IJNRD.ORG ISSN : 2456-4184
INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG
An International Open Access, Peer-reviewed, Refereed Journal

STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF HYDRALAZINE, METYL PARABEN AND PROPYL PARABEN HYDRALAZINE HYDROCHLORIDE INJECTION

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INTRODUCTION :

A vasodilator, hydralazine was used to treat severe hypertension, congestive heart failure, myocardial infarction, and preeclampsia [(1)].

Hydralazine HCl was primarily used to treat hypertension. It is beneficial in the treatment of hypertension individuals who are in the chronic stage. Hydralazine's importance in antihypertensive medication has grown in recent years, according to research that the substance is particularly effective [(3,4)].

Mechanism of Action Hydralazine exerts its hypotensive action by reducing vascular resistance through direct relaxation of arteriolar smooth muscle[(2)].Excessive or habitual hydralazine use can result in toxic symptoms such headaches, joint or muscle pain, swollen ankles, nausea, perspiration, tachycardia, arrhythmia, and angina precipitation [(5)].

Hydralazine passes the placenta with ease. It is a frequently used antihypertensive medicine in pregnancy-induced hypertension due to its relative safety and effectiveness, as well as considerable clinical experience. Coronary artery disease is the main contraindication due to higher cardiac output and effort [(6)].

Parabens have antibacterial properties against a wide range of bacteria. Their antibacterial mechanism of action, however, is unknown. In some bacterial species, they impair membrane transport mechanisms or impede DNA and RNA synthesis, as well as several important enzymes including ATPases and phosphotransferases. Propylparaben has a higher level of antibacterial activity than methylparaben. Because of its increased solubility in the bacterial membrane, it has a stronger antibacterial effect [(7)].

Methyl paraben and Propyl Paraben are both parabens, a class of chemical compounds with antifungal and antibacterial properties that are extensively employed as preservatives in pharmaceutical, food, and cosmetic products [(8-11)]. These parabens work across a wide pH range [(9)]. Because of the nature of the component, liquid solutions are particularly vulnerable to microbial development. Preservatives are added to such preparations to keep the items from deteriorating [(10)].

The survey of various literatures discloses the method development and validation of the simultaneous estimation of HYDRALAZINE, METHYL PARABEN & PROPYL PARABEN IN HYDRALAZINE HYDROCHLORIDE INJECTION by using RP-HPLC has some limitations like long run time, low resolution and few analytical works found on stability indicating studies. Thus, a new study was developed for the precise, rapid, accurate analysis of analytes compared to subsisting methods. This newly developed found to be simple, precise, accurate, sensitive and cost effective with good reproducibility and recovery.

Nowadays RP-HPLC is most widely preferred analytical technique which could be applied for the separation of wide range of molecules that requires of small size.

METHODS:

1. The active pharmaceutical ingredients of Hydralazine HCL, Methyl paraben and propyl paraben are obtained from Aurobindo Pharma Ltd, Hyderabad.
2. Injection of Hydralazine HCL (Aurobindo Pharma Ltd, Hyderabad)
3. Chemicals: Acetonitrile (HPLC grade)

STRUCTURES:

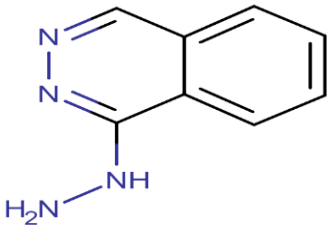

Figure1: structure of Hydralazine [(12)]

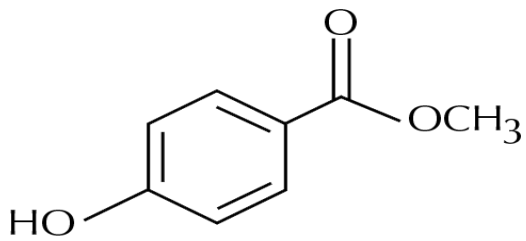

Figure2: structure of methyl paraben [(12)]

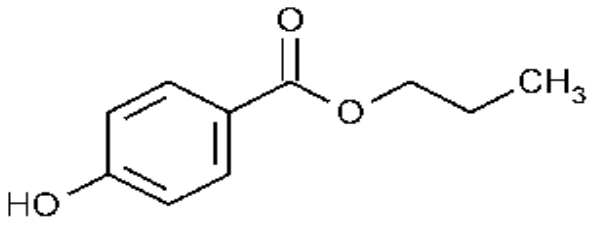

Figure3: structure of propyl paraben (12)

MATERIALS AND METHODS:

Instrumentation:

The separation was done on SHIMADZU 2010 CHT system equipped with quaternary pumps, UV detector and injector. The column of Inertsil ODS-3V C_{18} column of 250 x 4.6 mm, 5 µ dimensions was used for the separation. Uv spectrophotometer (SHIMADZU 1280), Analytical Balance (Mettler Toledo XS20504), Sonicator (VWR 97043-938), Vacuum/ Pressure pump (Millipore BM2EA9672R), Fume Hood (Mott 5 feet hv), Hot air oven (Thermo Scientific Herat herm), Refrigerator (Thermo Scientific) were used for study.

HPLC optimized conditions

Analyte's separation was achieved with 0.1% Orthophosphoric, Methanol & Acetonitrile in (50:50 v/v) in the proposed gradient mode using Inertsil ODS-3V (250 x 4.6 mm, 5 µ) C18 column as stationary phase with flow rate of 1.0 mL/min and column temperature of 30^o at a wavelength of 254 nm. Before injecting the solution, the column was equilibrated with mobile phase. The injection had a capacity of 15µl.

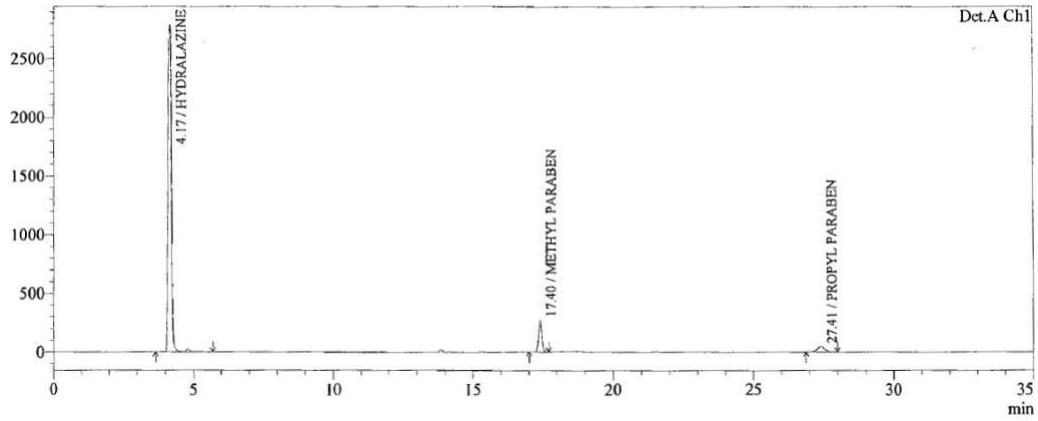

Figure 4 optimized chromatogram

Composition of analytical solutions

Diluent:

0.1% Orthophosphoric, Methanol & Acetonitrile in (50:50) was used as diluent.

Mobile phase:

Mobile phase A: 0.1% Orthophosphoric

Mobile phase B: Methanol & Acetonitrile in (50:50 v/v) was utilized.

Standard solution 1 of Hydralazine:

Weighed and transferred 200mg Hydralazine standards into a10 ml of clean and dry volumetric flask. Add about 5ml of diluent and sonicate to dissolve. Dilute with diluent and mix well.

Standard solution 1of methyl and propyl paraben:

Weighed and transferred 65mg Methyl paraben, 35mg Propyl paraben standards into a10 ml of clean and dry volumetric flask. Add about 2ml of diluent and sonicate to dissolve. Dilute with diluent and mix well.

Standard solution 2 of Hydralazine:

2ml of solution was taken from Hydralazine standard stock into a 100 ml of clean and dry volumetric flask. Dilute to volume with diluent and mix well.

Standard solution 2 of methyl and propyl paraben:

1ml of solution was taken from methyl and propyl paraben standard stock solution solution-1 into 10 ml clean and dry volumetric flask. Dilute to volume with diluent and mix well.

Validation parameters of HPLC method:

Method validation was performed according to the ICH guidelines of Q2(R1) [13]. The parameters include system suitability, linearity, precision, accuracy, sensitivity, robustness. Stability studies followed the ICH guidelines Q1A(R2) [14] includes degradation studies like acid, base, peroxide, thermal, UV, water degradation studies.

System suitability parameters

The system suitability parameters were determined by preparing the standard solutions of Hydralazine, Methyl paraben, Propyl paraben and the solutions were injected six times and the parameters like peak tailing, resolution, USP plate count were determined.

Specificity

Checking of interference in the optimized method. We should not find any interfering peaks in the blank, placebo of retention times of these drugs in this method. So, this method was said to be specific.

System precision

System precision or injection repeatability was estimated by injecting 15µl six replicate injection of standard solutions containing Hydralazine, Methyl paraben, Propyl paraben. The %RSD was calculated.

Method precision was determined by injecting six different of sample solutions of same concentration which was prepared separately. 15µl of above solutions was injected and chromatograms were recorded. Precision of the method was estimated by the RSD of the percentage of the analytes.

In method precision the %RSD for assay of six sample solution should not be more than 2.0.

Ruggedness (intermediate precision)

The procedure followed for assay method in method precision was repeated of two different days, different batch number, by two different analytes and using different HPLC systems. The overall %RSD of % assay from twelve determinants (six from method precision and six from intermediate precision data) should not be more than 5.0%.

Linearity

By constructing calibration curves of peak area versus concentration range, we can assess linearity of drugs in concentration range of 40-600µg/ml for Hydralazine and 1.3-19.5µg/ml for Methyl paraben and 0.7-10.5µg/ml for Propyl paraben. The linearity can be determined by calculating the regression equation from the calibration curve constructed by using six standard concentrations.

Accuracy

It was closeness of observed value of the approach to the true value. It was estimated as percentage of analyte assay after adding a known number of respective drugs. To the formulation (pre analysed sample), the

reference standards of the drugs were added at the level of 50%, 100%, 150%. The recovery studies were carried out three two and the percentage recovery and percentage mean recovery.

Robustness

Robustness conditions like flow rate (±20%), temperature (5℃) wavelength (5nm) was maintained and samples were injected in a duplicate manner. System suitability parameters were not much effected, all the parameters were passed. %RSD was within the limit.

Limit of detection and limit of quantification

LOD and LOQ were calculated from standard deviation of response from precision and slope from linearity.

LOQ = 10 σ/S

LOD = 3.3 σ/S

Where σ is standard deviation from standard deviation from response

S is slope from calibration curve

Forced degradation studies

The specificity of the method was demonstrated through forced degradation studies conducted on the standard and sample using acid degradation (1M HCL, heated at 85℃ for 60 min), alkaline degradation (1M NaOH, heated at 85℃ for 60 min), peroxide degradation (10% hydrogen peroxide heated at 85℃ for 60 min), and thermal degradation (samples are arranged in hot air oven at 105℃ for 24 hours). The standard and sample were exposed to these conditions and the main peak was studied for peak purity, indicated that the method effectively separated the degradation products from the analyte under investigation. Regulatory guidelines in ICH Q2A, Q2B, Q3B and FDA 21 CFR section 211 requires the development and validation of stability-indicating potency assays.

RESULTS AND DICUSSION:

System suitability:

The retention time of hydralazine, methyl paraben, propyl paraben was found to be 4.17 min, 17.40 min, 27.41 min respectively. For the drugs, no interfering peaks were observed in the graphical data of blank and placebo at retention time. The results of system suitability parameters given in the below. Table no.1

S. No	**Hydralazine**			**Methyl paraben**			**Propyl paraben**		
Injection	Retention Time (min)	USP Plate Count	Tailing	Retention Time (min)	USP Plate Count	Tailing	Retention Time (min)	USP Plate Count	Tailing
1.	4.16	3906	1.8	17.71	95936	1.1	28.66	45591	1.0
2.	4.15	3896	1.8	17.70	95655	1.1	28.62	45729	1.0
3.	4.16	3931	1.8	17.69	96099	1.1	28.57	45655	1.0
4.	4.16	3966	1.8	17.68	96037	1.1	28.53	45656	1.0

5.	4.16	3900	1.8	17.68	96332	1.1	28.24	45640	1.0
6.	4.15	3961	1.7	17.68	96210	1.1	28.57	45653	1.0

TABLE 1 Results of system suitability

Acceptance criteria: Six consecutive injections of the standard showed uniform retention time, theoretical plate count, tailing factor and resolution for the three analytes which indicated that this method was validated foe system suitability.

Specificity

The method was highly specific the chromatographic peak does not interfere with any peak. This proves that, excipients have no effect on the analytical method. On the other hand, blank peak did not overlap drug peak. So, the method is highly selective.

Precision

System precision

For all the drugs mean, standard deviation, %RSD were calculated. The %RSD for hydralazine, methyl paraben, propyl paraben were found to be 0.11, 0.20, 0.48 respectively. The acceptance of system precision is less than 2% and the data obtained was within the limits.

S.NO	**Hydralazine**		**Methyl paraben**		**Propyl paraben**	
	t_R (min)	**Area**	**t_R (min)**	**Area**	**t_R (min)**	**Area**
1.	3.92	18135244	17.42	1159658	27.49	508824
2.	3.93	18139589	17.41	1157963	27.48	503910
3.	3.93	18156362	17.41	1158243	27.49	506382
4.	3.92	18152338	17.41	1158385	27.49	505682
5.	3.92	18148215	17.42	1158589	27.49	504348
6.	3.92	18102162	17.41	1153195	27.49	501781
Mean	3.92	18138985	17.41	1157672	27.49	505154
%RSD	0.08	0.11	0.01	0.20	0.02	0.48

Table 2 Results of system precision

Acceptance criteria: %RSD was calculated for various run and was found to be less than 2% which proves that system precision was précised.

Intermediate precision

Preparation of multiple samples from a sample stock solution was done and then and six working sample solutions of same concentrations were prepared. Each injection from working sample solution was given on the next day of the sample solution and obtained areas were mentioned in table no.3. Average, standard

deviation, % RSD for hydralazine, methyl paraben, propyl paraben were obtained as 0.83, 0.79, 0.94 respectively and the results obtained were within the limits. The results were reported in table 3

Preparation	Hydralazine	Methyl paraben	Propyl paraben
Average	100.485	100.905	100.415
SD	0.83	0.795	0.95
%RSD	0.83	0.79	0.94

Table 3: Results of ruggedness

Acceptance criteria: The cumulative % RSD for ruggedness was showed in above table for Hydralazine, Methyl paraben and propyl paraben respectively.

Accuracy

According to the ICH guidelines, accuracy must be determined on nine determinations covering minimum of three concentrations. The % recoveries of known amount of analytes are shown in table for Hydralazine, methyl paraben, propyl paraben respectively as calculated by the addition of known amounts of standard Hydralazine, methyl paraben, propyl paraben to the formulation, indicating accuracy of the method and good recovery of the analytes.

Level in %	**Amount Spiked (µg/ml)**	**Amount Recovered (µg/ml)**	**Average % Recovery**	**% RSD**
Hydralazine				
50%	200	198.5	99.95	0.21
100%	400	397.5	100.1	0.12
150%	600	604.5	101.45	0.10
Methyl paraben				
50%	6.5	6.5895	98.9	0.11
100%	13	13.323	100.7	0.09
150%	19.5	19.942	99.8	0.02
Propyl paraben				
50%	3.5	3.417	97.55	0.18
100%	7	7.0415	100.5	0.02
150%	10.5	10.666	101.5	0.02

Table 4 Results of accuracy

Acceptance criteria: The recovery rates between 50, 100, 150 levels were showed in the above table for Hydralazine, Methyl paraben and Propyl paraben respectively as calculated by the addition of known amounts of standard Hydralazine, Methyl paraben and Propyl paraben to the formulation, indicating accuracy of the method and good recovery of the analytes.

Linearity

Seven linear concentrations of Hydralazine (40-600), methyl paraben (1.3-19.5), propyl paraben (0.7-10.5) were injected in a duplicate manner. From this data, linearity equations obtained for Hydralazine, Methyl paraben, Propyl paraben were found to be Y= 42507x + 490837, Y= 90056x - 7734.2 and Y = 77279x - 3741.7 respectively. The correlation coefficient obtained was 0.999 for the drugs. The results reported in table no.5. calibration graphs shown in the below figure 5, 6, 7.

Concentration of Hydralazine (µg/ml)	Peak area of Hydralazine	Concentration of methyl paraben (µg/ml)	Peak area of Methyl Paraben	Concentration Of propyl paraben (µg/ml)	Peak area of propyl Paraben
40	1721473	1.3	109872	0.7	50907
100	4546893	3.25	284521	1.75	131230
200	9185005	6.5	576747	3.5	266142
320	14586969	10.4	927452	5.6	428620
400	18064947	13	1167387	7	537640
480	21150431	15.6	1395284	8.4	646105
600	25145597	19.5	1747977	10.5	807248

Table 5 Results of linearity

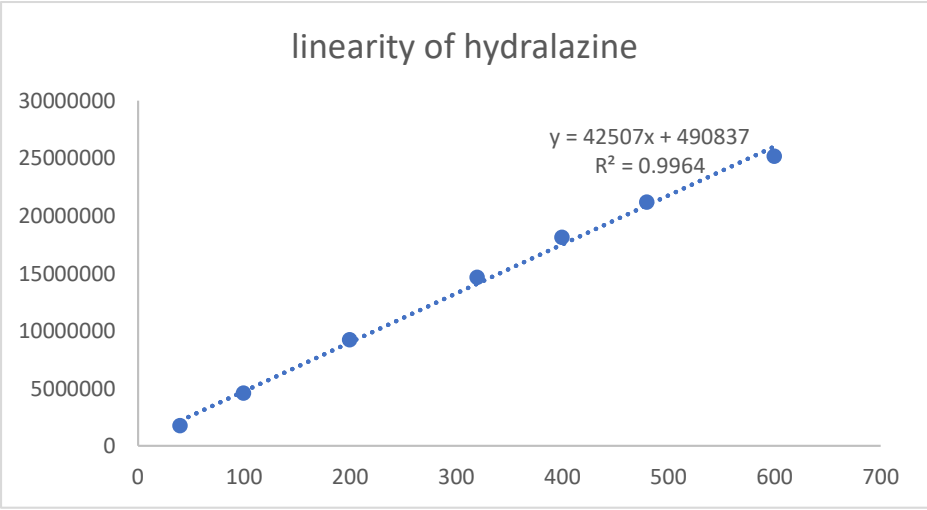

Figure 5: Linearity graph of hydralazine

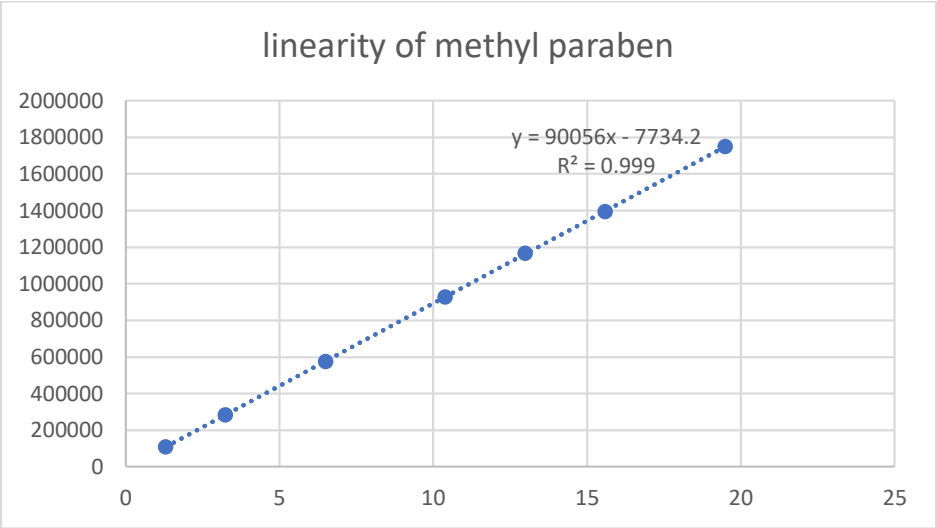

Figure 6: Linearity of methyl paraben

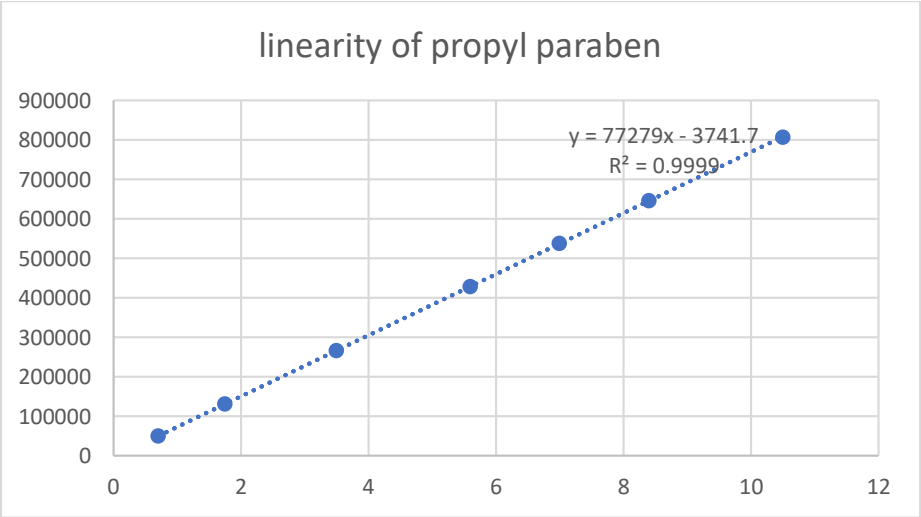

Figure 7 Linearity graph of propyl paraben

Acceptance criteria: a linear relationship peak area versus concentration was observed of Hydralazine, Methyl paraben and Propyl paraben in the range of 10% to 150% of nominal concentration. Correlation coefficient was 0.99 for the three which prove that method was linear in the range of 10% to 150%.

SENSITIVITY:

Analyte name	**Limit of detection µg/ml**	**Limit of quantification µg/ml**
Hydralazine	2.66	8
Methyl paraben	0.23	0.7
Propyl paraben	0.21	0.64

Table 6 Results of LOD and LOQ

The LOD for this method was found to be 2.66 µg/ml for Hydralazine, 0.23 µg/ml for methyl paraben and 0.21 µg/ml for propyl paraben. The LOQ for this method was found to be 8 µg/ml for Hydralazine, 0.7 µg/ml for methyl paraben and 0.64 µg/ml for propyl paraben.

Acceptance criteria: The LOD and LOQ result were showed in the above table which indicates that the proposed method can be used for detection and quantification of Hydralazine, Methyl paraben and Propyl paraben in a very wide concentration range.

ROBUSTNESS

Robustness was determined by small but deliberate variations in conditions like flow rate, composition of eluent, column temperature. Results due to variance in the conditions were compared with that of optimized chromatographic conditions. Results of robustness are tabulated below table no.7

s.no	**parameters**	**System suitability parameters**		**System suitability results**		
				hydralazine	**Methyl paraben**	**Propyl paraben**
1.	**Flow rate(ml/min)**	0.8	Area (µv*sec)	21053637	1392782	627928
			Rt (mins)	5.16	19.72	33.15
			USP Plate count	3870.5	86141.5	45425.5
			USP Tailing	1.7	1.1	1.0
		1	Area (µv*sec)	16937040	1111898	512559
			Rt (mins)	4.18	17.69	28.58
			USP Plate count	4091.5	96217.4	45700.6
			USP Tailing	1.5	1.1	1.1
		1.2	Area (µv*sec)	14118333	927680	428224
			Rt (mins)	3.55	16.43	25.78
			USP Plate count	4226.7	104421.5	46916.9
			USP Tailing	1.2	1.1	1.0
2.	**Column oven temperature (℃)**	25	Area (µv*sec)	16714994	1121069	461013
			Rt (mins)	4.39	18.19	30.35
			USP Plate count	5243.3	85765.8	44225.7
			USP Tailing	1.7	1.1	1.1
		30	Area (µv*sec)	16937040	1111898	512559

			Rt (mins)	4.18	17.69	28.58
			USP Plate count	4091.5	96217.4	45700.6
			USP Tailing	1.5	1.1	1.1
		35	Area (µv*sec)	16310886	1108465	512628
			Rt (mins)	3.93	17.27	27.15
			USP Plate count	4787.9	105312.3	50670.5
			USP Tailing	1.7	1.1	1.1
3.	**Detection Wavelength (nm)**	249	Area (µv*sec)	17151722	121439	460994
			Rt (mins)	4.19	17.77	28.88
			USP Plate count	4292.5	95254.5	45824.1
			USP Tailing	1.4	1.1	1.0
		254	Area (µv*sec)	16937040	1111898	512559
			Rt (mins)	4.18	17.69	28.58
			USP Plate count	4091.5	96217.4	45700.6
			USP Tailing	1.5	1.1	1.1
		259	Area (µv*sec)	16430363	1050733	299926
			Rt (mins)	4.17	17.70	28.60
			USP Plate count	4039.5	96055.6	63124.5
			USP Tailing	1.5	1.1	1.2

Table 7 Results of robustness

Acceptance criteria: The results of the robustness study indicates that the method was robust and was unaffected by small variations in the chromatographic conditions.

Forced degradation studies

Standards and degraded samples are injected and calculated. The percentage of drug degraded in solution by applying different conditions like acid, alkali, photolytic, thermal analysis.

Stress conditions	Hydralazine			Methyl paraben			Propyl paraben		
	% Assay	% Degradation	Pass / Fail	% Assay	% Degradation	Pass / Fail	% Assay	% Degradation	Pass / Fail
Control	99.1	NA	NA	98.9	NA	NA	95	NA	NA
Acid	99.1	0.00	pass	98.9	0.00	Pass	92.3	2.7	Pass
Base	80.1	19.9	pass	81	19	Pass	85	15	Pass
Thermal	89.5	10.5	pass	93.7	6.3	Pass	90.9	9.1	Pass
Peroxide	98.6	1.4	pass	99	NA	Pass	94.3	0.7	Pass

Table 8 Results of forced degradation studies

Acceptance criteria: the forced degradation study showed that there is no interference from degradants with all the three analytes. The three analytes were highly sensitive to base hydrolysis and partially sensitive to temperature.

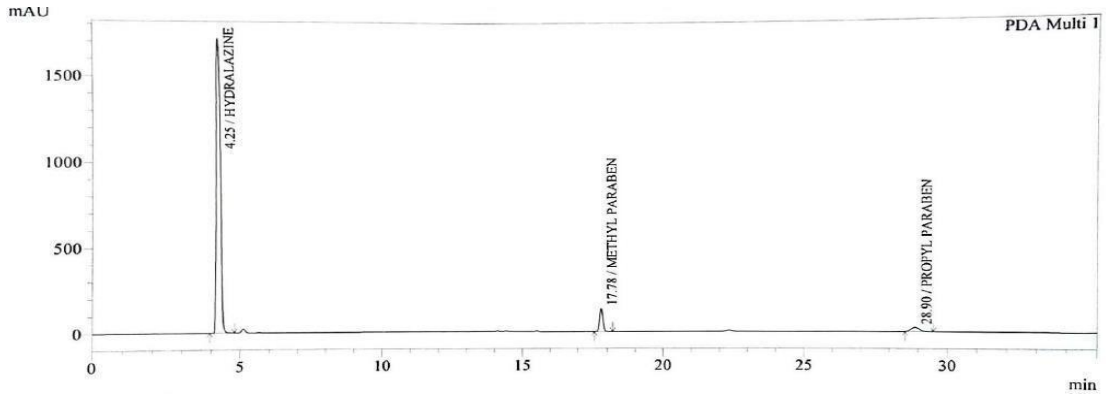

Figure 8 Sample chromatogram of forced degradation (control)

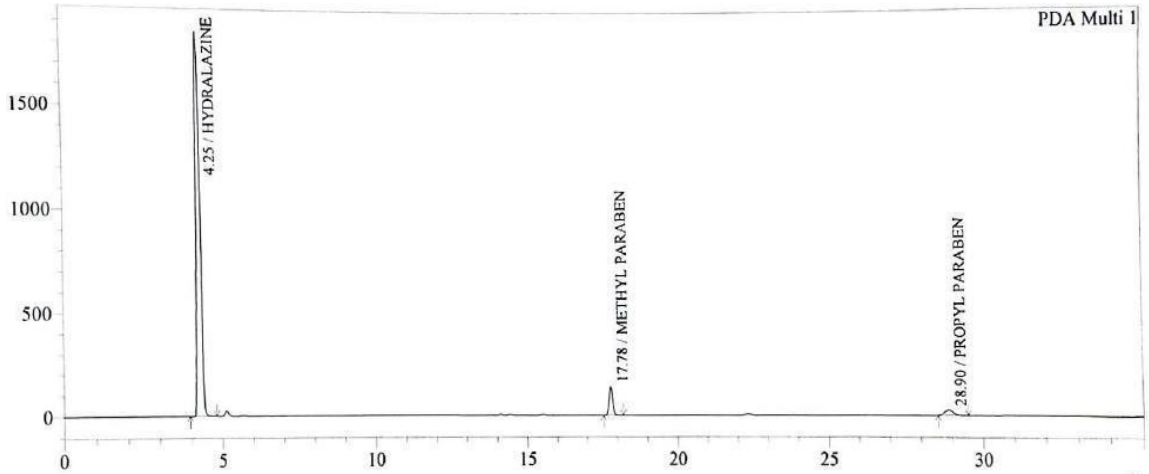

Figure 9 Sample chromatogram of forced degradation (acid degradation)

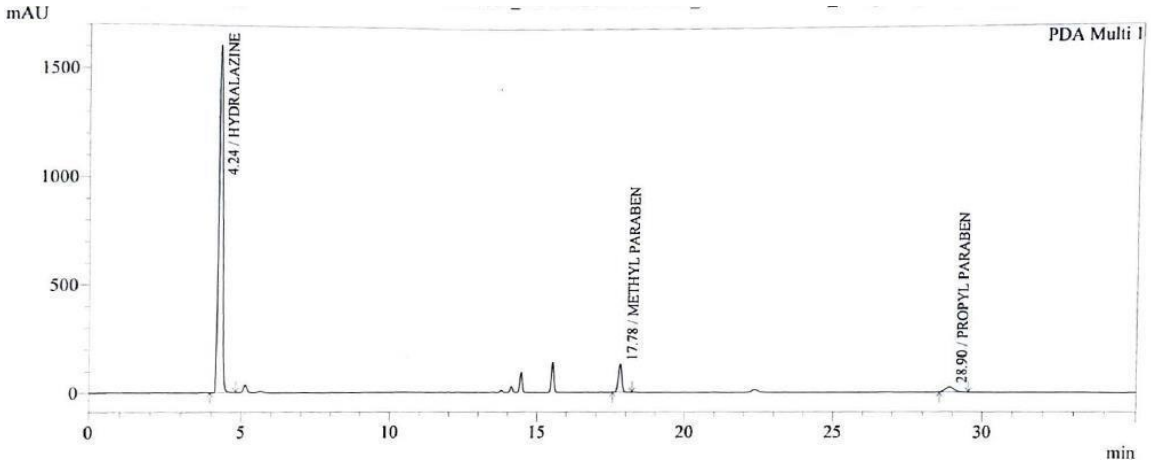

Figure 10 Sample chromatogram of forced degradation (base degradation)

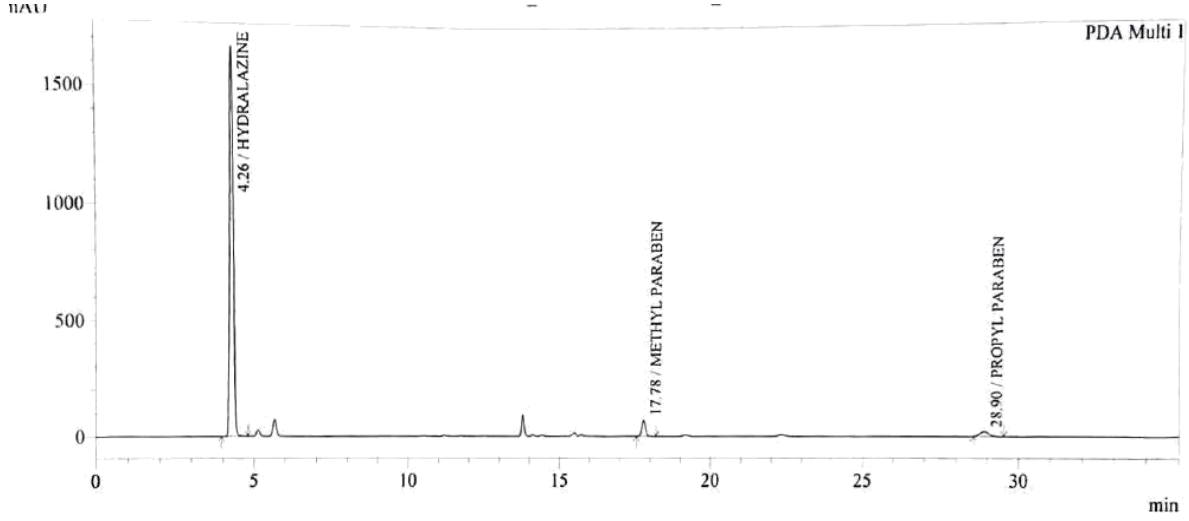

Figure 11Sample degradation of forced degradation (thermal degradation)

CONCLUSION:

The study was focussed to develop and validate RP-HPLC method for simultaneous estimation of Hydralazine, Methyl paraben, Propyl paraben in Hydralazine HCL injection. The developed method was useful for routine analysis and is capable of analysing huge number of samples in a short period with good robustness, accuracy and precision.

This RP-HPLC method generates large amount of quality data, which serve as highly powerful and convenient analytical tool. From the experimental data obtained it was concluded that, the chromatographic method

developed for simultaneous estimation of Hydralazine, Methyl paraben, Propyl paraben was found to be simple, precise, accurate, sensitive and cost effective with good reproducibility and recovery. All the parameters were validated as per ICH guidelines and found to be within the acceptance criteria

So, the developed method may be recommended for routine analysis in research institutions and quality control departments in industries for the simultaneous estimation of Hydralazine, Methyl paraben and propyl paraben in Hydralazine HCL injection.

CONFLICTS OF INTEREST:

The author have no conflicts of interest regarding the investigation.

ACKNOWLEDGMENTS:
The authors would like to thank AUROBINDO PHARMA Pvt. Ltd. Hyderabad for their kind support during Method development and Validation studies.

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