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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF HYDRALAZINE, ISOSORBIDE DINITRATE IN BULK AND TABLET FORMULATION BY RP-HPLC

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ABSTRACT: A simple, sensitive, linear, precise and accurate RP-HPLC method for simultaneous estimation of Hydralazine, Isosorbide dinitrate in bulk and tablet formulation was developed and validated. The chromatographic separation of the three drugs was achieved on Zodiac C18 (250 mm × 4.6 mm) 5µ column in an isocratic mode. The mobile phase consisting of 0.01M 0.01 M Ammonium acetate : Acetonitrile : Methanol in the ratio of 50:30:20 v/v and pH adjusted to 3 using ortho phosphoric acid was delivered at a flow rate of 1ml/ min and effluents were monitored at 270 nm. The retention time of Hydralazine, Isosorbide Dinitrate was found to be 2.337 and 3.413 min, respectively. Calibration curves were linear with a correlation coefficient of 0.994 for HYD , and 0.997 for ISD over the concentration range of 45-105µg/ml for HYD, and 24-56 µg/ml for ISD and precise with (%RSD<2). The method was validated as per the ICH guidelines and can be employed for routine quality control analysis.

INTRODUCTION: Chemically, Isosorbide dinitrate is known as 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 [1] (**Figure 1**). 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.

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The vasodilating 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 [2].

Hydralazine, 1-hydrazinylphthalazine (**Figure 2**) 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 acts by increasing cyclic guanosine mono-phosphate (cGMP) levels which causes an increase in the activity of protein kinase G (PKG). Active PKG adds an inhibitory phosphate to myosin light-chain kinase which is a protein involved in the activation of cross-bridge cycling (i.e. contraction)

in smooth muscle. This results in blood vessel relaxation and causes dilation of arteries and arterioles. It also functions as an antioxidant. It inhibits membrane-bound enzymes that form reactive oxygen species, such as superoxides. Excessive superoxide counteracts nitric oxide-induced vasodilation [3, 4].

Isosorbide dinitrate and Hydralazine 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.

Literature survey reveals that few analytical methods were reported for determination of Isosorbide dinitrate and Hydralazine individually. Gas chromatography [5] and HPLC [6] methods were reported for Isosorbide dinitrate and spectrophotometric methods [7-9], electrochemical determination [10] and HPLC [11] methods were reported for Hydralazine alone in pharmaceutical products.

However, no method is reported for simultaneous estimation of these two drugs by reverse phase HPLC in combined dosage form.

MATERIALS AND METHODS:

Chemicals and reagents: The reference samples of HYD, ISD were provided by Dr. Reddy labs, Hyderabad. Tablet used for analysis was ISOLAZINE (Label claim: HYD 75mg, ISD 40 mg) is procured from the local market. Acetonitrile, methanol, and water used were of HPLC grade.

Potassium dihydrogen phosphate used is of Analytical grade.

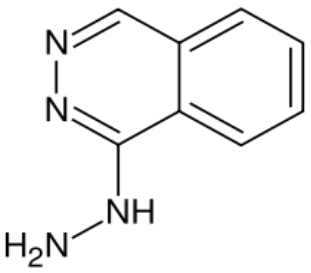

FIGURE 1: MOLECULAR STRUCTURE OF HYDRALAZINE

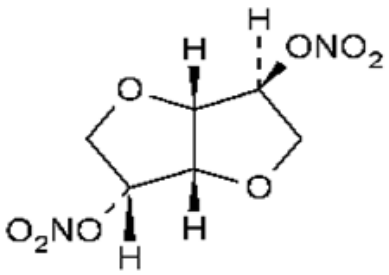

FIGURE 2: MOLECULAR STRUCTURE OF ISOSORBIDE DINITRATE

Instrument and Chromatographic conditions: The liquid chromatographic system consisted of Shimadzu, Pump (LC-10AT), fitted with UV-Visible detector (SPD-20A) with manual injector. The chromatogram was recorded using Spinchrome software. HPLC separations were carried out on Zodiac C18 (250 mm × 4.6 mm i.d.) packed with 5 µ diameter particles. The mobile phase was a mixture of 0.01 M Ammonium acetate, Acetonitrile and Methanol in the ratio of 50:30:20 v/v and pH adjusted to 3 using ortho phosphoric acid. The aqueous phase consisted of 0.01 M Ammonium acetate buffer, pH of the aqueous phase was adjusted to 3 with orthophosphoric acid and filtered through 0.45µm membrane filter and degassed before use. The flow rate was 1 ml/ min. The detection wavelength was set at 270 nm. The injection volume was 20 µl.

Preparation of Buffer for mobile phase: Weighed 3.85gm of Ammonium acetate into a 1000 ml beaker added 2 ml of triethylamine, dissolved and diluted to 1000 ml with HPLC water. The pH was adjusted to 3 with orthophosphoric acid and filtered through 0.45µm membrane filter and degassed.

Preparation of Mobile Phase: Mixed a mixture of 0.01 M Ammonium acetate buffer 500 ml (50%) and 300 ml of Acetonitrile (30%) and 200 ml methanol (20%) of HPLC grade, and degassed in ultrasonic water bath for 15 minutes, filtered through 0.45µm membrane filter.

Diluent Preparation*:* Mobile phase is used as diluents.

Preparation of Standard Solution: Stock solutions of standard drugs HYD and ISD were prepared by weighing accurately 75 mg of HYD and 40 mg of ISD into a 100 ml clean dry volumetric flask.

About 70 ml of the mobile phase was added and sonicated to dissolve the drugs completely. The volume was made upto 100 ml with the mobile phase and filtered through 0.45 µm membrane filter. From the above prepared standard stock solution, 5 ml was taken to 50 ml volumetric flask and the volume was made up with the mobile phase to obtain a concentration of 75 µg/ml and 40 µg/ml for HYD and ISD respectively.

Preparation of Sample Solution: Twenty tablets of Isolazine were weighed and powdered. Powder weight equivalent to 75 mg of HYD and 40 mg of ISD was weighed and transferred into a 100 ml clean dry volumetric flask. About 70 ml of the mobile phase was added and sonicated to dissolve the drugs completely. The volume was made upto 100 ml with the mobile phase and filtered through 0.45 µm membrane filter. From the above prepared standard stock solution, 5 ml was taken to 50 ml volumetric flask and the volume was made up with the mobile phase to obtain a concentration of 75 µg/ml and 40 µg/ml for HYD and ISD respectively

Method Validation: The proposed method was validated in compliance with ICH guidelines for linearity, accuracy, precision, selectivity, sensitivity, robustness, and system suitability parameters by the following procedures.

Linearity: Linearity of developed HPLC method was studied by obtaining calibration curves of HYD, ISD at six different concentration levels in triplicate ranging from 45-105 µg/ml for HYD, and 24-56 µg/ml for ISD. **Table 1** shows the linearity data of HYD and ISD. The linearity regression co-efficient (R^2) values were found to be 0.999 and 0.999 for HYD, ISD each. Linearity equation obtained for HYD, ISD were y = 35.61x+12.47, and y = 8.598x-97.03 respectively. **Figure 3 and 4** shows calibration curves for HYD, ISD respectively. High level of correlation coefficient indicates good linearity.

TABLE 1: LINEARITY DATA OF HYDRALAZINE, ISOSORBIDE DINITRATE

Hydralazine		Isosorbide Dinitrate	
Concentration (µg/mL)	Peak area	Concentration (µg/mL)	Peak area
45	1598.165	24	110.89
60	2141.807	32	170.979
75	2677.099	40	253.116
90	3318.275	48	318.275
105	3680.663	56	381.164

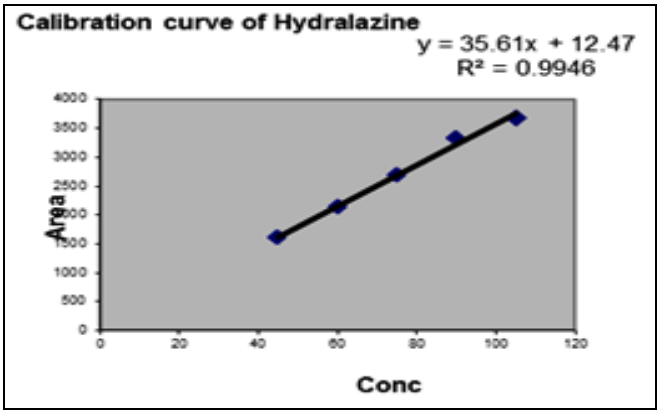

FIGURE 3: CALIBRATION CURVE OF HYDRALAZINE

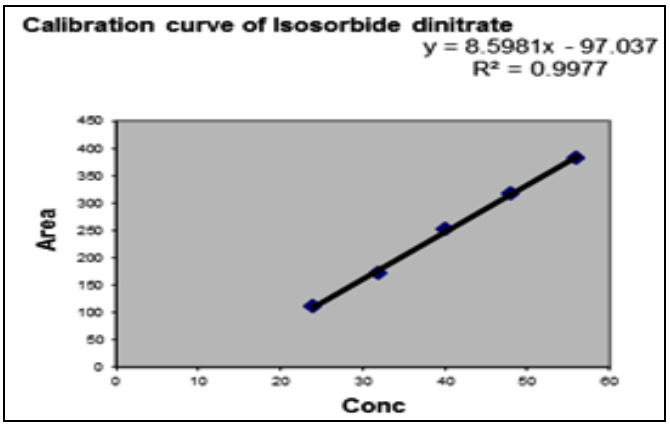

FIGURE 4: CALIBRATION CURVE OF ISOSORBIDE DINITRATE

Accuracy: The accuracy of the developed method was evaluated in triplicates by recovery studies at three different concentration levels of 80%, 100 %, and 120% for HYD, ISD respectively. Known amounts of standard drug concentrations were added to the sample and peak area was determined. The mean percentage recovery values are shown in **Table 2**. The mean recovery of the drugs was found to be in the range of 99- 101% indicating a high degree of accuracy for the developed method.

TABLE 2: ACCURACY RESULTS

Drugs	Conc.	Amount Present	Amount Spiked	Conc. after spiking	% recovery	Mean
HYD	80 %	60 µg/ml	15 µg/ml	75 µg/ml	100.49	100.02
	100%	75 µg/ml	15 µg/ml	90 µg/ml	100.83	
	120%	90 µg/ml	15 µg/ml	105 µg/ml	98.75	
ISD	80 %	32 µg/ml	8 µg/ml	40 µg/ml	99.29	99.67
	100%	40 µg/ml	8 µg/ml	48 µg/ml	100.72	
	120%	48 µg/ml	8 µg/ml	56 µg/ml	99.01	

Precision: The precision at 100 % concentration of the assay method was evaluated by six replicate injections and measurement of peak areas by determining the % RSD of Hydralazine, ISD. The calculated values of % RSD for HYD and ISD are mentioned in **Table 3**. The results indicated a high degree of repeatability.

TABLE 3: RESULTS FOR PRECISION

Injection	Hydralazine		Isosorbide Dinitrate	
	RT	Area	RT	Area
1	2.337	2767.579	3.413	222.354
2	2.273	2728.926	3.323	222.081
3	2.26	2710.928	3.303	221.739
4	2.273	2728.926	3.323	222.081
5	2.24	2700.010	3.270	210.357
6	2.233	2689.708	3.260	218.775
Average	2.2693	2721.013	3.315	219.565
STD	0.0371	27.628	0.055	4.703
%RSD	**0.93**	**1.02**	**0.98**	**1.45**

Selectivity: Selectivity test determines the effect of excipients on the assay result. To determine the selectivity of the method, standard sample of HYD, and ISD were injected first. Then commercial product, blank solutions were run in the instrument one after another. The results of the tests proved that the components other than the drug did not produce a detectable signal at the retention time of HYD, and ISD as shown in **Figure 5 and 6**.

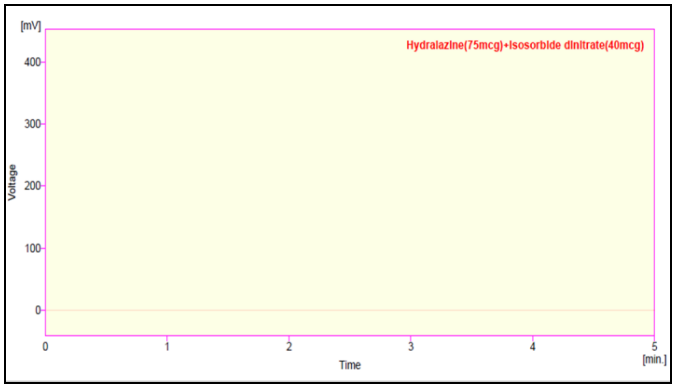

FIGURE 5: CHROMATOGRAM OF PLACEBO TABLET

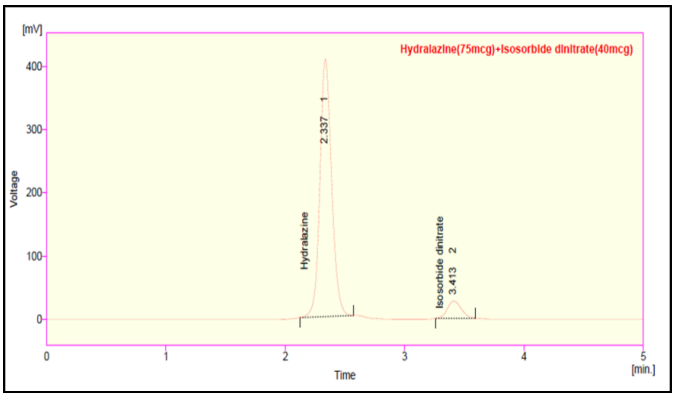

FIGURE 6: CHROMATOGRAM SHOWING PEAKS OF HYDRALAZINE AND ISOSORBIDE DINITRATE

LOD and LOQ: LOD and LOQ for HYD, ISD by this method were evaluated on the basis of signal-to-noise ratio method described in ICH guidelines. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit. A typical signal-to-noise ratio required for LOQ is 10:1.Using the proposed HPLC method, the LOD and LOQ values were calculated and are given in **Table 4**.

TABLE 4: LOD AND LOQ VALUES OF HYD AND ISD

	HYD	ISD
LOD(µg/mL)	2.20 µg/ml	4.89 µg/ml
LOQ(µg/mL)	6.66 µg/ml	14.83 µg/ml

Robustness: To evaluate the robustness of the developed RP-HPLC method, small deliberate variations in the optimized parameters were made in chromatographic conditions like of flow rate and wavelength.

The effect of change in flow rate and wavelength of detection on retention time and tailing factor were examined. The values obtained are mentioned in **Table 5**. The method was found to be unaffected by the small changes like ± 0.2 ml/min in flow-rate of mobile phase and ± 2 nm in detection wavelength.

TABLE 5: RESULTS OF ROBUSTNESS BY VARIATIONS IN FLOW RATE AND WAVELENGTH

Parameters	Value	HYD		ISD	
		RT	TF	RT	TF
Flow-rate	0.8 ml/min	2.800	1.281	4.107	1.324
	1.0 ml/min	2.337	1.259	3.413	1.219
	1.2 ml/ min	1.873	1.167	2.743	1.241
Wavelength	268 nm	2.333	1.450	3.320	1.478
	270 nm	2.337	1.259	3.413	1.219
	272 nm	2.300	1.500	3.303	1.417

System suitability: Six replicate of sample containing HYD, ISD were given to evaluate equipment, electronics, analytical operations and samples suitability. Parameters calculated for system suitability were %RSD of retention time and area, number of theoretical plates and Resolution. The results are given in **Table 6**.

TABLE 6: SYSTEM SUITABILITY RESULTS FOR ISD AND HYD.

S. No.	Parameters	Hydralazine	Isosorbide Dinitrate
1	Theoretical plates	2659	3819
2	Tailing factor	1.259	1.219
3	Resolution	--	5.345
4	Relative retention time (minutes)	2.337	3.413

RESULTS AND DISCUSSION:

Optimized chromatographic conditions: Chromatographic conditions were screened for mobile phase composition, mobile phase proportion, pH and flow rate Finally, mobile phase of 0.01M 0.01 M Ammonium acetate buffer (pH 3): Acetonitrile: Methanol in the ratio of 50:30:20 v/v was optimized to give symmetric peak with short runtime at UV detection wavelength of 270 nm and flow rate at 1ml/min was found to be appropriate with adequate separation between the two drugs. Chromatogram of HYD, ISD at optimized chromatographic condition was recorded, the runtime was 5 min and the retention times of HYD, ISD were found to be 2.337 and 3.413 min as shown in **Figure 6.**

Assay: The proposed method was applied for the analysis of tablet sample of Isolazine and the results of the assay were obtained within the specification limit. From the peak area obtained for HYD, ISD, the amount of the drug in the sample was calculated and was found to be 99.64% for HYD, and 99.01% for ISD.

CONCLUSION: The proposed HPLC method was found to be economical, simple, sensitive, accurate, precise, specific and robust and can be used for the routine quality control analysis of HYD, ISD in bulk as well as in tablet formulation.

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