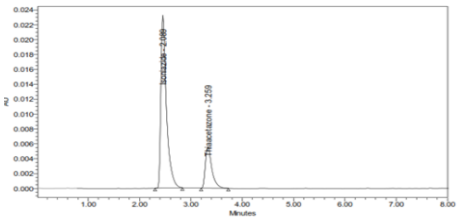

Figure 8: Calibration graph for Isoniazide

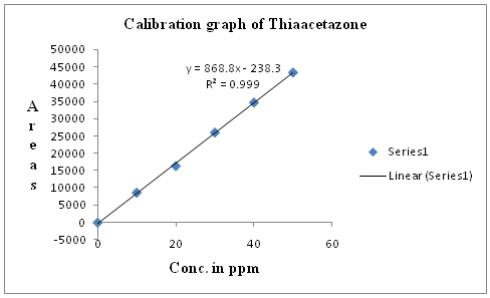

Figure 9: Calibration graph for Thiaacetazone

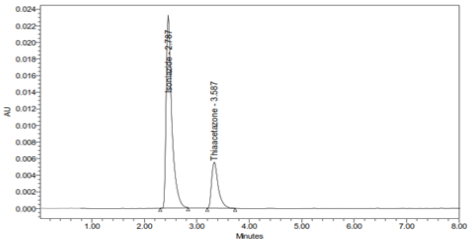

Figure 10: Precision Chromatogram

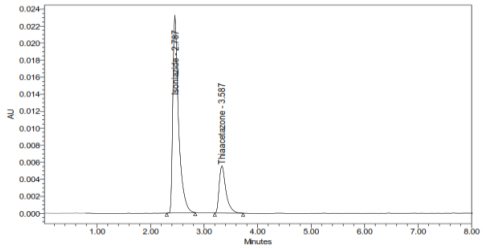

Figure 11: ID Precision Chromatogram

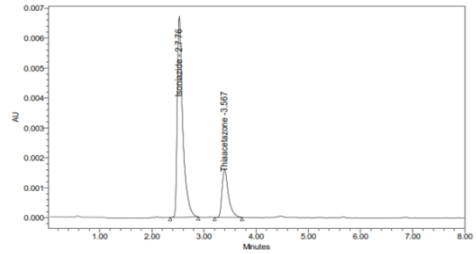

Figure 12: Accuracy 50%-3 Chromatogram

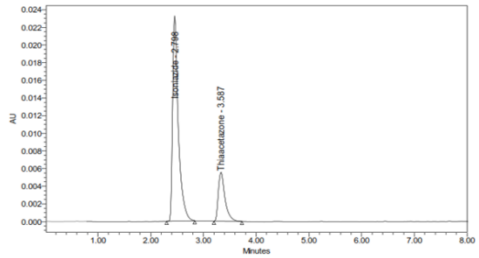

Figure 13: Accuracy 100%-3 Chromatogram

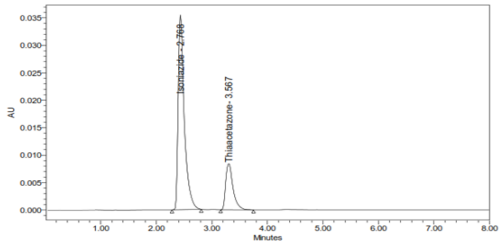

Figure 14: Accuracy 150%-3 Chromatogram

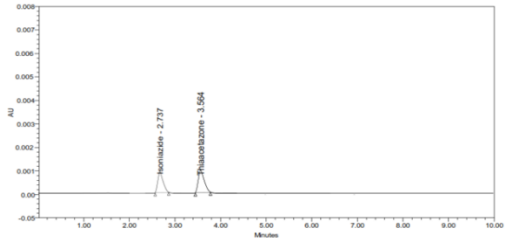

Figure 15: Thiaacetazone &Isoniazide depicting LOD Chromatogram

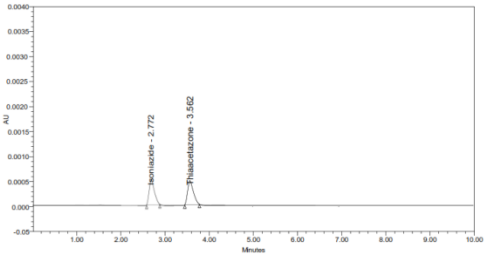

Figure 16: Thiaacetazone & Isoniazide depicting LOQ Chromatogram

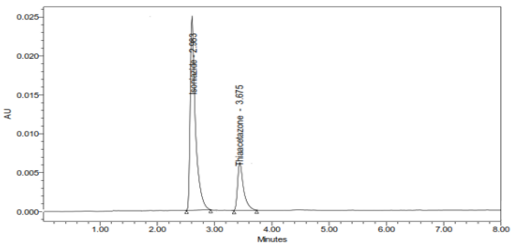

Figure 17: less flow Chromatogram

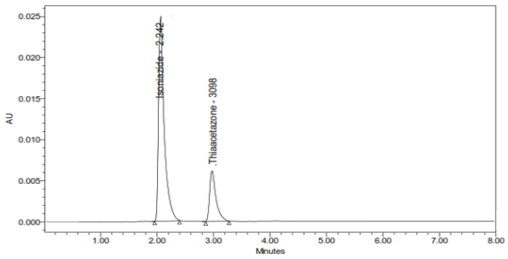

Figure 18: more flow Chromatogram

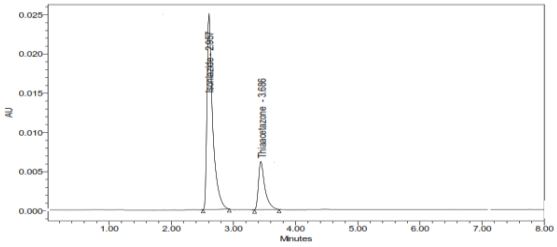

Figure 19: less organic composition Chromatogram

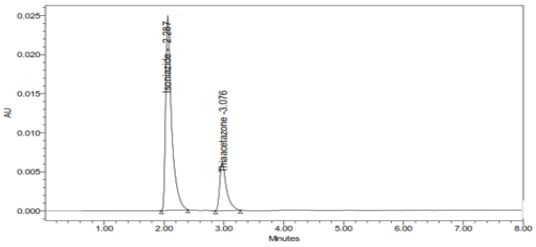

Figure 20: more organic composition Chromatogram

Table 1: Areas of various conc. of Thiaacetazone

S.NO	Concentration (µg per ml)	Areas of Isoniazide
1	20	17320
2	40	34641
3	60	51962
4	80	69282
5	100	86803

Table 2: Areas of various conc. of Thiaacetazone

S.NO	Concentration (µg per ml)	Areas of Thiaacetazone
1	10	8659
2	20	17319
3	30	25979
4	40	34638
5	50	43298

Table 3: Regression equation parameters of Thiaacetazone &Isoniazide

Parameters	Isoniazide	Thiaacetazone
Slope(m)	868.89	868.82
Intercept(c)	276.43	238.33
Coefficient of Correlation (R^2)	0.9998	0.9994

Table 4: Outcomes of Precision for Thiaacetazone & Isoniazide

Injection	Isoniazide Are's	Thiaacetazone Area's
1 Injection	51962	25979
2 Injection	50962	24979
3 Injection	51962	25979
4 Injection	51962	25979
5 Injection	51862	25979
6 Injection	50962	25979
Avg	51612	25812.33
Standard Deviation	504.97	408.24
%RSD	0.9	1.5

Table 5: ID precision outcomes for Thiaacetazone & Isoniazide

Injection	Isoniazide Area	Thiaacetazone Area
1 Injection	51962	25979
2 Injection	50962	25979
3 Injection	51962	24979
4 Injection	51862	25979
5 Injection	50862	25979
6 Injection	50962	25979
Avg	51428.67	25812.33
Std. Deviation	550.15	408.24
%RSD	1.06	1.5

4. Conclusion

An analytical technique was advancement & verification for the parallel identification of Thioacetazone& Isoniazid (INH) in dosage forms using RP-HPLC). The research was conducted following the ICH rules, ensuring the technique reliability, reproducibility, & robustness. A suitable MP, column, & detection λmax were selected to achieve excellentl separation of Thioacetazone& Isoniazid in a short runtime. Once method developed, the technique was verified according to ICH rules covering parameters such as SST, specificity, linearity, precision, accuracy, robustness, & LOD and LOQ. The results of the verification demonstrated that the method was suitable for regular QC testing of dosage forms containing Thioacetazone& Isoniazid. The created RP-HPLC technique was successfully verified for the parallel quantification of Thioacetazone& Isoniazid in dosage forms. It can be effectively applied in regular analysis for QC purposes, ensuring the accurate& reliable measurement of these compounds in combined dosage forms. This verified technique ensures product quality & consistency, contributing to the safe use of these drugs in therapeutic applications.

5. References

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