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Stability indicating analytical method validation for hydralazine hydrochloride related substances method-I by Reverse Phase High Performance Liquid Chromatography in drug substances

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ABSTRACT

A simple, rapid, precise, accurate and cost effective stability-indicating reversed phase (RP) HPLC related substance method-1 was validated for Hydralazine Hydrochloride (HYD HCl) in Active pharmaceutical ingredient. All the analytical parameters were determined as per ICH Q2B guidelines. Good chromatographic separation was achieved with Inertsil ODS 3V column (4.6 mm x 250 mm, 5 µm particle size) at a wavelength of 230 nm using phosphate buffer pH 2.5 and acetonitrile as mobile phase A and Methanol as mobile phase B with gradient programming with a flow rate of 1.0 ml/ min. The Resolution between Hydralazine peak and impurity-A should not be less than 3.0. From the statistical treatment of the linearity data of Hydralazine HCl, it is clear that the response of Hydralazine HCl is linear between 50 % to 150 % level. The correlation coefficient is greater than 0.998. The developed method showed good linearity, Accuracy, reproducibility, precision and robustness and can be suitably applied for the routine quality control analysis in the estimation of commercial formulations.

Keywords: Hydralazine hydrochloride, HPLC, Validation, Estimation.

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INTRODUCTION

Hydralazine HCl 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[1-3].

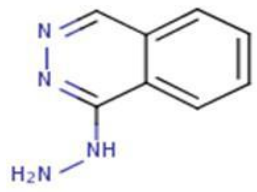


Figure 1: Chemical structure of Hydralazine HCl

Objective of Study

Literature survey revealed that Methods for the determinations of Hydralazine HCl include HPLC, Gas chromatography, simultaneous spectrophotometric determination and other methods. Literature survey reveals that different assay methods like spectrophotometry, spectrofluorometry, oxidimetry, and HPLC are available for the validation of Hydralazine hydrochloride in drug substances, But none of these methods are found suitable for routine quality control studies due to the following reasons like poor sensitivity, longer run time, using costly solvent, suitable at higher concentration only, extraction procedure involved in sample preparation [4-6]. Based on this, it was felt necessary to develop a validated simple, selective and sensitive HPLC method for the determination of Hydralazine hydrochloride in drug substances. The proposed method has been demonstrated superior to the existing procedures due to its sensitivity, speed, accuracy and it is suitable for routine quality control analysis. This proposed method can be successfully employed for quality control during

manufacture and for assessment of the stability of drugs in drug substances [6-10].

EXPERIMENTAL WORK:

Chromatographic Conditions:

Column: Inertsil ODS-3V, 250 x 4.6mm, 5.0µm
Detector wavelength: UV at 230 nm
Flow rate: 1.0mL / min.
Temperature: 30°C
Sample temperature: 10°C
Injection volume: 10µL
Run time : 70 minutes
Diluent: Water pH adjusted to 3.2 with Orthophosphoric acid.
Rinsing solution: Water: Acetonitrile (1:1) v/v
Preparation of Mobile phase:

Mobile phase A: Weigh and transfer about 0.68 g of potassium dihydrogen phosphate in 2000mL Water, sonicate to dissolve and adjust the pH to 2.5 with dilute Orthophosphoric acid, filter thorough 0.45µ.

Mobile phase B: Methanol

Gradient Program:

Time (minutes)	Solution A	Solution B
0	90	10
10	90	10
45	35	65
50	30	70
60	30	70
61	90	10
70	90	10

Standard Stock solution-A: Weigh and transfer accurately 15.0 mg of Impurity-A reference standard, 15.0 mg of Impurity-B reference standard, 15.0 mg of Impurity-C reference standard into a 100 mL volumetric flask, add 10 mL of Methanol. sonicate to dissolve, add diluent and make up to volume with diluent and mix.

Standard Stock solution-B: Weigh and transfer accurately 10.0 mg of Impurity-D reference standard in to 100mL volumetric flask, add approximately 50 mL of 10 % v/v Orthophosphoric acid solution in water and sonicate to dissolve and make up to mark with 10 % v/v Orthophosphoric acid solution.

Standard Stock solution-C: Weigh and transfer accurately 25.0 mg of Hydralazine Hydrochloride reference standard in to 100 mL volumetric flask, add about 50 mL of diluent and sonicate to dissolve and make up to mark with diluent.

Standard stock solution-D: Transfer 5.0 mL of standard stock solution-A, B and 2.0 mL of standard stock solution-C in to a 50 mL volumetric flask and dilute to mark with diluent.

Standard solution: Transfer 5.0 mL of standard stock solution-D in to a 50 mL volumetric flask and dilute up to mark with diluent.

System suitability solution: Weigh and transfer accurately 20.0 mg of Hydralazine Hydrochloride reference standard in 20 mL volumetric flask add standard solution, sonicate to dissolve and dilute up to the mark with standard solution.

Test Sample solution: Weigh and transfer accurately 50.0 mg of sample in to 50 mL volumetric flask add diluent, sonicate to dissolve and dilute up to the mark with diluent.

Impurity E Stock solution preparation: Weigh and transfer accurately 10.0 mg of Impurity-E reference standard in to 100 mL volumetric flask, add about 50 mL of diluent and sonicate to dissolve and make up to mark with diluent.

Impurity F Stock solution preparation: Weigh and transfer accurately 10.0 mg of Impurity-F reference standard in to 100 mL volumetric flask, add about 50 mL of solvent mixture (Acetonitrile: methanol) and sonicate to dissolve and make up to mark with Solvent mixture.

Hydrochloric acid, Impurity E and Impurity F peak Identification Solution: Transfer 4.5mL of conc. Hydrochloric acid in to 10 mL volumetric flask and add 1.0 mL each Impurity E and Impurity F stock solution and diluted up to mark with diluent.

Note: Inject freshly prepared system suitability solution, standard solution and Test solution

Procedure: Inject Blank (diluent), system suitability preparation, standard solution six replicates and Test preparation in duplicate. Hydrazine peak is eluting at the retention time of about 6.0 minutes under the given chromatographic condition. The relative retention times of all components are as follows.

Table 1: Name of component with relative retention time

Name of the component	Relative retention time (RRT)
Hydralazine Hydrochloride	1.00
Impurity-A	3.0
Impurity-B	4.9
Impurity-C	5.5
Impurity-D	6.8
Impurity-E	7.1
Impurity-F	8.3

Evaluation of System suitability: The system is suitable for analysis, if and only if, (a). The Resolution between Hydralazine peak and impurity-A should not be less than 3.0 and (b). %RSD for area of six replicate injections of standard solution for each component should be not more than 5.0

Note: Disregard the peaks due to blank, Hydrochloric acid, Impurity E and Impurity F.

Calculation: Calculate impurity-A, impurity-B, impurity-C, impurity- D and any other individual impurity and total impurities by the following formula

%Impurity A /%Impurity B/%Impurity C/%Impurity D

$$= \frac{\text{Area of impurity in sample X Wt. of impurity.std (mg) X 5 X 5X 50 X P1}}{\text{Avg.area of Impurity Std. X100 X 50 X 50 X Sample Weight (mg)}}$$

% any other individual impurity:

$$= \frac{\text{Area of any other individual impurity X wt of Hydralazine.std (mg) X 2 X 5 X 50 X P2}}{\text{Avg.peak area of Hydralazine in Standard X 100 X 50 X50 X Sample Weight(mg)}}$$

Where,

P1=Potency of Impurity standard

P2=Potency of Hydralazine HCl standard

%Total impurities = %Impurity A+% of Impurity B+% Impurity C+%Impurity D +% Total other impurities

Table 2: Specification limit of impurities and standard details

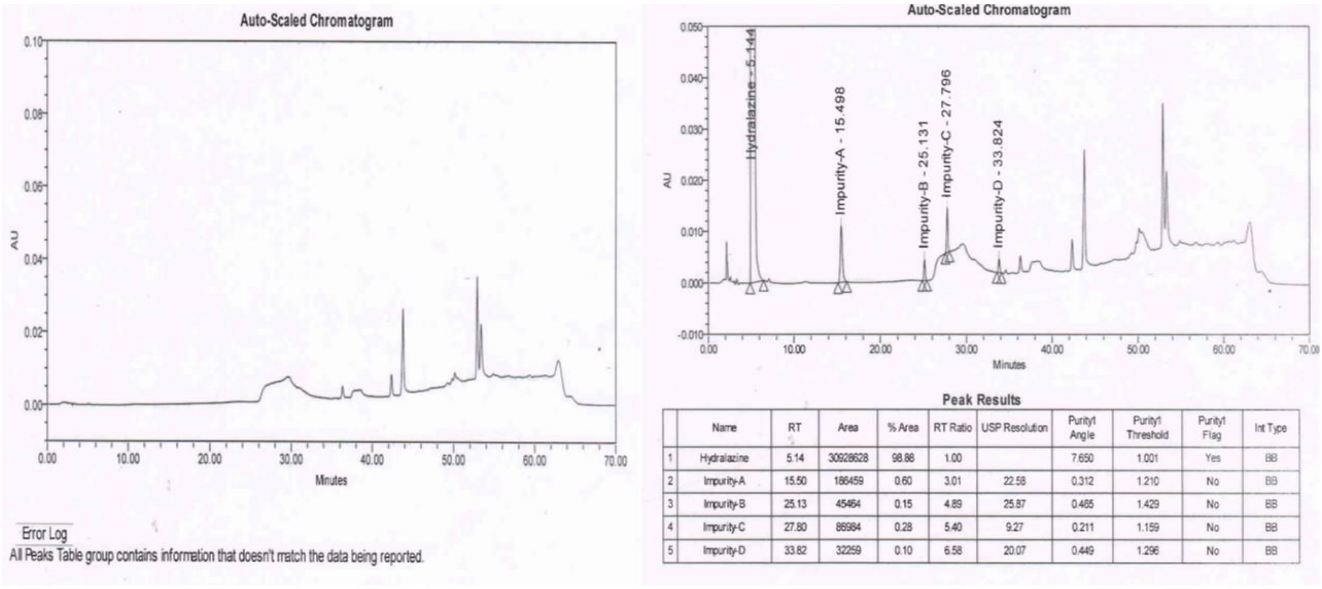
Sr. No	Name of the Component	Specification
1	Impurity-A	Not more than 0.15%
2	Impurity-B	Not more than 0.15%
3	Impurity-C	Not more than 0.15%
4	Impurity-D	Not more than 0.10%
7	Any other individual impurity	Not more than 0.10%
8	Total impurities	Not more than 1.0 %

Standard Details:

Standard Name	Potency
Hydralazine Hydrochloride	99.7
Impurity-A(Phthalazine)	98.8
Impurity-B	96.7
Impurity-C	99.6
Impurity-D	96.6
Impurity-E	94.3
Impurity-F	94.2
Hydralazine Hydrochloride	NA
Hydrazine dihydrochloride	100.0

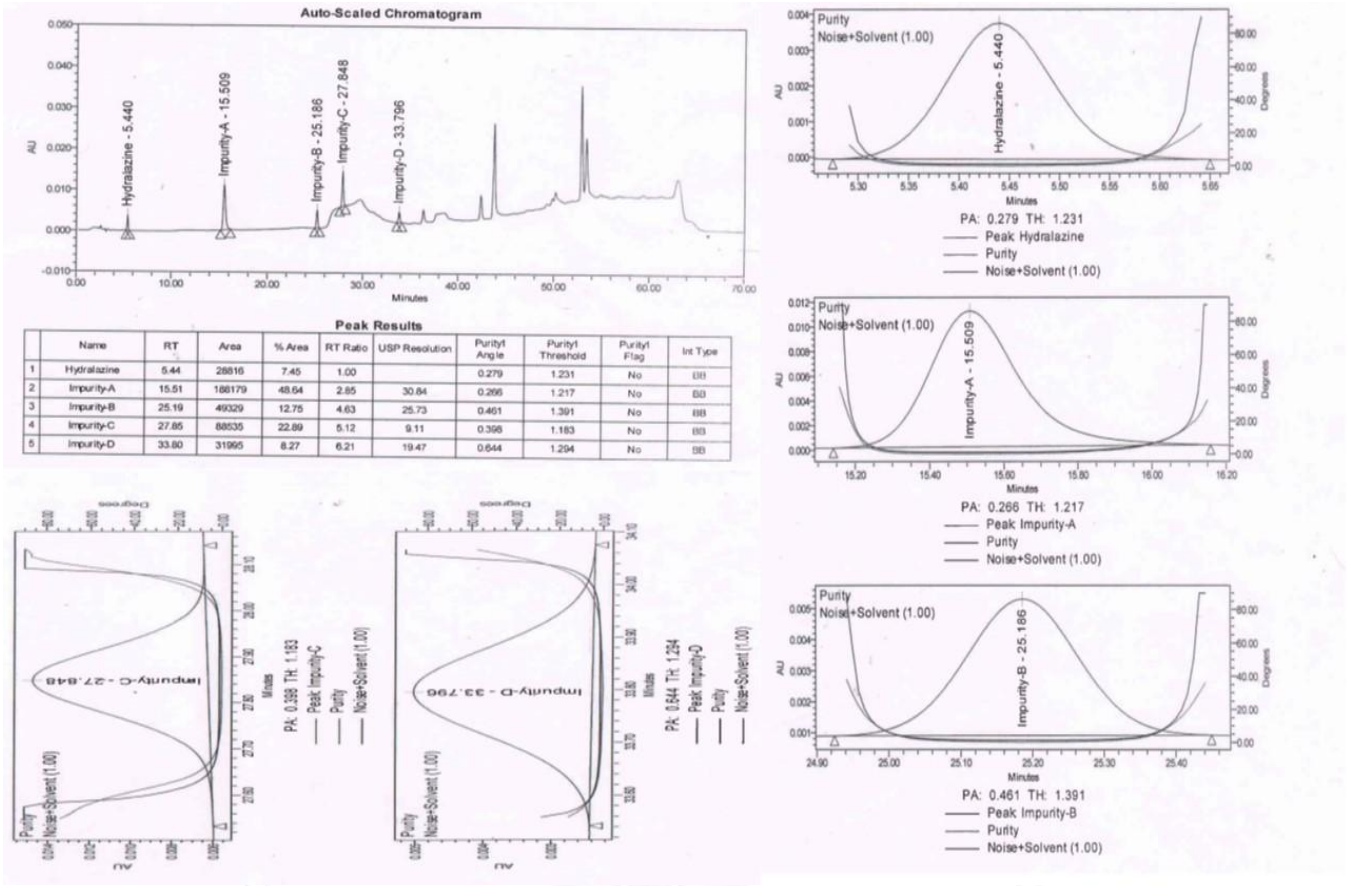
RESULTS AND DISCUSSION

Specificity: Blank (diluent), system suitability solution, diluted standard solution, all known impurity solutions individually, sample solution and sample solution spiked with all known impurities at specification level were prepared and injected into the HPLC equipped with a photodiode array detector and analysed. Peak purity passed for Hydralazine and its related impurities in control sample and spiked sample. Data is reported in Table 3 and Figure 2, 3,4 and 5.



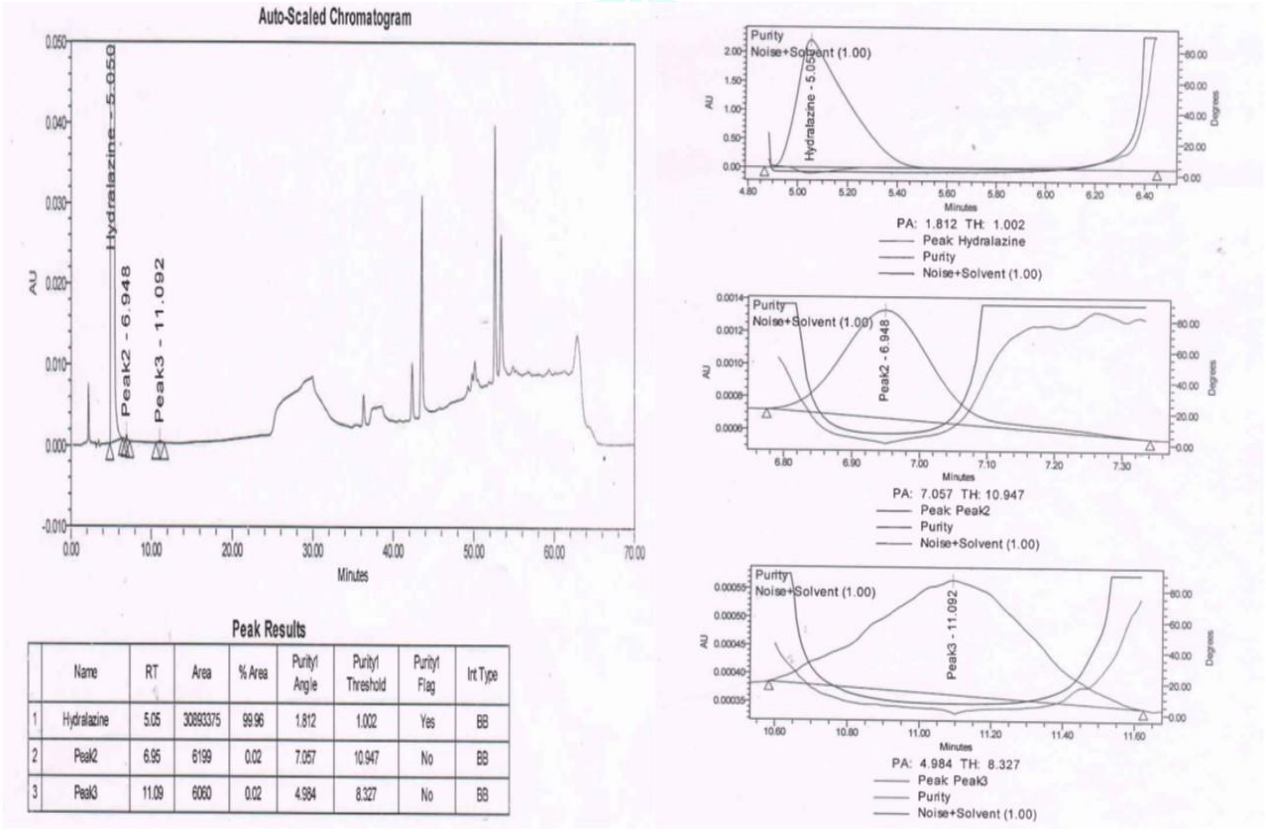
Blank Chromatogram **System Suitability**

Figure 2: Blank and System Suitability chromatogram



	Name	RT	Area	% Area	RT Ratio	USP Resolution	Purityl Angle	Purityl Threshold	Purityl Flag	Int Type
1	Hydralazine	5.44	28816	7.45	1.00		0.279	1.231	No	BB
2	Impurity-A	15.51	188179	48.64	2.85	30.84	0.266	1.217	No	BB
3	Impurity-B	25.19	49329	12.75	4.63	25.73	0.461	1.391	No	BB
4	Impurity-C	27.85	88535	22.89	5.12	9.11	0.399	1.183	No	BB
5	Impurity-D	33.80	31995	8.27	6.21	19.47	0.644	1.294	No	BB

Figure 3: Standard Solution with peak purity chromatogram



	Name	RT	Area	% Area	Purityl Angle	Purityl Threshold	Purityl Flag	Int Type
1	Hydralazine	5.05	30893375	99.96	1.812	1.002	Yes	BB
2	Peak2	6.95	6199	0.02	7.057	10.947	No	BB
3	Peak3	11.09	6060	0.02	4.984	8.327	No	BB

Figure 4: Sample Solution with peak purity chromatogram

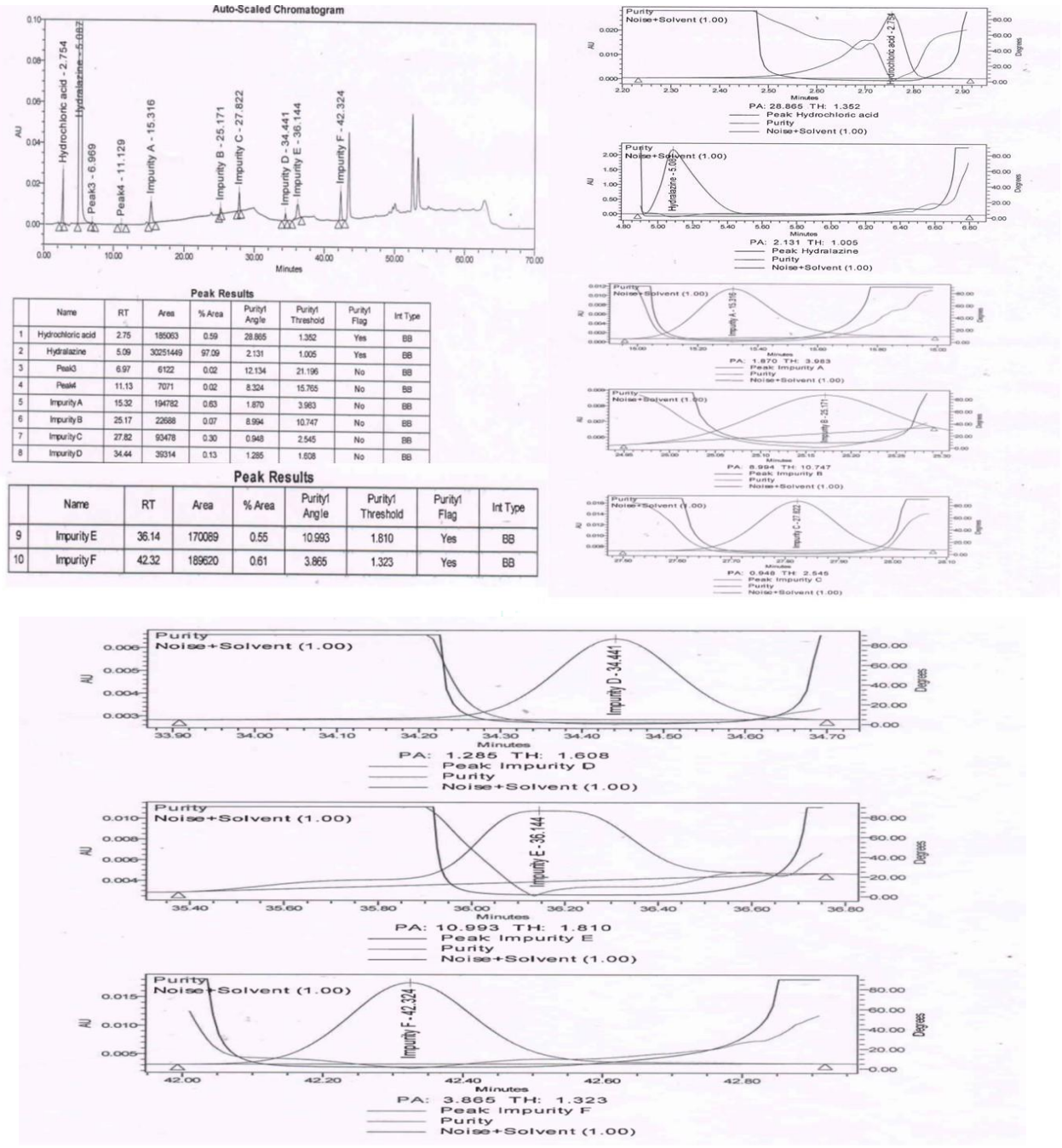


Figure 5: Spike Solution with peak purity chromatogram

Table: 3 Peak purity information (For Spiked solution)

Name of the compound	Purity angle	Purity Threshold	Peak Purity Result
Impurity A	1.870	3.983	Pass
Impurity B	8.994	10.747	Pass
Impurity C	0.948	2.545	Pass
Impurity D	1.285	1.608	Pass

From the above data, it is clear that, Hydralazine, Impurity-A, Impurity-B, Impurity-C and Impurity-Dare well separated from each other and Hydralazine peak. There is no interference of Blank at the retention time of all known impurities and unknown Impurities. Peak Purity is passes for Hydralazine peak and all known impurities. Based on the above data method is Specific.

Solution Stability: From the below given data it is clear that, spiked solution is not stable at sample cooler temperature of 10°C since Impurity B area is decreasing. So it is recommending that spiked solutions should be prepared freshly. Data reported in table no. 4.

Table 4: solution stability data for spiked solution at 10°C Sample cooler temperature:

S. N.	Sample ID	Impurity A Area	% Diff with initial	Impurity B Area	% Diff with initial
1	Initial	198105	---	51493	---
2	Sample solution after 1hrs	197877	-0.12	23719	-14.02
3	Sample solution after 2hrs	195653	-1.12	12680	-5.57
4	Sample solution after 5hrs	195102	-0.28	4795	-3.98
5	Sample solution after 12hrs	191853	-1.64	3235	-0.79
6	Sample solution after 18hrs	194241	1.21	2990	-0.12
7	Sample solution after 24hrs	193899	-0.17	2956	-0.02

Limit of Detection and Limit of Quantification: Based on determination of Prediction linearity, six replicate injections were made for LOD & LOQ. Details summarized in the given Table 5.

Table 5: for LOD and LOQ Establishment

Solution name	Concentration (%)				
	Impurity A	Impurity B	Impurity C	Impurity D	Hydralazine
Linearity at 1% solution	0.002	0.001	0.002	0.005	0.001
Linearity at 5% solution	0.008	0.007	0.008	0.010	0.005
Linearity at 10% solution	0.015	0.015	0.015	0.015	0.010
Linearity at 15% solution	0.023	0.022	0.023	0.019	0.015
Linearity at 20% solution	0.030	0.029	0.030	0.024	0.020
Linearity at 25% solution	0.038	0.037	0.038	0.005	0.025
Slope of calibration curve(S)	**1303819.957**	**359622.915**	**616594.628**	**312992.951**	**290112.650**
Standard Deviation of Response STEYX (σ)	**778.732**	**119.039**	**163.076**	**98.753**	**55.039**
LOD (in %)	**0.002**	**0.001**	**0.001**	**0.001**	**0.001**
LOQ (in %)	**0.006**	**0.003**	**0.003**	**0.003**	**0.002**

The predicted LOD and LOQ values of Hydralazine, impurity B, impurity C and Impurity D are Low and not reproducible. So LOD and LOQ Values are considering from 5% linearity and 10% Linearity respectively. These values shall be further confirmed by precision and accuracy studies.

LOD Confirmation and LOQ Precision: The Resolution between Hydralazine peak and Impurity A peak is 23.4 (NLT 3.0). %RSD of six replicates of standard solution is Complies (NMT 5.0%). System suitability parameter Complies. From the below given results, it is concluded that method is precise at LOQ Level. All individual known impurities were detectable at LOD level concentration.

Table 6: Standard solution and all individual known impurities:

Inj. No	Area of Hydralazine	Area of Impurity-A	Area of Impurity-B	Area of Impurity-C	Area of Impurity-D
1	27413	202224	50291	90922	27597
2	27600	203548	50552	90880	27855
3	27343	203811	50771	91184	27707
4	27700	201418	50873	90847	28099
5	27539	202023	51930	90900	27691
6	27385	202071	50935	90583	27634
Avg.	27497	202516	50892	90886	27764
STDEV	139.21	945.94	560.55	191.66	186.48
%RSD	0.51	0.47	1.10	0.21	0.67

Table 7: LOD and LOQ for All impurities and API:

S.No	Name of the compound	LOD in %	LOQ in %
1	Hydralazine	0.005	0.015
2	Impurity-A	0.002	0.006
3	Impurity-B	0.0075	0.025
4	Impurity-C	0.0075	0.025
5	Impurity-D	0.005	0.015

Linearity & Range:

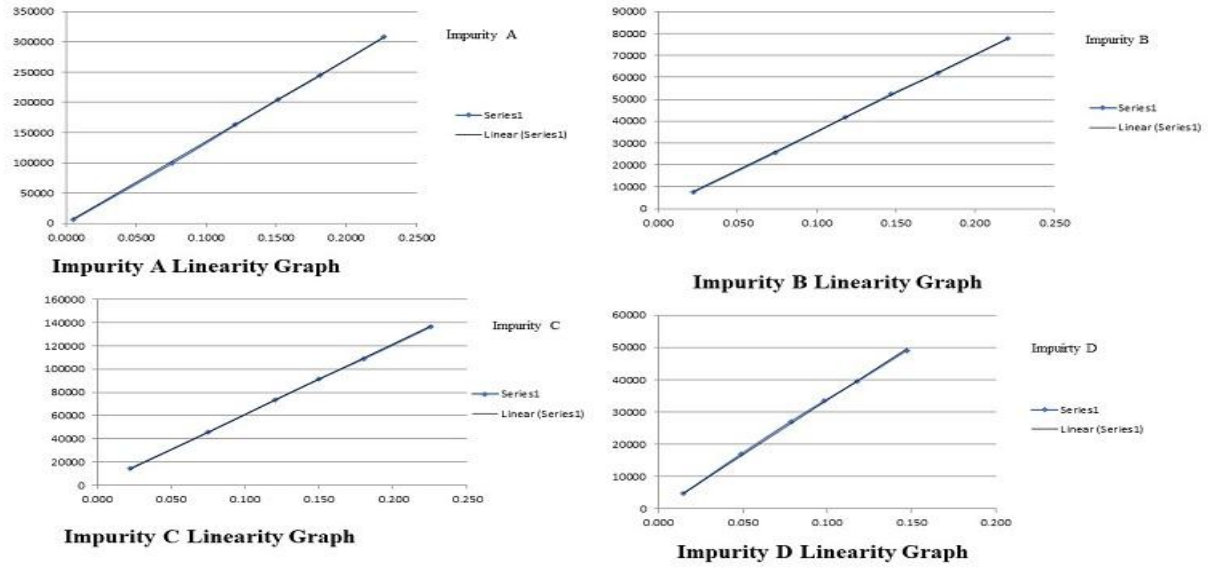
A series of Standard preparations (minimum of five preparations) in triplicate of hydralazine hydrochloride and Impurity A, B, C and D working standards were prepared over a range of the LOQ to 150% of specification limits. The Correlation coefficient for hydralazine hydrochloride and Impurity A, B, C and D is more than 0.99. Therefore, HPLC Method for the determination of related substances of hydralazine hydrochloride is linear. Linearity reported in Table 8-9.

Table 8: Linearity of Impurity A, B, C, D and hydralazine

Linearity levels	Impurity-A		Impurity-B		Impurity-C	
	Conc. in %	Avg. Area	Conc. in %	Avg. Area	Conc. in %	Avg. Area
Linearity at LOQ	0.0054	6693	0.022	7699	0.023	14712
Linearity at 50%	0.076	99369	0.074	25606	0.075	45728
Linearity at 80%	0.121	162812	0.118	41694	0.120	73594
Linearity at 100%	0.151	204689	0.147	52357	0.150	91800
Linearity at 120%	0.181	244911	0.176	62011	0.180	109165
Linearity at 150%	0.227	308449	0.221	77619	0.225	136569
STEYX		**1264.111**	**344.810**		**381.002**	
Slope		**1359996.5**	**354232.938**		**601870.424**	
Correlation coefficient		**1.000**	**1.000**		**1.000**	

Table 9: Linearity of Impurity A, B, C, D and hydralazine

Linearity levels	Impurity-D		Hydralazine	
	Conc. in %	Avg. Area	Conc. in %	Avg. Area
Linearity at LOQ	0.015	4835	0.015	4163
Linearity at 50%	0.049	17041	0.126	14202
Linearity at 80%	0.079	27065	0.202	22929
Linearity at 100%	0.098	33386	0.253	28580
Linearity at 120%	0.118	39474	0.304	33792
Linearity at 150%	0.147	49068	0.379	41516
STEYX		**450.771**	**588.799**	
Slope		**336738.026**	**104104.048**	
Correlation coefficient		**1.000**	**0.999**	



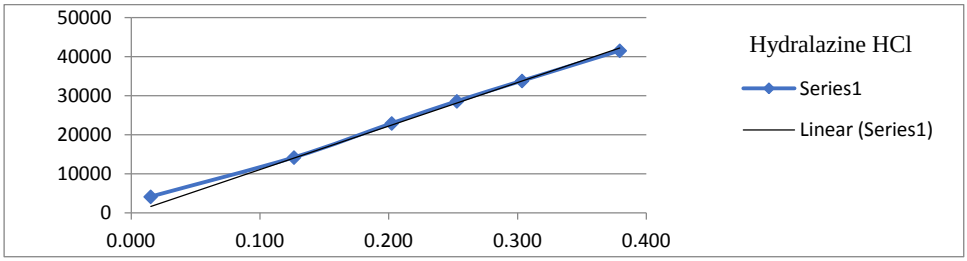


Figure 6: Linearity Graph of Impurity A, B, C, D and hydralazine

Accuracy: Sample of Hydralazine hydrochloride drug substances, were spiked with Impurities A, B, C and D at four different levels: LOQ, 50%, 100%, and 150% of specification limits (in triplicate (in total twelve determinations) and analysed. The Mean Recovery for known impurities is within limits. Therefore, the HPLC Method for the determination of related substances method-1 of Hydralazine hydrochloride in Hydralazine hydrochloride drug substances is accurate. Accuracy reported in Table 10.

Table 10: Accuracy of Impurity A, B, C and D at LOQ to 150%

Name of the component	%Recovery			
	LOQ	50%	100%	150%
Impurity-A	89.4	98.9	99.8	101.0
Impurity-B	92.7	96.4	95.0	92.5
Impurity-C	101.2	100.6	99.9	100.3
Impurity-D	93.7	96.9	98.1	101.0
Average				

System precision Method precision and intermediate precision:

System Precision: Six replicate injections of the standard solution were made & injected. RSD should not be more than 5.0% and The Resolution between Hydralazine peak and Impurity A peak is 22.1 (NLT 3.0). The RSD of system precision is 2.43 %.

Method Precision: Six Sample solutions of hydralazine hydrochloride spiked with Known impurities was prepared and injected into the HPLC, along with standard solution. RSD should not be more than 10.0%. RSD is less than 10.0%. Therefore, the HPLC Method for the determination of related substances of hydralazine hydrochloride (Method-1) is precise.

Ruggedness (Intermediate Precision): Six Sample solutions of the same lot of hydralazine hydrochloride, spiked with known impurities was made by a different analyst and analysed using different column on a different day and injected into a different HPLC, along with Standard solution. Overall RSD is less than 10.0%. Therefore, the HPLC Method for the determination of related substances of hydralazine hydrochloride (Method-1) is rugged. Based on the above data it is clear the method is Precise &Rugged. Precision and ruggedness data summarized in Table 11.

Table 11: Overall RSD for method precision and intermediate precision:

Sample ID	Impurity-A (% w/w)	Impurity-B (% w/w)	Impurity-C (% w/w)	Impurity-D (% w/w)
Method precision-1	0.15	0.14	0.15	0.10
Method precision-2	0.15	0.14	0.15	0.10
Method precision-3	0.15	0.14	0.15	0.10
Method precision-4	0.15	0.14	0.15	0.10
Method precision-5	0.15	0.14	0.15	0.10
Method precision-6	0.15	0.14	0.15	0.09
Intermediate precision-1	0.15	0.14	0.15	0.10
Intermediate precision-2	0.15	0.14	0.15	0.10
Intermediate precision-3	0.15	0.12	0.16	0.10
Intermediate precision-4	0.15	0.15	0.16	0.10
Intermediate precision-5	0.15	0.14	0.15	0.09
Intermediate precision-6	0.15	0.16	0.15	0.09
Average	0.15	0.14	0.15	0.10
STDEV	0.0012	0.0093	0.0025	0.0014
% RSD	0.8	6.7	1.7	1.4

Robustness: System suitability results meet as per criteria. The % RSD for content of each impurity in as such condition and changed condition should not be more than 10.0. The % RSD for Contents of each impurity in spiked sample under test with each variable condition (mentioned in below table) along with as such condition is complies.

Table 12: Robustness of different variable conditions

Conditions	Impurity % w/w			
	Imp. A	Imp. B	Imp. C	Imp. D
As Such sample	0.149	0.139	0.151	0.096
Low Ph	0.158	0.126	0.150	0.105
Average	**0.15**	**0.13**	**0.15**	**0.10**
STDEV	**0.0059**	**0.0090**	**0.0004**	**0.0063**
% RSD	**3.8**	**6.8**	**0.3**	**6.3**
As Such sample	0.149	0.139	0.151	0.096
High Ph	0.150	0.139	0.148	0.102
Average	**0.15**	**0.14**	**0.15**	**0.10**
STDEV	**0.0005**	**0.0001**	**0.0016**	**0.0041**
% RSD	**0.3**	**0.1**	**1.1**	**4.2**
As Such sample	0.149	0.139	0.151	0.096
Low Temp.	0.150	0.140	0.159	0.110
Average	**0.15**	**0.14**	**0.15**	**0.10**
STDEV	**0.0006**	**0.0009**	**0.0063**	**0.0100**
% RSD	**0.4**	**0.6**	**4.0**	**9.7**
As Such sample	0.149	0.139	0.151	0.096
High Temp.	0.146	0.132	0.142	0.084
Average	**0.15**	**0.14**	**0.15**	**0.09**
STDEV	**0.0026**	**0.0047**	**0.0059**	**0.0088**
% RSD	**1.7**	**3.5**	**4.0**	**9.8**
As Such sample	0.149	0.139	0.151	0.096
Increase Flow	0.151	0.124	0.150	0.110
Average	**0.15**	**0.13**	**0.15**	**0.10**
STDEV	**0.0008**	**0.0109**	**0.0005**	**0.0097**
% RSD	**0.5**	**8.3**	**0.3**	**9.5**
As Such sample	0.149	0.139	0.151	0.096
Decrease Flow	0.149	0.146	0.153	0.086
Average	**0.15**	**0.14**	**0.15**	**0.09**
STDEV	**0.0004**	**0.0050**	**0.0015**	**0.0070**
% RSD	**0.3**	**3.5**	**1.0**	**7.7**

CONCLUSION

The Analytical Method for determination of Related substances (Method-I) by HPLC of Hydralazine Hydrochloride is validated as per method described. The validated method is found Specific, Linear, Precise, Accurate, Robust and Rugged for determination of Related substances (Method-I) by HPLC. Hence it is concluded that determination of Related substances (Method-I) for Hydralazine Hydrochloride by HPLC can be used for Routine release analysis of API at Quality control department.

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