METHOD DEVELOPMENT FOR SIMULTANEOUS ESTIMATION OF HYDROCHLOROTHIAZIDE, RESERPINE, AND HYDRALAZINE IN PHARMACEUTICAL DOSAGE FORM INCLUDING STUDIES ON STABILITY PARAMETERS.

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ABSTRACT:
For the simultaneous detection of hydrochlorothiazide, hydralazine hydrochloride, and reserpine, a new stability indicating method was created in this work using the RP-HPLC method.
Acetonitrile and potassium dihydrogen phosphate buffer were chosen as the mobile phases, while water:methanol (65:35) was used as the diluent.
In spite of using other columns that had issues with retention duration, run time, and peak resolution for 7 minutes was used. used in this investigation. Final optimisation of the method resulted in optimised wavelength of the three drugs found at 235 nM.
Key words: Reserpine, Hydralazine, Potassium dihydrogen phosphate

1. INTRODUCTION

As a diuretic medication used to treat hypertension, hydrochlorothiazide works by acting on the kidney to release Na+ ions for reabsorption in the distal converting tubule.
Myocardis (Boehringer Ingelheim) and "Cresar" (Cipla) are some of the trade names used to promote it.
Vascular Monoamine Transporter is permanently blocked by reserpine (VMAT-2).
Methyl(1,16,17,18,20)11,17-dimethoxy-18-(3,4,5-trimethoxybenzoyl)oxylyohimbine-16carboxylate is its chemical name.
It was created in collaboration between the Japanese companies Fuji Viscera Pharmaceutical Corporation and Ajinomoto, and was given the go-ahead to be sold for the first time in 1995 as a treatment for high blood pressure. Hydralazine is a smooth muscle relaxant management of hypertension. Chemically it is known as 1-Hydrazinylpthalazine. It is originally marketed as Hygroton in the USA.
According to a review of the literature, there aren't many analytical techniques available for estimating hydrochlorothiazide, respin, and hydralazine.

The disclosed spectrophotometric methods can be used to estimate Hydrochlorothiazide, Reserpine, and Hydralazine separately.

We had intended to design a straightforward, exact, economical, For the simultaneous quantification of Hydrochlorothiazide, Reserpine, Hydralazine, and an accurate stability indicating RP-HPLC technique, combination because there are so few approved high-performance liquid chromatographic methods, in a pharmaceutical dosage form.

2. EXPERIMENTAL

Equipment's Used

Sl. No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module, PDA detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000+
3	P^H meter	Adwa – AD 1020
4	Sonicator	Ultrasonic cleaner power sonic 420
5	Vacuum oven	Wadegati
6	Constant temperature water bath	Thermolab GMP
7	Single tube Vortexer	Spinix
8	Refrigerated Centrifuge	Thermo scientific
9	Deep Freezer (≤-20°C)	Haier pharmaceutical refrigerator
10	Micro pipettes	Eppendorf
11	Weighing machine	Sartorius ME235P
12	Pipettes and Burettes	Borosil
13	Beakers	Borosil

Chemicals and Standards Used List

S.No	Chemicals	Manufacturer Name	Grade
1.	Water	In- house	HPLC grade
2.	Methanol	Merck	HPLC grade
3.	Acetonitrile	Merck	HPLC grade
4.	Ortho phosphoric acid	Merck	G.R
5.	KH_2PO_4	Merck	G.R
6.	K_2HPO_4	Merck	G.R
7.	Sodium perchlorate	Merck	G.R
8.	Perchloric acid	Merck	G.R
9.	0. 22µ Nylon filter	Advanced lab	HPLC grade
10.	0.45µ filter paper	Millipore	HPLC grade

METHODOLOGY

Preparation of solutions

Preparation of Sample stock solutions:

The Following an estimation of the average weight of 10 tablets, they were finely ground. Powder from one pill was precisely measured and added to a volumetric flask with a volume of 10 mL.

The metric flask received a dose of 5–6 mL methanol before being subjected to 10 minutes of sonication.

Methanol was added to the volume after sonication, and the solution was then filtered.

The HPLC system received the filtered solution.

KH2PO4 buffer preparation (0.1% OPA)

A 1000 mL volumetric flask containing 1.36 gr of KH2PO4 should also include roughly 100 ml of milli-Q water. The pH should then be adjusted to 3.6 using a diluted Ortho phosphoric acid solution.

Creating functional standard solutions

Working standards of 25 mg of hydrochlorothiazide, 5 mg reserpine, and 25 mg hydralazine respectively 25 mL, 100 mL, and 25 mL clean, dry volumetric flasks after being precisely weighed.

The combination was then given a sonication for 30 minutes before the final volume was made up using diluents.

Accuracy

The conventional addition method is used to measure accuracy at three different levels: 50%, 100%, and 150%.

Calculations were made to determine the % pharmacological dose form recovery of hydrochlorothiazide, reserpine, and hydralazine.

The 125 mg tablet powder was Accuracy 50% Sample stock solution was created, diluted with diluents, filtered using HPLC filters, and put into a 10 mL volumetric flask.

Accuracy 50% Sample stock solution
To make 1mL of each standard stock solution was pipetted into a 10mL volumetric flask along with 1mL of spiked and diluted Accuracy 100% standard stock solution.
A volumetric flask with a 10 mL capacity and 250 milligrammes of tablet powder was filled with diluents, filtered via HPLC filters, and labelled with the phrase "Accuracy 100% Sample stock solution" before being used to create the 100% spiked solution.
To make a 1mL 100% filtered Accuracy
1mL of each standard stock solution was pipetted into a 10mL volumetric flask after being spiked and diluted with diluents.

Making a 150% spiked solution
A 10mL volumetric flask containing 375 mg of tablet powder was prepared using diluents, filtered through Branded as an accuracy 150% HPLC filters and sample stock solution.
To make a 1mL filtered Accuracy 100% Standard stock solution that was spiked and diluted with diluents, 1mL of each standard stock solution was pipetted out into a 10mL volumetric flask. **Forced degradation studies:** The dosage form was subjected to a variety of Acid, basic, peroxide, heat, light, and water are examples of stressors. The dose form was discovered to be stable, photolytic & hydrolytic circumstances, marginally deteriorated acid, base peroxide, and heat conditions.
Hydrogen peroxide (H2O2) degradation: Separately, 20% hydrogen peroxide was added to 1 mL of the hydrochlorothiazide, reserpine, and hydralazine stock solution (H2O2).

For 30 minutes, the solutions were kept at 600 C.
The resultant solution was diluted to obtain 80 g/mL, 20 g/mL, and 13 g/mL of each component in order to assess the sample's stability for the HPLC investigation.
The chromatograms were then recorded after ten litres were fed into the system.
Research on acid degradation: The mixture of 1mL of 2N hydrochloric acid and 1mL of a stock solution comprising hydrochlorothiazide, reserpine, and hydralazine was then refluxed for 30 minutes at 600°C.
To reach concentrations of 100 g/mL, 10 g/mL, and 100 g/mL of each component, the resulting solution was diluted.
The stability of the solution was then determined by injecting ten litres of solutions into the

system and recording the chromatograms.

Alkali Degradation Studies: Hydrochlorothiazide, Reserpine, and Hydralazine stock solution in 1 mL volume were combined 30 minutes of refluxing at 600°C with 1 mL of 2N sodium hydroxide.

The resulting solution was diluted to get 100 g/mL, 1 g/mL, and 100 g/mL of each component in order to assess the sample's stability.

The system was then injected with ten litres.

Studies on Dry Heat Degradation: The normal pharmaceutical solution was roasted at 1050°C for six hours to study dry heat deterioration.

The resultant solution was diluted to obtain 100 g/mL, 1 g/mL, and 100 g/mL of each component in order to assess the sample's stability for the HPLC investigation.

The chromatograms were then recorded after ten litres were fed into the system.

Photo Stability studies:

To assess the sample's stability, All components were introduced into the system at concentrations of 1g/mL and 100g/mL, together with 10 l, and the chromatograms were recorded.

The 80g/mL, 20g/mL, and 13g/mL solutions were exposed to UV Light for 7 days or 200 Watt hours/m2 in a photostability chamber for HPLC testing in order to further examine the drug's photochemical stability. The resulting solution was diluted to get 100g/mL.

Neutral Degradation Studies:

For the HPLC study, the drug was refluxed the resultant solution was diluted to obtain 100g/mL, 1g/mL, and 100g/mL of all components. Then, 10 l were injected into the system, and the chromatograms were recorded to determine the sample's stability. in water for 6 hours at a temperature of 60^{o}C.

Preparation of potassium dihydrogen orthophosphate :

Prepare potassium dihydrogen orthophosphate by dissolving 2.5 gm dissolved in 1000 mL of water that has undergone HPLC grading, with the pH being adjusted with orthophosphoric acid.

Optimised Chromatographic conditions:

Mobile phase : Acetonitrile: KH_2PO_4 (65:35 % v/v)
(pH- 3 Adjusted with 0.1% OPA)

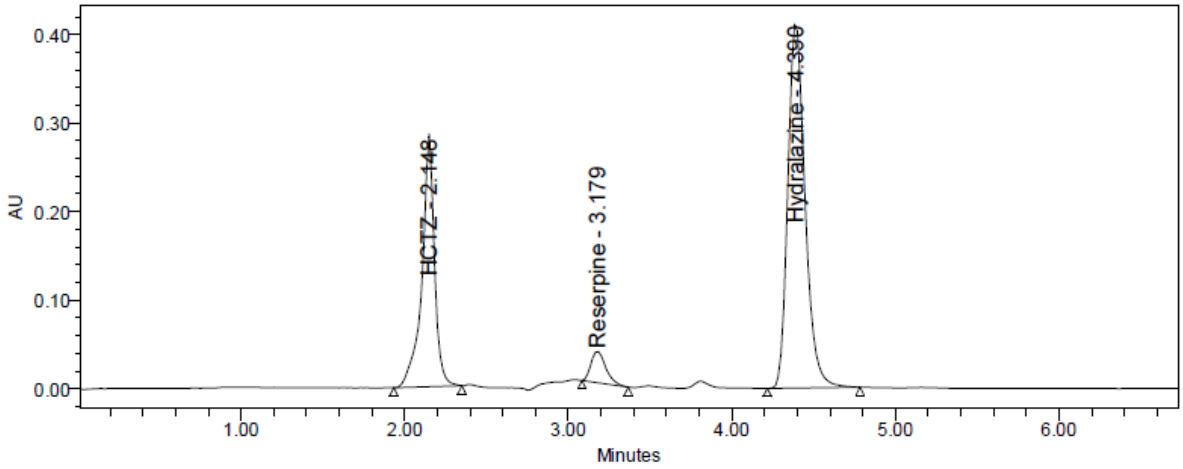
Diluent : Mobile phase.

Flow rate : 1.0 mL per min

Column : Agilent C_{18} (4.6 x 150mm, 1.7µm)

Detector wavelength : 235nm

Injection volume : 20 µl.

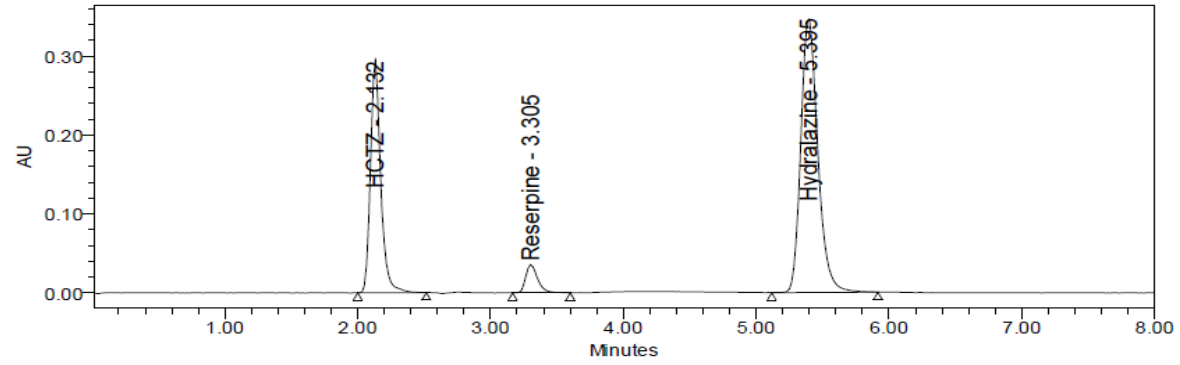


s. no	Peak Name	RT	Area
1	HCTZ	2.148	1502632
2	Reserpine	3.179	223173
3	Hydralazine	4.390	3007292

Parameters for system appropriateness

Before performing the validation, the system suitability tests were performed, in addition to the characteristics meeting the requirements for acceptance.

There were more than 2000 plates, there were two peak tailings, and the RSD of the peak regions from six injections was 2%. (Table1).



	Peak Name	RT	Area	USP Plate Count	USP Tailing	USP Resolution
1	HCTZ	2.119	2601678	3628	1.26	
2	Reserpine	3.296	218197	6696	1.29	7.71
3	Hydralazine	5.384	3066926	8850	1.22	10.39
	% RSD of Peak Area (n= 6)			0.4		

Linearity range

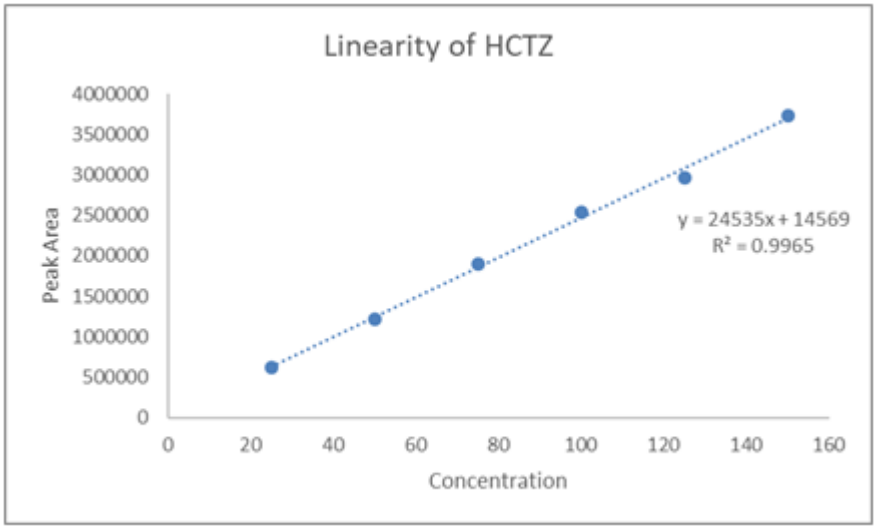
The range of linearity was between 25 and 150 g/mL for hydrochlorothiazide, 0.25 and respin is at 1.5 g/mL and hydralazine is at 25 to 150 g/mL, respectively. These following linear regression equations were used to describe them: Reserpine, Hydrochlorothiazide, and Hydrolazine all have y values of 24971x + 1456.6 (Hydrochlorothiazide), 221080x + 1778 (Hydralazine), and 31033x + 936.8 (Hydralazine), respectively (r2=0.999).

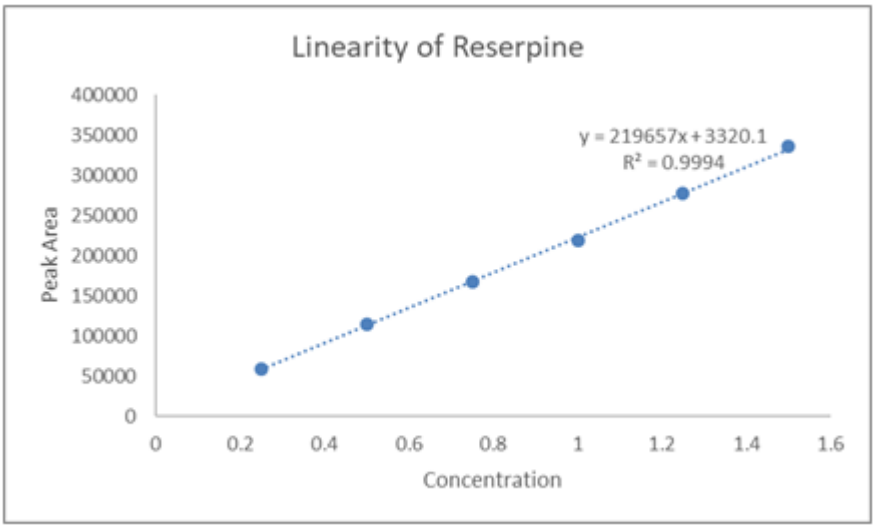
The After determining that the correlation coefficient (r2) for hydrochlorothiazide, resperpine, and hydralazine was greater than 0.999 regression line was built using the least squares approach.

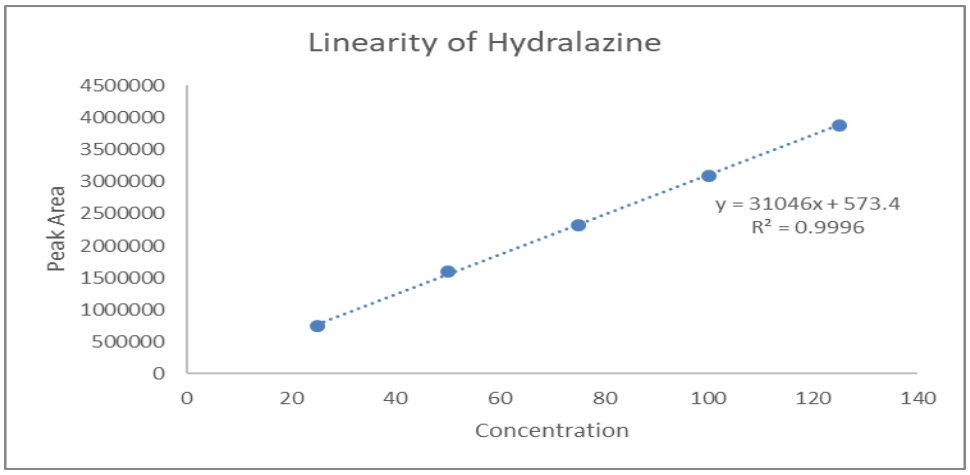
Hence, the established curves were linear.

Linearity table for Hydrochlorothiazide, Reserpine, Hydralazine

Hydrochlorothiazide		Reserpine		Hydralazine	
Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area	Conc (µg/mL)	Peak area
25	613191	.25	58630	25	751272
50	1216721	.5	115548	50	1590720
75	1899931	.75	167249	75	2327582
100	2543673	1	218708	100	3096154
125	5966714	1.25	277292	125	3879263
150	3728079	1.50	335691	150	


Linearity of HCTZ
Peak Area
Concentration
y = 24535x + 14569
R² = 0.9965
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
0
20
40
60
80
100
120
140
160


Linearity of Reserpine
Peak Area
Concentration
y = 219657x + 3320.1
R² = 0.9994
400000
350000
300000
250000
200000
150000
100000
50000
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6


Linearity of Hydralazine
Peak Area
Concentration
y = 31046x + 573.4
R² = 0.9996
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
0
20
40
60
80
100
120
140

Precision

In order to verify the variation in precision, the same day and two further days were used to analyse six replicate injections of the same concentration.

Hydrochlorothiazide, respin, and hydralazine all had % RSDs within the acceptable range of 2. As a result, procedure can be repeated on several days and with other analysts, and columns show that the method is accurate.

repeatability and moderate precision assessment

Drug Name	Repeatability			Intermediate		
	Peak Area	Std Dev	%RSD	Peak Area	Std Dev	%RSD
Hydrochlorothiazide	2613485	17390	0.7	2574909	6695.8	0.3
Reserpine	218708	1387.3	0.63	214077	805.8	0.4
Hydralazine	3080919	19939.9	0.6	3036457	10542.5	0.3

Accuracy

Reserpine, Hydralazine, and Hydrochlorothiazide all had percentage recoveries of respectively 100.48%, 100.14%, and 99.96% (Table 4, 5, 6). the results of recovery experiments unquestionably show how accurate the suggested strategy is.

Assessment of Hydrochlorothiazide Accuracy

% Level	Amount Spiked (µg/mL)	Total amount found (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean % Recovery
50%	50	149.76	49.92	99.83	
	50	152.52	50.84	101.68	
	50	151.60	50.53	101.07	
100%	100	203.52	101.76	101.76	101.02%
	100	201.23	100.61	100.61	
	100	202.45	101.22	101.22	

Assessment of Reserpine's Accuracy

% Level	Amount Spiked (µg/mL)	Total amount found (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean % Recovery
50%	0.5	1.51	0.50	101.31	100.64%
	0.5	1.50	0.50	100.52	
	0.5	1.49	0.49	99.59	
100%	1	2.03	1.01	101.96	
	1	1.96	0.98	98.03	
	1	1.99	0.99	99.94	
150%	1.5	2.53	1.52	101.42	
	1.5	2.54	1.52	101.92	
	1.5	2.52	1.51	101.08	

Evaluation of Hydralazine Accuracy

% Level	Amount Spiked (µg/mL)	Total amount found (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean % Recovery
50%	50	154.31	51.43	102.87	101.29
	50	152.92	50.97	101.94	
	50	152.51	50.84	101.68	
100%	100	198.98	99.49	99.49	
	100	202.95	101.47	101.47	
	100	202.33	101.16	101.16	
150%	150	255.11	153.06	102.06	
	150	250.62	150.37	100.24	
	150	251.60	150.96	100.64	

The limits of quantitation (LOQ) and detection (LOD) (LOQ)

The slope and Y-intercept were used to calculate the final values of LOD and LOQ.

The hydrochlorothiazide LOD and LOQ values were 0.04 and 0.13 g/mL, respectively, for

reserpine were 0.01 and 0.2 g/mL, and for hydralazine were 0.08 and 0.24 g/mL.

Hydrochlorothiazide, reserpine, and hydralazine sensitivity table

Molecule	LOD(µg/mL)	LOQ(µg/mL)
Hydrochlorothiazide	0.04 µg/mL	0.13 µg/mL
Reserpine	0.01 µg/mL	0.02 µg/mL
Hydralazine	0.08 µg/mL	0.024 µg/mL

Robustness

By The proposed method's robustness was determined by analysing the sample and standard solutions (Table 6).

Standard deviation, relative standard deviation, theoretical plates, retention period, and USP tailing factor are all variables to consider did not vary significantly after the circumstances were purposefully altered.

Data on the robustness of hydralazine, reserpine, and hydrochlorothiazide

S.no	Condition	%RSD of Hydrochlorothiazide	%RSD of Reserpine	%RSD of Hydralazine
1	Flow rate (-) 0.9mL/min	1	0.8	0.6
2	Flow rate (+) 1.1mL/min	0.6	0.7	0.3
3	Mobile phase (-) 38B:62A	0.6	3.8	0.3
4	Mobile phase (+) 26B:74A	0.3	1	0.3
5	Temperature (-) 25°C	1.4	1	0.6
6	Temperature (+) 35°C	0.3	0.7	0.4

Assay

The created method was used the amounts of hydrochlorothiazide, reserpine, and hydralazine in the pharmaceutical dosage form were calculated using the purity of these substances, which were found to be 99.93%, 100.65%, and 100.1%, respectively. all within the acceptable range of 2.

Forced degradation studies.

All of the values for reserpine, hydralazine, and hydrochlorothiazide were within the

acceptable ranges according to the forced degradation study.

Reserpine, Hydralazine, and Hydrochlorothiazide have all demonstrated notable sensitivity to the treatment of HCl, NaOH, and peroxide solutions.

As time, the medications degraded steadily, and noticeable degradation was seen.

During forced thermal degradation, photolytic degradation, and neutral degradation, hydrochlorothiazide, reserpine, and hydralazine remained stable.

The Hydrochlorothiazide, Reserpine, and Hydralazine peaks were homogeneous and pure in all of the stress samples analysed, according to peak purity test results from the PDA detector. These results came from the degradation investigations.

Stressed samples had a mass balance that was nearly 99.5% accurate.

Data on Hydrochlorothiazide Degradation

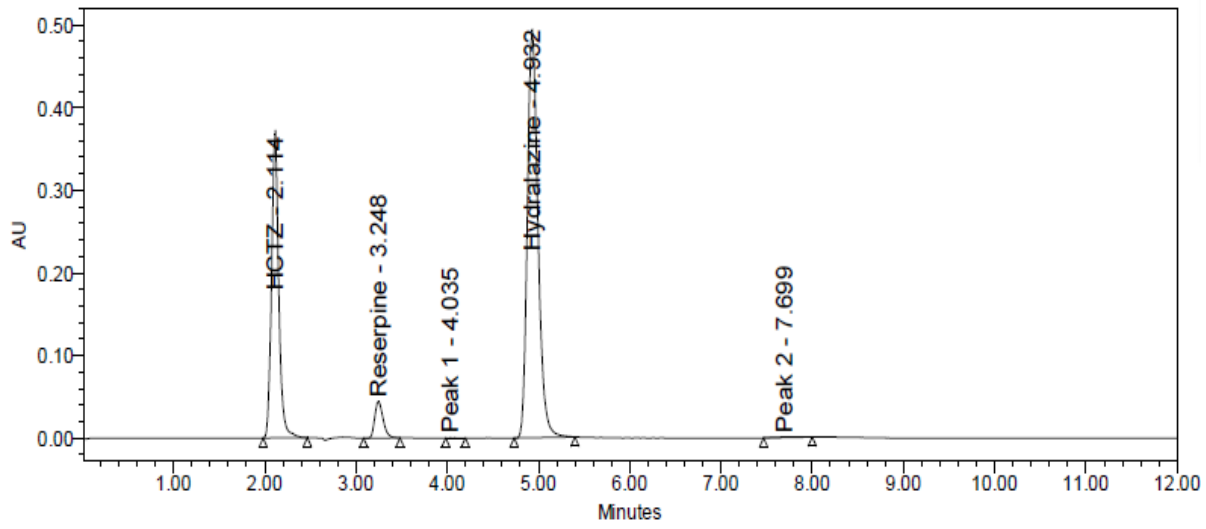
S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	4.6	0.239	0.482
2	Alkali	2.8	0.247	0.336
3	Oxidation	7.2	0.247	0.345
4	Thermal	1.7	0.262	0.480
5	UV	0.48	0.259	0.344
6	Water	0.47	0.267	0.334

Reserpine Degradation Information

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	4.2	0.463	0.712
2	Alkali	3.1	0.577	0.686
3	Oxidation	6.9	0.533	0.769
4	Thermal	1.6	0.429	0.656
5	UV	0.7	0.508	0.745
6	Water	0.6	0.528	0.739

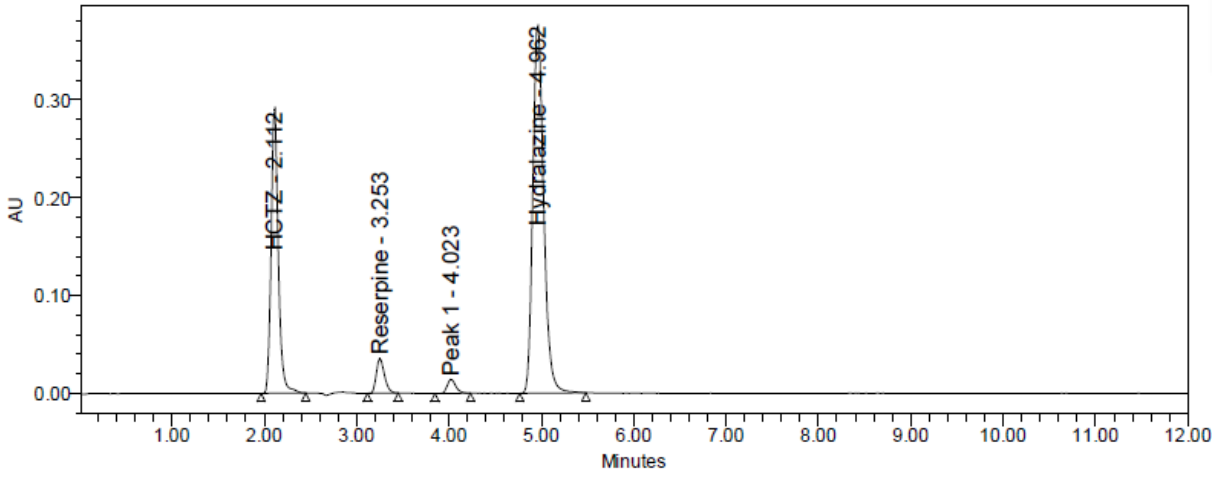
Hydralazine Degradation Data

S.NO	Degradation Condition	% Drug Degraded	Purity Angle	Purity Threshold
1	Acid	4.9	0.163	0.654
2	Alkali	2.3	2.021	2.686
3	Oxidation	3.4	0.110	0.386
4	Thermal	0.7	0.082	0.576
5	UV	1.3	0.075	0.392
6	Water	0.6	0.074	0.378



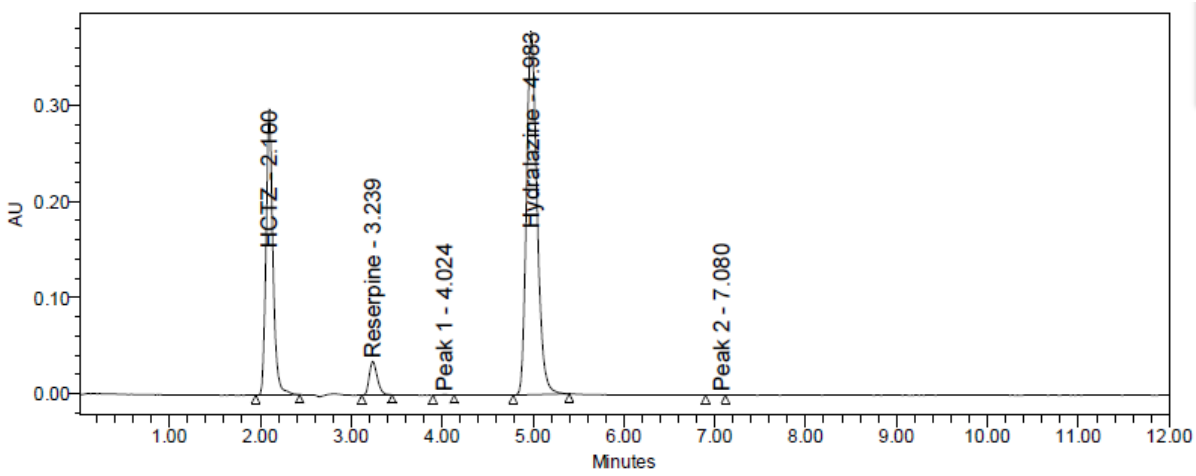
	Peak Name	RT	Area	Purity1 Angle	Purity1 Threshold
1	HCTZ	2.114	2481401	0.239	0.482
2	Reserpine	3.248	209780	0.463	0.712
3	Peak 1	4.035	1397	72.548	90.000
4	Hydralazine	4.932	2924358	0.163	0.654
5	Peak 2	7.699	12266	3.720	4.987

Acid degradation



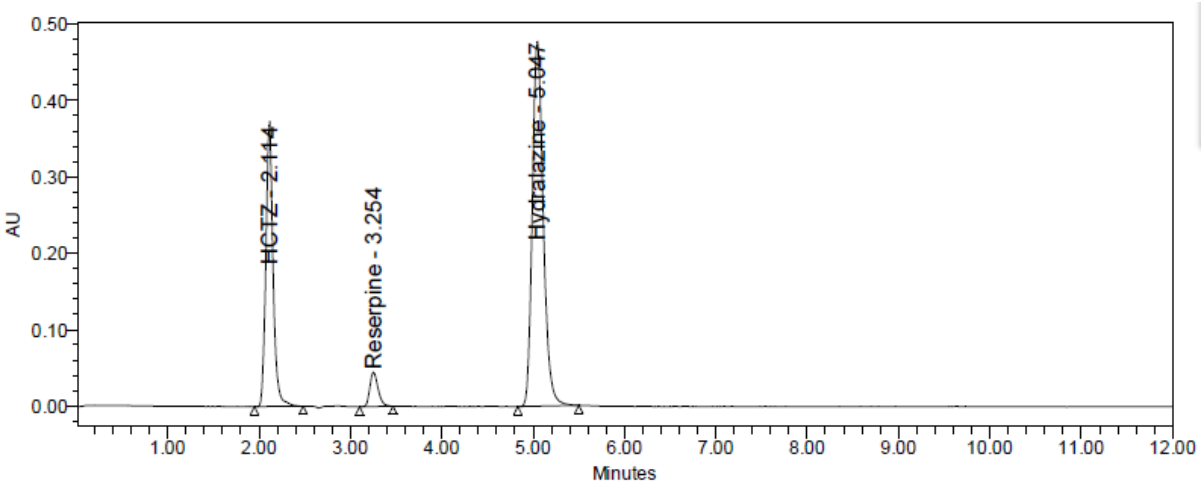
	Peak Name	RT	Area	Purity1 Angle	Purity1 Threshold
1	HCTZ	2.112	2527072	0.247	0.336
2	Reserpine	3.253	212233	0.577	0.686
3	Peak 1	4.023	91826	2.021	2.686
4	Hydralazine	4.962	3002776	0.110	0.382

Base degradation



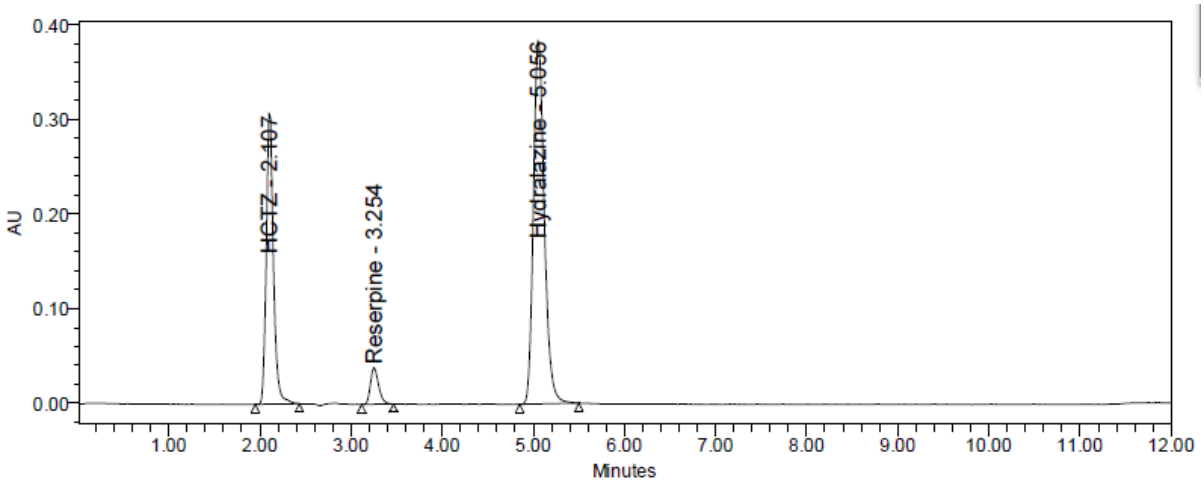
	Peak Name	RT	Area	Purity1 Angle	Purity1 Threshold
1	HCTZ	2.100	2414204	0.247	0.345
2	Reserpine	3.239	203983	0.533	0.769
3	Peak 1	4.024	2580	41.216	78.584
4	Hydralazine	4.983	2970095	0.110	0.386
5	Peak 2	7.080	453	84.268	90.000

Peroxide degradation



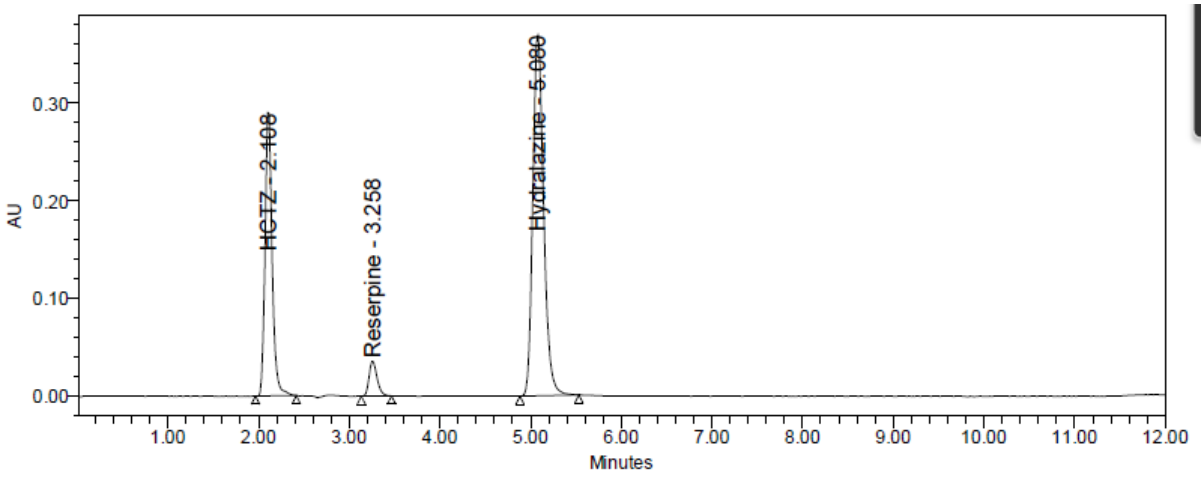
	Peak Name	RT	Area	Purity1 Angle	Purity1 Threshold
1	HCTZ	2.114	2555788	0.262	0.480
2	Reserpine	3.254	215572	0.429	0.656
3	Peak 1	4.000			
4	Hydralazine	5.047	3052864	0.082	0.576
5	Peak 2	7.000			

Thermal degradation



	Peak Name	RT	Area	Purity1 Angle	Purity1 Threshold
1	HCTZ	2.107	2589229	0.259	0.344
2	Reserpine	3.254	217435	0.508	0.745
3	Peak 1	4.000			
4	Hydralazine	5.056	3201763	0.075	0.392
5	Peak 2	7.000			

Photo degradation



	Peak Name	RT	Area	Purity1 Angle	Purity1 Threshold
1	HCTZ	2.108	2589449	0.267	0.334
2	Reserpine	3.258	217780	0.528	0.739
3	Peak 1	4.000			
4	Hydralazine	5.080	3056623	0.074	0.378
5	Peak 2	7.000			

Hydrolytic degradation

CONCLUSION:

The current paper's goal was to create an RP-HPLC method that could simultaneously estimate hydrochlorothiazide, reserpine, and hydralazine in pharmaceutical dosage forms

while also providing stability information.

The active pharmaceutical ingredient's solubility was investigated in a variety of ratios and solvents, including acetonitrile, methanol, and water, but the standard was finally found to be soluble in water: methanol (65:35), which led to its adoption as a diluent.

Acetonitrile and potassium dihydrogen phosphate buffer were investigated as alternate mobile phases in compositions with a flow rate of 1 ml/min, but because of insufficient peak resolution, retention period, and tailing factor, 0.1% ortho phosphoric acid and acetonitrile was ultimately chosen as a buffer.

The problem was initially solved using an ODS C18 column (150mm x 4.6mm x 5) maintained at 30°c for 7 minutes.

The retention time, run duration, and peak resolution of the columns "kromasil®" (250mm x 4.6mm x 5) and "BDS" (150mm x 4.6mm x 5) were initially employed, although they were not precise. The next stage in process optimization was to change the composition and ratio of the mobile phase, which was done, discovered that the optimal wavelength for three drugs hydrochlorothiazide, reserpine, and hydralazine was at 235 nm.

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