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Design And Validation Of A Combination Of Iso-Sorbide Dinitrate And Hydralazine Hydrochloride As An Rp-Hplc Stability Indicator Method

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ABSTRACT

This study established and validated an RP-HPLC technique for the simultaneous detection of Hydralazine Hydrochloride and Iso-sorbide dinitrate in combination tablet dosage forms. Reliability, accuracy, and precision characterise the method. To achieve chromatographic separation, a 55:45 v/v mixture of acetonitrile and 0.02 M phosphate buffer was used, along with a C18 column (250 mm × 4.6 mm, 5 µm particle size) that had its pH adjusted to 3.0 using orthophosphoric acid. With a constant flow rate of 1.0 mL/min, the UV detection was performed at 230 nm. Under these conditions, the peaks presented by iso-sorbide dinitrate (3.74 minutes) and hydralazine hydrochloride (5.58 minutes) were clearly visible. Complete method validation was carried out in line with ICH Q2(R1) guidelines. Linearity, accuracy, precision, specificity, robustness, limit of detection (LOD), limit of quantification (LOQ), and system adaptability were significant criteria in the validation process. Studies of accuracy and precision confirmed the method's reliability, while studies of forced degradation confirmed its specificity and stability-indicating capability by successfully separating the APIs from their degradation products under a range of stress conditions, including acidic, basic, oxidative, thermal, and photolytic pressures. This approved RP-HPLC method is a dependable tool for quality control laboratories. It may be used for routine pharmaceutical analysis and stability testing, ensuring that high-quality, safe, and effective combined formulations of Iso-sorbide dinitrate and Hydralazine Hydrochloride are produced

Keywords: *Stability-Indicating Method , Method Validation , Simultaneous Estimation , Iso-sorbide dinitrate , Hydralazine Hydrochloride , Fixed-Dose Combination , Quality Control ect*

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1. INTRODUCTION

Discovering and developing antidiabetic treatments that address the complicated biology of T2DM has been the focus of substantial research and development efforts in response to this increasing health concern. Many patients find that

monotherapy is not enough to achieve adequate glycemic control in type 2 diabetes since the disease includes many metabolic

pathways. This has led to the rise in popularity of combination therapies that aim to influence multiple physiological processes at once. Patients benefit greatly from fixed-dose combinations (FDCs) because they streamline medication regimens, increase adherence, and decrease the risk of adverse effects linked to higher doses of individual agents.[1]. FDCs contain two or more active pharmaceutical ingredients in a single dosage form. Due to their complementary action mechanisms, the combination of Hydralazine Hydrochloride and Iso-sorbide dinitrate has been the most talked-about of these combos. Because of its role as an inhibitor of sodium-glucose co-transporter-2 (SGLT2), hydroxyzine hydrochloride lowers blood sugar levels by increasing the rate of glucose excretion from the body through the urine and decreasing the risk of hyperglycemia. In addition to reducing blood glucose levels, this method aids in weight loss and has positive effects on cardiovascular health. By blocking the breakdown of incretin hormones like GLP-1 and GIP, iso-sorbide dinitrate, an inhibitor of dipeptidyl peptidase-4 (DPP-4), amplifies the incretin action. Postprandial and fasting glucose levels are both improved by these hormones. The synergistic effect of these two medications improves overall glucose control and clinical outcomes in type 2 diabetic patients when used together.[2,3]

There are considerable analytical hurdles in quantifying Hydralazine Hydrochloride and Iso-sorbide dinitrate in a single dosage form, despite the clinical advantages and the rising use of this combination in pharmaceutical formulations.[4] Measurements of individual drugs may be inaccurate when there are several excipients, possible contaminants, and degradation products present. In addition, in order to guarantee that drugs remain safe and effective throughout their shelf life, regulatory agencies need analytical procedures that can reliably differentiate between intact drug components and their degradation products. Consequently, a validated analytical approach that is sensitive, dependable, and capable of reliably quantifying both APIs even in complicated matrices is urgently required. Although there are various analytical methods available for the individual estimation of Hydralazine Hydrochloride and Iso-sorbide dinitrate, very few studies have documented the creation and validation of a single, reliable reversed-phase high-performance liquid chromatography (RP-HPLC) method that can analyze both drugs simultaneously in both normal and stress-induced degradation scenarios.[5] In light of this deficiency, the current research aims to close it by creating an easy-to-use, highly-specific RP-HPLC technique for routine quality control and stability indication. This method will effectively detect and separate degradation products produced under different artificial degradation conditions, including acid/base hydrolysis, oxidation, thermal stress, and photolysis. To guarantee accuracy, specificity, and reproducibility for pharmaceutical applications, the method was verified according to internationally established principles.[6].

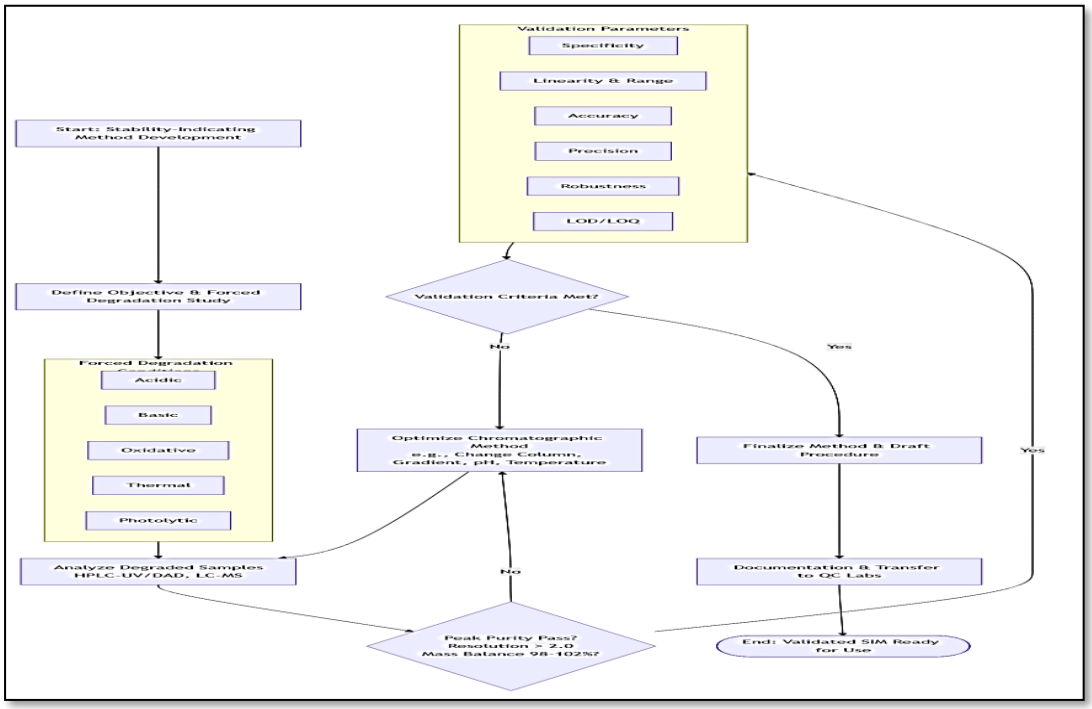


Fig.No.1 Flow diagram showing the stability indicating methods

2. MATERIALS AND METHODS[7]

Materials and Substances

Iso-sorbide dinitrate and **Hydralazine Hydrochloride** (API grade, >99% purity) were obtained from certified pharmaceutical suppliers.

Fixed-dose combination tablets labeled to contain 5 mg Iso-sorbide dinitrate and 10 mg Hydralazine Hydrochloride were procured from the local market.

Potassium dihydrogen phosphate and **orthophosphoric acid** (analytical grade) were used to prepare the phosphate buffer.

When making the aqueous solutions and mobile phase, Milli-Q water was utilised

Accredited pharmaceutical vendors supplied the iso-sorbide dinitrate and hydralazine hydrochloride, both of which are of API grade and have a purity level of over 99%. Iso-sorbide dinitrate and hydralazine hydrochloride fixed-dose combination tablets (5 mg and 10 mg, respectively) were acquired from the local market for the purpose of this analysis. The phosphate buffer was made with analytical-grade potassium dihydrogen phosphate and orthophosphoric acid. The mobile phase and all aqueous solutions were prepared using Milli-Q filtered water.[8]

3. INSTRUMENTATION USED IN STABILITY INDICATING METHOD

HPLC system: Shimadzu LC-20AT with UV detector and manual injector

Column: C18 column, 250 mm × 4.6 mm, 5 µm

Data software: LabSolutions

pH meter: Eutech Instruments, calibrated

Ultrasonicator: PCI Analytics

Analytical balance: Shimadzu AUW220D

A UV detector and a manual injector were used to conduct the chromatographic analysis on a Shimadzu LC-20AT HPLC system. To accomplish the separation, a C18 reversed-phase column of 250 mm × 4.6 mm and having a particle size of 5 µm was utilised. We processed and collected the data using LabSolutions software. To find the pH of the buffer solutions, we used a pH meter from Eutech Instruments that had been calibrated. A PCI Analytics ultrasonicator was used for sonication during sample preparation, and a Shimadzu AUW220D analytical balance was used for all weighings to guarantee precision.[9]

4. CHROMATOGRAPHIC CONDITIONS

The chromatographic separation was achieved under the following optimized conditions:

Mobile Phase: Acetonitrile: 0.02 M KH_2PO_4 buffer (pH 3.0) in a 55:45 v/v ratio

Flow Rate: 1.0 mL/min

Column Temperature: Ambient (25 ± 2°C)

Injection Volume: 20 µL

Detection Wavelength: 230 nm

Run Time: 10 minutes

The mobile phase for the chromatographic separation was prepared by mixing 55:45 v/v of acetonitrile and a pH 3.0 potassium dihydrogen phosphate (KH_2PO_4) buffer at a concentration of 0.02 M. While injecting 20 µL, the flow rate was kept constant at 1.0 mL/min. The detector employed a wavelength of 230 nm, while the column temperature was maintained at 25 2°C, in line with the surrounding conditions. The time limit for every analysis was ten minutes.

5. PREPARATION OF STANDARD AND SAMPLE SOLUTIONS[10]

Standard Stock Solutions

Iso-sorbide dinitrate: 10 mg was accurately weighed and dissolved in 100 mL mobile phase to obtain a 100 µg/mL solution.

Hydralazine Hydrochloride: 10 mg was similarly dissolved in 100 mL mobile phase for a 100 µg/mL solution.

In separate 100 mL volumetric flasks, 10 mg of Iso-sorbide dinitrate and 10 mg of Hydralazine Hydrochloride (both having a purity level of over 99%) were transferred. In order to create standard stock solutions with a concentration of 100 µg/mL, the filtered and degassed mobile phase was used to dissolve and dilute each constituent to volume. A 0.45 µm membrane filter was used to remove any remaining particles after sonicating the stock solutions for 10 minutes to guarantee full

dissolution. By gradually diluting the corresponding stock solutions with the mobile phase, working standard solutions of varied concentrations needed for method validation and calibration were created. To keep the solutions from going bad, they were made fresh every day and stored in an airtight container.[11]

Sample Preparation

Twenty uniformly weighed and finely powdered tablets of the fixed-dose combination formulation were used. A 100 mL volumetric flask was used to transfer a precisely weighed amount of the powdered sample that was equal to 5 mg of Iso-sorbide dinitrate and 10 mg of Hydralazine Hydrochloride. The active components were fully extracted by adding around 70 mL of the mobile phase to the flask and sonicating the mixture for 15 minutes. After adding mobile phase until the volume was the desired level, the solution was mixed well. Prior to chromatographic analysis, the solution was filtered using a 0.45 µm membrane filter to guarantee clarity and prevent column blockage. Particulate matter was thus eliminated.

6. VALIDATION PARAMETERS OF STABILITY INDICATING METHOD [12-14]

6.1 Linearity

In order to test the method's linearity, six different drug concentrations within the anticipated analytical range were prepared. Different quantities of Hydralazine Hydrochloride were utilized, including 10, 20, 30, 40, 50, and 60 µg/mL, and Iso-sorbide dinitrate at 2, 4, 6, 8, 10, and 12 µg/mL. A triple injection of each concentration allowed for the construction of calibration curves, which were then used to plot peak area against concentration. R^2, slope, and intercept were computed using least squares regression analysis to determine the linearity.[15]

6.2 `Accuracy

To verify the method's accuracy, recovery studies were carried out using the standard addition procedure. Each sample was spiked with 80%, 100%, or 120% of the labelled claim concentration of iso-sorbide dinitrate and hydralazine hydrochloride, respectively. In order to determine how close the measured values were to the actual values following triple analysis of each spiking sample, the percentage recovery was calculated. [16]

6.3 Precision

The variability between and between days was used to measure precision. In order to find the intra-day precision, we analyzed three distinct medication concentrations in triplicate on the same day. To determine the inter-day precision, the analysis was repeated three days in a row under the same experimental settings. A measure of the outcomes was the %RSD of the peak areas, which stands for percentage relative standard deviation.[17]

6.4 Specificity

Chromatograms of standard solutions, sample solutions, blank mobile phase, forced degradation samples treated with acid, base, oxidative, thermal, and photolytic stress, and standard solutions were analysed to establish specificity. Validating the method's specificity and capacity to distinguish the analytes from probable degradation products and excipients, the lack of interfering peaks at the retention periods of Iso-sorbide dinitrate and Hydralazine Hydrochloride was achieved. [18]

6.5 LOD and LOQ

Isosorbide dinitrate and HCl hydrochloride were used to determine the limits of detection (LOD) and quantitation (LOQ) for the developed RP-HPLC technique, which allowed us to evaluate its sensitivity. The response standard deviation and the calibration curve slope served as inputs. The response standard deviation and the calibration curve slope served as inputs. To an acceptable degree, these numbers represent the minimal concentrations of analytes that can be detected consistently and reliably.

6.6 Robustness

The method's robustness was checked by intentionally changing a few crucial chromatographic parameters and seeing how it affected the method's performance. The parameters that were changed were the flow rate (±0.1 mL/min), the detection wavelength (±2 nm), and the content of the mobile phase (±2% v/v). While holding all other variables constant, each variant was evaluated independently. Testing variations in theoretical plates, tailing factor, peak area, retention time, and robustness was done. If, under the changed circumstances, these parameters stayed within acceptable ranges, the approach was said to be resilient.

6.7 Forced Degradation Studies[20]

Drug solutions were subjected to stress under the following conditions:

An acidic degradative process involving 0.1 N HCl at 60°C for 1 hour

Aqueous breakdown: 0.1 N NaOH, 60°C for 1 hour

Subjected to 1 hour of room temperature oxidative degradation with 3% H_2O_2

Degradation by heat: 800 watts of dry heat for 60 minutes

Using ultraviolet light at 254 nm for a whole day causes photolytic deterioration.

After treatment, samples were neutralized (where needed), diluted appropriately, and analyzed.

The new RP-HPLC method's capacity to indicate stability was tested in forced degradation experiments of Iso-sorbide dinitrate and Hydralazine Hydrochloride under a variety of stress settings specified by the International Council for Harmonization (ICH). These tests attempted to determine how well the approach isolated APIs from their degradation byproducts, a measure of its specificity.

The drug substances were subjected to the following stress conditions:

Acidic degradation: Samples were treated with 0.1 N hydrochloric acid and maintained at 60°C for 1 hour.

Basic degradation: Samples were exposed to 0.1 N sodium hydroxide under the same thermal conditions (60°C for 1 hour).

Oxidative degradation: Samples were treated with 3% hydrogen peroxide and allowed to stand at room temperature for 1 hour.

Thermal degradation: Solid drug samples were exposed to dry heat in a hot air oven at 80°C for 6 hours.

Photolytic degradation: Samples were exposed to ultraviolet (UV) light at 254 nm for 24 hours in a photostability chamber.

Materials that had been hydrolysed acidically or basicly following degradation were neutralised using equimolar solutions of acid or base. The mobile phase was used to dilute all stressed samples to verify that the analyte concentrations were within the linear range. Using 0.45 µm membrane filters, the samples were filtered before being injected into the HPLC apparatus. The studies proved the method's specificity by demonstrating the separation of APIs from their degradation products and the absence of interference at the defined retention times.

7. RESULTS AND DISCUSSION

7.1 System Suitability

Table No.1 standard requirements indicating good chromatographic performance

Parameter	Iso-sorbide dinitrate	Hydralazine Hydrochloride
Retention Time (min)	3.74	5.58
Tailing Factor	1.02	1.05
Theoretical Plates	4980	5362
Resolution	—	4.7

All parameters met the standard requirements indicating good chromatographic performance.

To make sure the chromatographic system was efficient and reproducible, system suitability tests were run before analysis. Time of retention, tailing factor, theoretical plates, and resolution between the two analytes were some of the key factors that were investigated. Hydralazine hydrochloride had a retention time of 5.58 minutes and iso-sorbide dinitrate 3.74 minutes. Hydralazine hydrochloride had a tailing factor of 1.05 and iso-sorbide dinitrate had a tailing factor of 1.02, both

of which point to symmetrical peak morphologies. Good column efficiency was demonstrated by the theoretical plate counts of 4980 and 5362, respectively. Adequate separation was confirmed by a resolution of 4.7 between the two analytes, which is significantly higher than the minimal permitted value of 2.0. There was constant and dependable chromatographic performance, since all system suitability parameters were within the specified limits.

7.2 Linearity

Calibration curves for both drugs showed a linear relationship with high correlation coefficients ($R^2 > 0.999$).

Table No.2 Data showing the linear relationship with high correlation coefficients

Concentration	Iso-sorbide dinitrate Area	Hydralazine Hydrochloride Area
2 / 10 µg/mL	34562	125410
4 / 20 µg/mL	69234	251872
6 / 30 µg/mL	103123	377928
8 / 40 µg/mL	137654	502341
10 / 50 µg/mL	172103	630248
12 / 60 µg/mL	205812	754362

Over a concentration range of 2-12 µg/mL for Iso-sorbide dinitrate and 10-60 µg/mL for Hydralazine Hydrochloride, the linearity of the RP-HPLC technique that was developed was assessed. By graphing the peak area against the concentration for every analyte, calibration curves were generated. Excellent linearity was shown by the results, which showed a strong linear connection over the investigated ranges for both medicines (R^2 greater than 0.999).

7.3 Accuracy

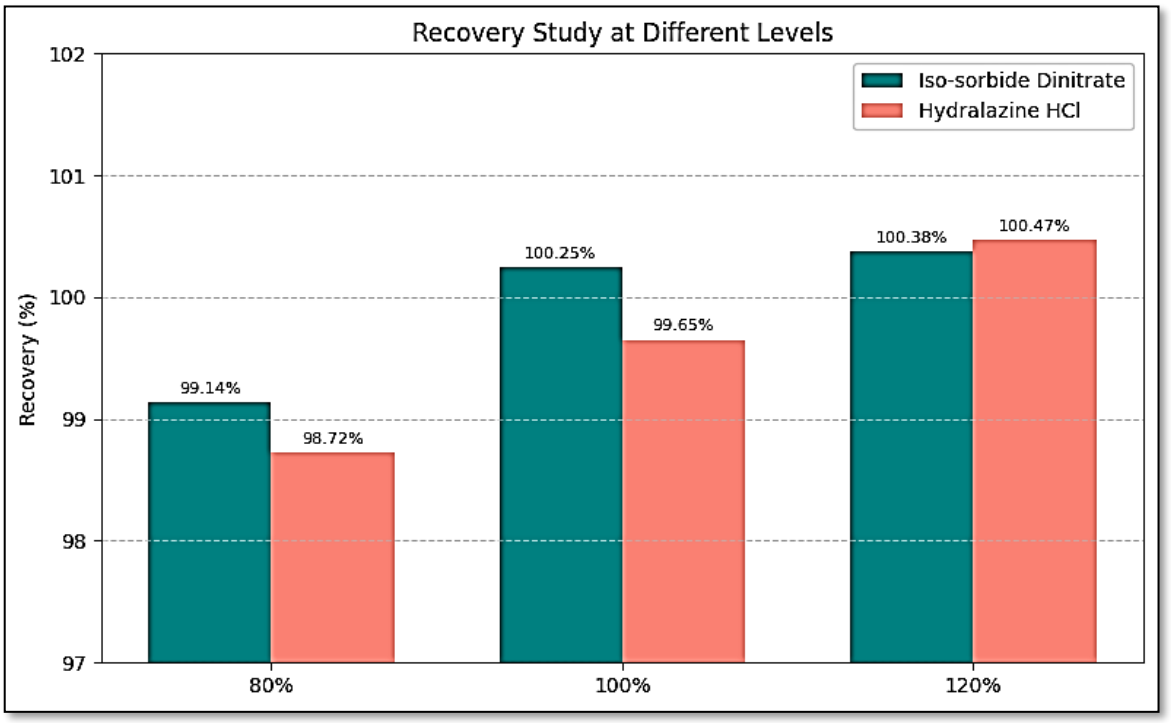
Table No.3 Standard method used to evaluate the new RP-HPLC method's accuracy

Level (%)	Iso-sorbide dinitrate Recovery (%)	Hydralazine Hydrochloride Recovery (%)
80	99.14	98.72
100	100.25	99.65
120	100.38	100.47

Standard addition recovery studies were used to evaluate the new RP-HPLC method's accuracy. Three concentration levels—80%, 100%, and 120% of the target concentration—of Iso-sorbide dinitrate and Hydralazine Hydrochloride were added to the pre-analyzed sample matrix. To determine how near the measured values were to the actual values, we performed triplicate analyses on each level and then computed the percentage recovery.

Fig.No.2 Flow Chart represents the recovery study at different levels

7.4 Specificity and Forced Degradation



The method could effectively separate drug peaks from degradation products. Percentage degradation observed under stress conditions is tabulated in Table no.4

Stress Condition	Iso-sorbide dinitrate Degradation (%)	Hydralazine Hydrochloride Degradation (%)
Acid	11.3	12.6
Base	15.4	18.9
Oxidative	9.7	13.2
Thermal	7.2	6.8
Photolytic	6.5	7.4

The stability-indicating capability was confirmed by the chromatograms, which clearly separated all contaminants. The new RP-HPLC technique was shown to be highly specific by testing its capacity to clearly detect the analytes even when excipients, degradation products, and other potential interferences were present. This was accomplished by inducing deterioration and determining the stability-indicating nature of the procedure by subjecting Iso-sorbide dinitrate and Hydralazine Hydrochloride to different stress settings in accordance with ICH Q1A(R2) recommendations.

The drug substances were exposed to the following stress conditions:

Hydrolysis in an acidic environment with 0.1 N HCl at 60°C for 1 hour A one-hour basic hydrolysis reaction with 0.1 N NaOH at 60°C Stress caused by oxidation when exposed to 3% hydrogen peroxide for one hour at room temperature Caused by six hours of dry heat at 80 degrees Celsius, thermal stress Degradation through photolysis induced by twenty-four hours of exposure to ultraviolet light at 1925 nm

Preparation for HPLC analysis included neutralising materials as needed, diluting them with mobile phase properly, and filtering them through 0.45 µm membrane filters after stressful treatment.

7.5 Robustness

In order to test the developed RP-HPLC method's dependability and consistency under slightly changed conditions, tiny purposeful variations in critical chromatographic parameters were introduced. Some of the parameters that changed were:

Flow rate: ±0.1 mL/min from the optimized 1.0 mL/min

Detection wavelength: ±2 nm from the selected 230 nm

Mobile phase composition: ±2% variation in acetonitrile to buffer ratio

Buffer pH: Adjusted ±0.2 units from pH 3.0

The other parameters were held constant while each modified condition was examined separately. The chromatographic parameters that were measured included retention time, peak area, resolution, and tailing factor. The relative standard deviation (%RSD) of peak areas was also computed. Regardless of the analytical conditions, the method consistently produced dependable and repeatable results with iso-sorbide dinitrate and hydrochloride concentrations, as the %RSD values for both substances were less than 2.0.

7.6 Limits of Detection and Quantification

Iso-sorbide dinitrate: LOD = 0.18 µg/mL, LOQ = 0.54 µg/mL

Hydralazine Hydrochloride: LOD = 0.32 µg/mL, LOQ = 0.96 µg/mL

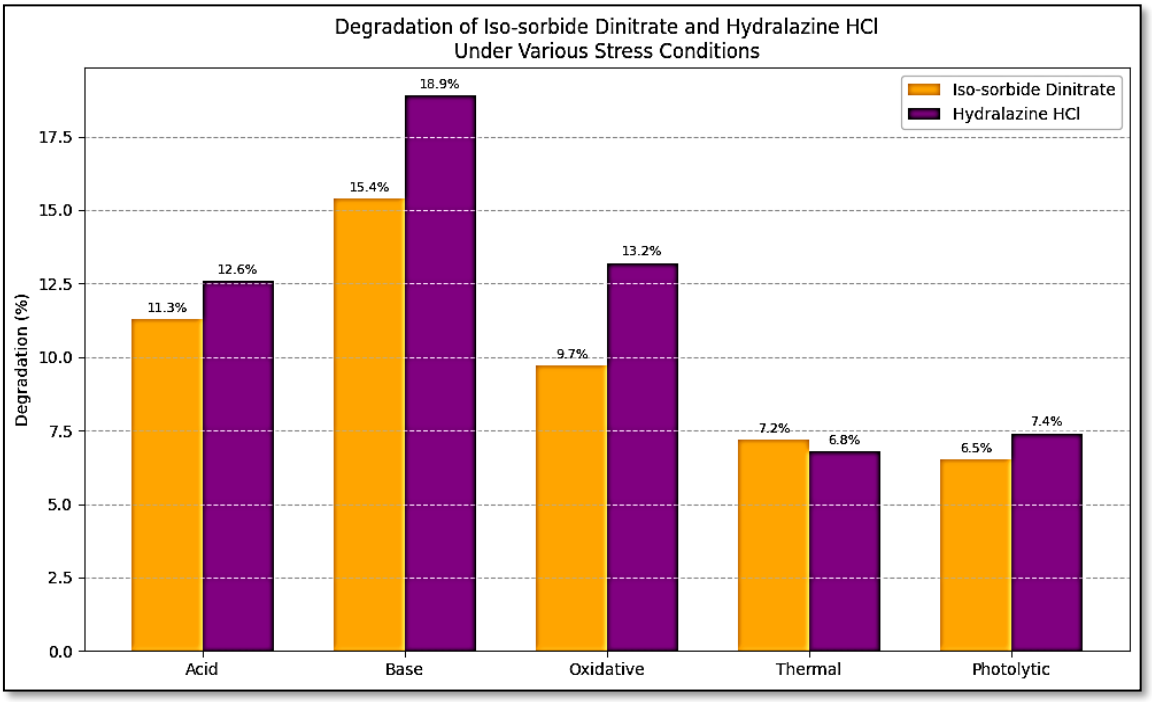


Fig.No.3 Bar graph of degradation of Iso-sorbide Dinitrate and Hydralazine HCL under various stress conditions.

8. CONCLUSION

Combination tablet dosage forms of Iso-sorbide dinitrate and Hydralazine hydrochloride were successfully measured using an RP-HPLC method that was developed and validated. This approach is meticulous, thorough, and accurate. As shown by thorough forced degradation investigations in acidic, basic, oxidative, thermal, and photolytic conditions, the approach was meant to be stability-indicating. The specificity and robustness of the devised approach were confirmed by the successful separation of the APIs from their degradation products. Method validation was conducted in accordance with the guidelines of ICH Q2(R1), which included the evaluation of important metrics such as linearity, accuracy, precision, specificity, LOD, LOQ, robustness, but also system adaptability. Analytical performance that meets all validation parameters' specified acceptance criteria is characterised by correlation coefficients (R^2) greater than 0.999, recoveries within 98-102%, and %RSD values less than 2.0. Due to its accuracy, reproducibility, and capacity to signal stability, the developed method is perfect for routine quality control studies, assay results, and stability testing of fixed-dose pharmaceutical formulations including Iso-sorbide dinitrate and Hydralazine Hydrochloride.

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