Research Article

Stability Indicating Validated RP-HPLC Method for Simultaneous Determination of Hydralazine Hydrochloride and Isosorbide Dinitrate in Bulk and Pharmaceutical Dosage Form

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ABSTRACT

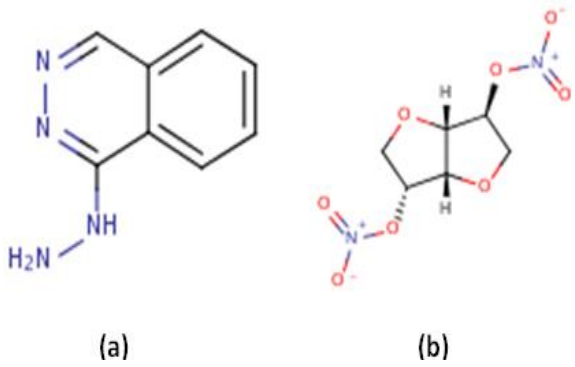
A simple, rapid, precise, accurate and economical stability-indicating reversed phase (RP) HPLC assay method was developed and validated for simultaneous estimation of Hydralazine Hydrochloride (HYD HCl) and Isosorbide Dinitrate (ISD) in bulk drugs and their combined commercial tablets. The method has shown adequate separation of HYD HCl and ISD from their degradation products. Separation was achieved on a Zorbax C18 (250mm×4.6mm I.D; 5 µm) column at a detection wavelength of 278nm, using a mobile phase consists of Orthophosphoric acid (0.1%) pH 2.1 and Methanol (60/40) in a isocratic elution mode at a flow rate of 1 ml/min. The retention times for Hydralazine HCl and Isosorbide Dinitrate were found to be 3.7 and 4.7 min respectively. Hydralazine HCl and Isosorbide Dinitrate their combination drug products were subjected to acid, base, neutral hydrolysis, thermal and photolytic stress conditions. Thus stressed samples were analyzed by the proposed analytical method. Validation of the proposed analytical method was carried out as per ICH guidelines Q2R1. Quantitation was achieved with UV detection at 278 nm based on peak area with linear calibration curves at concentration ranges 18.75-112.5µg/ml for HYD HCl and 10-60µg/ml for ISD (R2 > 0.999 for both drugs). The limits of detection were 0.0179µg/ml and 0.763µg/ml for Hydralazine HCl and Isosorbide Dinitrate respectively. The method was found to be specific and stability indicating as no interfering peaks of degradens and excipients were observed. The proposed method is hence suitable for application in quality-control laboratories for quantitative analysis of both the drugs individually and in combination dosage forms, since it is simple and rapid with good accuracy and precision.

Keywords: Stability indicating assay, RP-HPLC, Hydralazine Hydrochloride, Isosorbide Dinitrate, Forced degradation studies.

INTRODUCTION

Hydralazine HCl[1-2] is chemically 1-hydrazinylphthalazine.With molecular formula- $C_8H_8N_4$ and 160.17 mg molecular weight. It is freely soluble in water and sparingly soluble in methaline chloride. Hydralazine is a direct-acting smooth muscle relaxant. It is used as an antihypertensive agent in cases like preeclampsia (a condition in pregnancy characterized by high blood pressure). Hydralazine HCl acts by increasing cyclic guanosine mono-phosphate (cGMP) levels which causes an increase in the activity of protein kinase G (PKG).This results in blood vessel relaxation and causes dilation of arteries and arterioles.

Isosorbide Dinitrate (ISD)[3-4] is 1,4:3,6-dianhydro-2,5-di-O-nitro-D-glucitol or (3R,3aS,6S,6aS)-6-(nitrooxy)-hexahydrofuro [3,2-b]furan-3-yl nitrate. Its molecular weight 236.1363mg. It is slightly soluble in water and propanol, sparingly soluble in ethanol and freely soluble in methanol. Isosorbide Dinitrate is a moderate to long acting oral organic nitrate which acts as a vasodilator profoundly used in the treatment of angina pectoris, a condition which occurs when the oxygen supply to the myocardium is insufficient for its needs. The vasodilatation action of Isosorbide dinitrate is through the relaxing action in blood vessels of nitrates, particularly nitric oxide. This will decrease the oxygen demand of the heart and preventing chest pain. Hydralazine HCl and Isosorbide dinitrate in combination are used with other medications to treat heart failure. As both the drugs are vasodilators they work by relaxing and widening blood vessels so blood can flow more easily to the heart.



(a) (b)

Figure 1: Chemical structure of (a) Hydralazine HCl (b) Isosorbide Dinitrate

Objective of Study

Literature survey revealed that Methods for the determination of include Isosorbide Dinitrate [HPLC] Method available and for the determinations of Hydralazine HCl include HPLC[5-12], Gas chromatography[15-16], simultaneous spectrophotometric[13-14] determination and other methods[17-18]. However, these analytical methods lack stability indicating nature. Also, there was no reported analytical method for the estimation of both the drugs in pharmaceutical dosage forms in presence of their degradation products. In the present investigation, an attempt was made to develop a simple, rapid, precise and accurate stability indicating RP-HPLC assay method for simultaneous estimation of HYD HCl and ISD in

presence of their degradation products. The major advantage of the proposed method is that Hydralazine HCl and Isosorbide Dinitrate can be determined on a single chromatographic system with the same detection wavelength.

This proposed method can be successfully employed for quality control during manufacture and for assessment of the stability of both drugs in bulk samples and their combined tablet dosage forms.

MATERIALS AND METHODS

Drug Substance

Hydralazine HCl and Isosorbide Dinitrate (working standard 99.10 and 99.70) ware obtained as gift sample from Rainbow pharma training lab, Hyderabad, India. Pharmaceutical tablet formulation of ISOLAZINE was purchased from local pharmacy.

Methanol (HPLC Grade; MERCK), Orthophosphoric acid (HPLC grade, MERCK), Hydrochloric acid(AR), sodium hydroxide(AR), hydrogen peroxide (AR) and HPLC grade water ware used for the entrained study.

Instrumentation

All HPLC experiments were carried out on a Waters Alliance 2695 separation module, with waters 2996 photodiode array detector in isocratic mode using Auto sampler. Data collection and processing was done using EMPOWER PDA 2 software. The analytical column used for the separation was Zorbax C18, 250× 4.6 mm I.D., 5µm particle size. Other equipment's used were ultra-sonicator (model 3210, BransonUltrasonics Corporation, Connecticut, USA), Analytical balance (contech balance).

Preparation of Solutions

Diluent

Diluents were prepared by mixing solvent A and solvent B (a: b, 60/40)

Preparation of 0.1% OPA Buffer Solution

0.1% OPA was prepared by taking 1ml of OPA in 1000ml HPLC grade water.

Mobile Phase

Mobile phase was prepared by mixing OPA (pH-2.1, 0.1%) and Methanol (60/40). It was filter to 0.45µ membrane filter to remove the impurities otherwise they may interfere in the final chromatogram and it was sonicated for 15min to remove the undissovable gases and air bubbles.

Preparation of 0.1N HCL

0.1 N HCL was prepared by taking 0.08ml of conc. HCL in 100ml of HPLC grade water.

Preparation of 0.1N NaoH

0.1N NaoH was prepared by taking 0.4mg of NaoH in 100ml of HPLC grade water.

Preparation of Hydrogen Peroxide

Hydrogen peroxide was prepared by taking 3ml of hydrogen peroxide in 100ml of HPLC grade water.

Standard Solution

Standard solutions of Hydralazine HCl and Isosorbide Dinitrate ware prepared by dissolving 37.5mg of Hydralazine HCl and 20mg of Isosorbide Dinitratein two separate 100ml volumetric flasks contain 10ml of HPLC grade water in each flask, sonicate for 5min and final volume ware made up to the mark with HPLC grade water. From these stock solutions take 5ml from each flask and transfer into two separate 25ml volumetric flasks, the final volumes were made up to the mark with HPLC grade water to get the concentrations of 75µg/ml of HYD HCl and 40µg/ml of ISD respectively.

Chromatographic Condition

Zorbax C18 (250mm×4.6mm I.D; 5 µm) column at a detection wavelength of 278nm, using a mobile phase consists of Orthophosphoric acid (0.1%) pH 2.1 and Methanol (60/40) in a isocratic elution mode. The contents of the mobile phase was degassed with a helium spurge for 15 min and pumped from the respective solvent reservoirs to the column at a flow rate of 1 ml/min. The column temperature was maintained at 30°C and run time 10mins. The injection volume of samples was 10 µl. The retention times for Hydralazine HCl and Isosorbide Dinitrate were found to be 3.701 and 4.7 mins.

Method Development

Table 1: Optimized Chromatographic Conditions

Column	Zorbax 250*4.6mm,5µm
Flow rate	1ml/min
Wavelength	278nm
Column temperature	30°c
Injection volume	10µl
Run time	10min
Diluents	HPLC grade water
Elution	Isocratic
Mobile phase	0.1%OPA (pH 2.1),Methanol(60/40 v/v)
Remarks	Good peak

To saturate the column, the mobile phase was pumped for about 30 minutes thereby to get the base line corrected. The separate standard calibration lines were constructed for each drug. A series of aliquots were prepared from the above stock solutions using HPLC grade water to get the concentrations 18.75-112.5µg/ml HYD HCl and 10-60µg/ml ISD. Each concentration 6 times was injected in to chromatographic system. Each time peak area and retention time ware recorded separately for both the drugs. Calibration curves were constructed by taking average peak area on Y-axis and concentration on X-axis separately for both the drugs. From the

calibration curves regression equations ware calculated. The obtained optimized chromatographic conditions were shown in the Table 1.

Estimation of HYD HCL and ISD in Tablet Dosage Form

For the analysis of drugs, 20 tablets were weighed and triturated in a glass mortar and quantity of powder equivalent to 37.5mg of Hydralazine HCl was transferred to 100ml volumetric flask and dissolved in sufficient quantity of HPLC grade water. It was sonicated for 5mins and volume was made up to 100ml HPLC grade water. It was filter to 0.45µ membrane filter. From this solution transfer 5ml into 25ml volumetric flasks, the final volume were made up to the mark with HPLC grade water to get the concentrations of 75µg/ml of HYD HCl and 40µg/ ml of ISD respectively. The test concentration is injected 6 times in to chromatographic system. Each time peak area and retention time was recorded and the results obtain for as shown in the Table 2.

Method Validations

The analytical method was validated for various parameters as per ICH guidelines.

Linearity

The linearity of the method was determined in concentration range of 18.75-112.5 µg/ml for HYD HCl and 10-60 µg/ml for ISD. Each solution was injected in triplicate. The average peak area versus concentration data of both drugs was treated by least squares linear regression analysis. Linearity was checked over the same concentration range on three consecutive days and the results obtained from as shown in Table 3.

Specificity and Selectivity

Specificity is the degree to which the procedure applies to a single analyte and is checked in each analysis by examining blank matrix samples for any interfering peaks. The specificity of the method was evaluated with regard to interference due to presence of any other excipients. Two different samples were injected and studied with respective excipients. The HPLC chromatograms recorded for the drug matrix (mixture of the drug and excipients) showed almost no interfering peaks with in retention time ranges. Thus, the HPLC method proposed in this study was selective.

Accuracy and Recovery

Accuracy was evaluated in triplicate, at three different concentrations equivalent to 50, 100, and 150% of the target concentration of active ingredient, by adding a known amount of each of the Standard to a sample of known concentration of both drugs and calculating the % of recovery, And the results obtained from as shown in Table 4.

Precision

Precision is the degree of repeatability of an analytical method under normal operational conditions. Precision of the method was determined with the standard and test sample.

The precision of the method was verified by repeatability (intraday) and the intermediate precision studies.

Repeatability studies were performed by analysis of the concentrations of working standard for HYD HCl and ISD.

Method repeatability was achieved by repeating the same procedure of preparation of solution six times and injecting.

Intermediate precision was performed by performing the same procedure on the same day for intra-day precision.

The inter day precision of the method was checked by performing same procedure on different days under same experimental conditions.

The repeatability of sample application and measurement of peak area were expressed in terms of relative standard deviation (%RSD) and results obtained from as shown in Table 5.

LOD and LOQ

LOD

It is lowest amount of analyte in a sample that can be detected but not necessarily quantities as an exact value under the stated, experimental conclusions.

The detection limit is usually expressed as the concentration of analyte. The standard deviation and response of the slope.

LOD = 3.3*standard deviation (б)/s

LOQ

The quantitation limit of an analytical procedure is the lowest amount of an analyte of a sample which can be quantitatively determined with suitable precision and accuracy.

The standard deviation and response of the slope and the results obtained from as shown in the Table 3.

LOQ= 10* standard deviation (б)/s

Robustness

To evaluate the robustness of the method, the chromatographic conditions were deliberately altered and degree of reproducibility was evaluated.

During robustness testing each condition was varied separately, all other conditions being held constant at the optimized values. Robustness of the proposed method was assessed with respect to small alterations in the flow rate (1.0 ± 0.2ml/min), and Temperature (30°C ± 2°C) and the results obtained from as shown in the Table 6.

System Suitability Parameters

For assessing system suitability, six replicates of working standards samples of HYD HCl and ISD were injected and studied the parameters like plate number (N), tailing

factor (K), resolution, relative retention time and peak asymmetry of samples.

The results were tabulated in Table 7.

Degradation Sample Preparation

Weigh accurately 20 tablets and crush into fine powder transfer powder into petridish. Weigh accurately 100mg (18.75mg HYD HCL and 10mg ISD) of powdered sample into a 100ml volumetric flask dissolve and dilute to volume with HPLC grade water and filter the solution using 0.45µ Nylon filter.

Acid Hydrolysis

Transfer 1ml (100ug/ml HYD HCl) of above stock solution to10ml volumetric flask and add 1ml of 0.1N HCL and reflux for 30min at 60°C. Cool to room temperature and neutralize with 1ml of 0.1N NaOH and makeup volume with HPLC grade water.

Base Hydrolysis

Transfer 1ml (100ug/ml HYD HCl) of above stock solution to10ml volumetric flask and add 1ml of 0.1N NaOH and reflux for 30min at 60°C. Cool to room temperature and neutralize with 1ml of 0.1N HCl and makeup volume with HPLC grade water.

Peroxide Hydrolysis

Transfer 1ml (100ug/ml of HYD HCl) of above stock solution to10ml volumetric flask and add 1ml of 3%v/v of H_2O_2 and reflux for 30min at 60°C. Cool to room temperature and makeup volume with HPLC grade water.

Thermal Degradation

Weigh accurately 20tablets and crush into fine powder and transfer powder to 200mg (37.5mg HYD HCl and 20mg ISD) powder into petridish.

Heat the sample in oven for about 6hrs at 105°C. From this weigh accurately 100 mg of powdered sample into a 100ml volumetric flask dissolve and dilute to volume with HPLC grade water.

Transfer 1ml of above stock solution to10ml volumetric flask and filter the solution using 0.45µ Nylon filter.

Photolytic Degradation

Photolytic degradation study was carried out by exposing the accurately weighed 200mg (37.5mg HYD HCl and 20mg ISD) of tablet powder to UV light in a photolytic chamber at 2600 lux for 24 hr, After 24hrs weigh accurately 100 mg of powdered sample into a 100ml volumetric flask dissolve and dilute to volume with HPLC grade water.

Transfer 1ml of above stock solution to10ml volumetric flask and filter the solution using 0.45µ Nylon filter. Using the peak purity test, the purity of the drugs peaks were checked at every stage of above-mentioned studies.

Forced Degradation Study

Stress-degradation studies of the drug substances can help identifying the possible degradation products which can in turn help establishing the degradation pathways and the intrinsic stability of the molecule and validate the stability-indicating power of the analytical procedures used. The chromatograms of Hydralazine HCl and Isosorbide dinitrate, after being subjected to different mild and drastic degradation conditions, were compared with blank solutions injected in a similar manner and with recently prepared solutions. These results showed the specificity of the developed method clearly. The results indicated that in the stressed degradation studies using the optimized methods, degradation peaks for HYD HCl and ISD did not affect the drug peak.

The mass balance of hydralazine HCl and Isosorbide Dinitrate under each stress condition was found 100% and, moreover, assay of each unaffected compound in the tablets confirmed the stability indicating nature of the method. ISD was relatively more labile than HYD HCl in neutral degradative conditions while HYD HCl was more susceptible than ISD in acidic, basic, oxidative, and thermal stress conditions.

The percentage of degradation of photolytic stress condition was same for the both the drugs.

The results from forced degradation studies were summarized in Table 7. Representative Chromatograms obtained from forced degradation studies are shown in Fig no. 2 (a-e).

The degradation behavior and degradation products of HYD HCL and ISD were found to be similar in combination drug product and in bulk drugs under various stress conditions assessed.

The study was not intended to identify degradation products but merely to show they would not interfere if and when present. To conclude, the results of stress testing studies indicate a high degree of specificity of this method for both HYD HCl and ISD.

Table 2: Results of Marketed Formulation Analysis

Drug Name	Labeled Claim(mg)	Test Concentration (µg/ml)	Mean Amount Found (µg/ml)	% Estimated Amount	%RSD
Hydralazine HCl	37.5	75	112	100.4	0.25
Isosorbide Dinitrate	20	40	59	101.6	0.11

Table 3: Results of Linearity Studies

Parameters	Hydralazine HCl	Isosorbide Dinitrate
Linearity range (µg/ml)	37.5-112.5	20-60
Regression line equation	y = 14278x + 999.6	y =18112.x + 353.4
Correlation coefficient (*r*)	0.999	0.999
No of data points	5	5
LOD (µg/ml)	0.179	0.0763
LOQ (µg/ml),	0.597	0.2543

Table 4: Accuracy Results

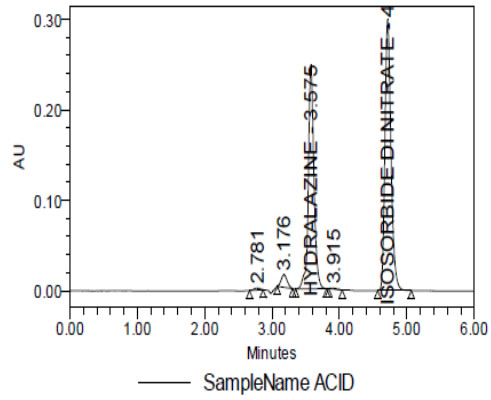
Drug Name	Pre analysed Concentration Taken (µg/ml)	Recovery Level (%)	Amt of Drug Added (µg/ml)	Amt of Drug Found (µg/ml) (n=3)	% Recovery	Acceptance Criteria
Hydralazine HCl	37.5	50	18.75	56.24	100.01	97-103%
		100	37.5	74.9	100.13	97-103%
		150	56.2	93.69	100	97-103%
Isosorbide Dinitrate	20	50	10	29.9	100.33	97-103%
		100	20	39	102.56	97-103%
		150	30	49.9	100.2	97-103%

Table 5: Precision Results

Day of Analysis		% Recovery ± SD; (n=3) % RSD		
Intraday Precision				
HYD HCl (µg/ml)	18.75	37.5	56.2	0.25
Day 0	100.01 ± 0.17	100.33 ± 0.77	100.01 ± 0.03	
Day 1	100.13 ± 0.09	102.56 ± 1.46	100.13 ± 0.09	
Day 2	100 ± 0.2	100.2 ± 0.9	100 ± 0.04	
ISD (µg/ml)	10	20	30	
Day 0	100.33 ± 0.71	100.33 ± 0.78	100.33 ± 0.29	0.11
Day 1	102.56 ± 1.53	102.56 ± 1.53	102.56 ± 1.53	
Day 2	100.2 ± 0.83	100.2 ± 0.83	100.2 ± 0.83	
Interday Precision				
HYD HCl (µg/ml)	108.75	37.5	56.2	
Day 0,1,2	100.13 ± 0.09	100.2 ± 0.9	100.13 ± 0.9	0.25
ISD (µg/ml)	10	20	30	
Day 0,1,2	102.56 ± 1.53	102.56 ± 1.53	100.2 ± 0.83	0.11

Table 6: Robustness Results

Method Parameters	Conditions	Retention Time(R_t)		Area		%Recovery	
		HYD HCl	ISD	HYD HCl	ISD	HYD HCl	ISD
Temp1	28	3.631	4.697	3588044	3680877	100.01	100.33
Temp2	30	3.647	4.739	3592919	3669072	100.13	102.56
Temp3	32	3.641	4.641	3588041	3680878	100	100.2
Flow1	0.8	3.653	4.735	3601543	3686219	99.12	100.33
Flow2	1	3.660	4.744	3586529	3687693	102.56	102.56
Flow3	1.2	3.610	4.731	3601542	3686218	98.1	100.2



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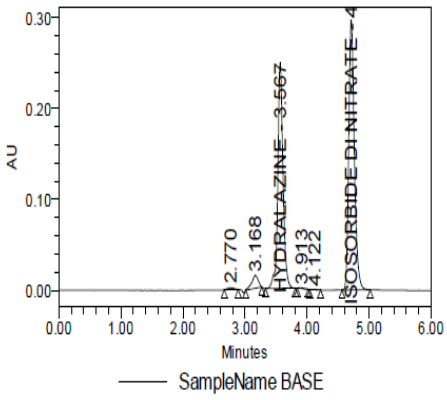

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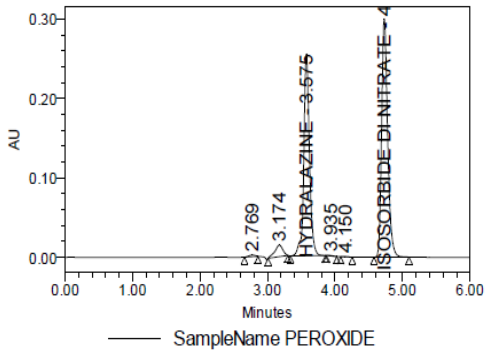

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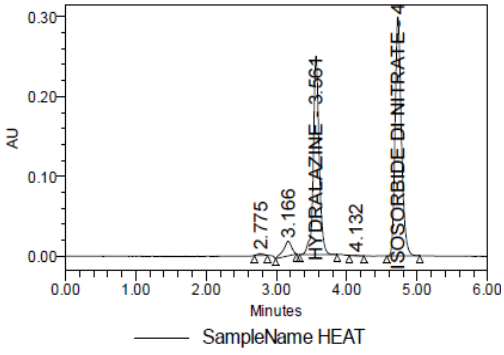

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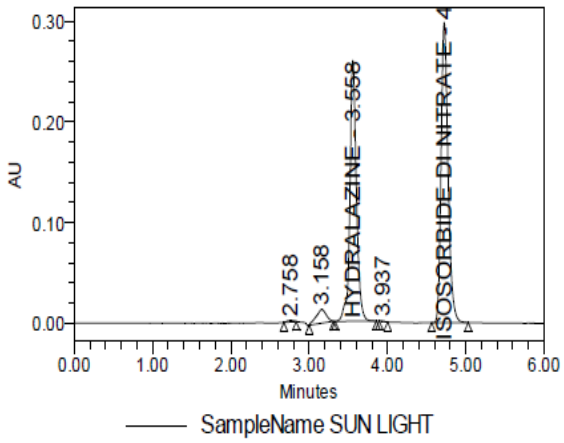

(e)

Figure 2 (a-e): Chromatograms of degradation-(a) acid degradation (b) Alkali degradation (c) peroxide degradation (d) thermal degradation (e) photo degradation sample.

Table 7: Forced Degradation Studies

Stress Conditions	Hydralazine HCl	Isosorbide Dinitrate
	% Degradation	% Degradation
Acidic/0.1 M HCl/60°C reflux/48 h	92	97
Basic/0.1 M NaOH/60°C reflux/48 h	87	91
Oxidizing/3% H2O2/cool at RT/30min	88	97
Thermal/105°C/6hr	86	97
Photolysis/UV light	90	90

Solution Stability

The stock solution showed no significant change in analyte composition, retention time and peak areas of HYD HCl and ISD after 1 weeks of storage at room temperature which was sufficient for the whole analytical process.

RESULTS AND DISCUSSION

Optimized Chromatographic Conditions

Most of all reported HPLC methods till date use C-8 or C-18 columns. Most of this use complex mobile phase compositions. Hence, attempts were directed towards development of a Simple and better method on commonly used C18 column with good resolution. Different logical Modifications were tried to get good separation among the drugs and the degraded products. These changes included change in mobile phase composition in isocratic elution as well as gradient modes on different C18 columns.

The optimized chromatographic conditions (Figure 1). The best peak shape and maximum separation was achieved with mobile phase composition of 0.1% OPA (pH 2.1) and methanol (a:b, 60/40). The best separation, peak symmetry and reproducibility were obtained on Zorbax C18 250mm×4.6mm I.D; 5 µm).

The optimum wavelength for detecting the analyte was found to be 278nm, a flow rate of 1ml/min yielded optimum separation and peak symmetry. As shown Table 1.

Degradation Studies

Results are tabulated in Table 7.

Acid Hydrolysis (Figure 2a)

Upon performance of acid degradation studies 92% of Hydralazine HCl and 97% of Isosorbide Dinitrate was degraded.

Base Hydrolysis (Figure 2b)

Upon performance of base degradation studies 87% of Hydralazine HCl and 91% of wasIsosorbide Dinitrate degraded.

Peroxide Hydrolysis (Figure 2c)

Upon performance of peroxide degradation studies 88% of Hydralazine HCl and 97% of Isosorbide Dinitrate was degraded.

Thermal Degradation (Figure 2d)

Upon performance of Thermal degradation studies 86% of Hydralazine HCl and 97% of Isosorbide Dinitrate was degraded.

Photolytic Degradation (Figure 2e)

Upon performance of Photolytic degradation studies 90% of Hydralazine HCl and 90% of Isosorbide Dinitratel was degraded.

Linearity, LOD and LOQ

The calibration plot was linear over the concentration range investigated (18.75-112.5µg/ml; *n* = 3) and (10-60µg/ml; *n* = 3) for HYD HCl and ISD respectively.

Average correlation co-efficient =0.999 for both drug candidates with %RSD values ≤2.0 across the concentration range studied was obtained from regression analysis. The LOQ that produced the requisite precision and accuracy was found to be 0.597µg/ml for HYD HCl and 0.2543µg/ml for ISD.

The resultant %RSD values were ≤ 1.00 % (Table 3). The LOD for HYD HCl and ISD were found to be 0.179µg/ml and 0.076 µg/ml respectively.

The regression results indicate that method was linear in the concentration range studied (Table 3) and can be used for detection and quantification of HYD HCl and ISD in a very wide concentration range.

Accuracy and Precision

Accuracy as recovery was evaluated by spiking previously analysed test solution with additional standard drug at three different concentration levels (Table 4).

Recovery of standard drugs added was found to be 101.03% for Hydralazine HCland 100.04% for Isosorbide Dinitrate with the value of RSD less than 1% indicating that the proposed method is accurate for the simultaneous estimation of both drugs from their combination drug products in presence of their degradation products. The low RSD values indicate the repeatability and reproducibility of the Method (Table 4).

Robustness

Results of the robustness study are shown in Table 6. The elution order and resolution for both components were not significantly affected. RSD of peak areas were found to be well within the limit of 2.0%.

CONCLUSION

Stress testing (or forced degradation studies) is an important part of drug development process and the pharmaceutical industry has much interest in this area.

A simple, rapid, accurate and precise stability-indicating HPLC analytical method has been developed and validated for the quantitative analysis of Hydralazine HCl and Isosorbide Dinitrate in bulk drugs and combined dosage forms.

The results of stress testing undertaken according to the ICH guidelines reveal that the method is specific and stability-indicating. The proposed method has the ability to separate these drugs from their degradation products in tablet dosage forms and hence can be applied to the analysis of routine quality control samples and samples obtained from stability studies.

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