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Stability Indicating Forced Degradation Studies and a Newly Developed Validated Analytical Method for the Simultaneous Estimation of Amitryptaline and Perphenazine by RP-HPLC in Pure Bulk and Pharmaceutical Dosage Form

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ABSTRACT

A simple and selective LC method is described for the determination of Amitryptaline and perphenazine in bulk and Pharmaceutical dosage forms. Chromatographic separation was achieved on a C_{18} column using mobile phase consisting of a mixture of 50 volumes of acetonitrile and 50 volumes of TEA buffer with detection of 247 nm. Linearity was observed in the range 110-190 µg /ml for Amitryptaliner^2 =0.990) and 4-12µg /ml for Perphenazine (r^2 =0.990) for the amount of drugs estimated by the proposed methods was in good agreement with the label claim. The proposed methods were validated. The accuracy of the methods was assessed by recovery studies at three different levels. Recovery experiments indicated the absence of interference from commonly encountered pharmaceutical additives. All the validation parameters were done and the results were found to be within the desired limits. Amitryptaline and perphenazine stock solutions were subjected to acid and alkali hydrolysis, chemical oxidation and dry heat degradation. The degraded product peaks were well resolved from the pure drug peak with significant difference in their retention time values. The procedures were strictly followed according to the ICH guidelines. All statistical data proves validity of the methods and can be used for routine analysis of pharmaceutical dosage form.

INTRODUCTION

Analytical chemistry is the study of the separation, identification and quantification of the chemical components of natural and artificial materials. Analytical chemistry is divided into two parts.

Qualitative analysis and Quantitative analysis

Analytical methods can be separated into following types

1. Chemical methods
2. Microbiological methods
3. Biological methods
4. Instrumental or physico-chemical methods

Chromatography is a procedure by which solutes are separated by a dynamic differential migration process in a system containing two migration phases, one of which moves continuously in a given direction, other is stationary and in which the individual substances exhibit different mobilities due to difference in absorption, partition, solubility, vapour pressure, molecular size or ionic charge density.

HPLC is defined as high performance liquid chromatography or high pressure liquid chromatography.

In HPLC separations are achieved by partition, adsorption or ion exchange depending on the stationary phase. HPLC is advantageous over Gas chromatography in analysis of organic compounds because the compounds are dissolved in organic liquid and most of the separations take place at room temperature. Non-volatile and thermally unstable drugs can be chromatographed without decomposition or necessity of making volatile derivatives.

The basic principle of HP-LC is Adsorption. In this a small volume of liquid sample is injected into the column packed in a suitable stationary phase. The sample is then forced through the column by delivering the mobile phase with high pressure. The sample gets separated based on their relative affinities towards the column. The sample which shows

higher affinity towards the mobile phase elutes first than the sample which shows higher affinity towards the column.[1-5]

Method Validation:

It is defined as the process of proving (through scientific studies) that an analytical method is acceptable for its intended use. Recent guidelines for method development and validation for new non compendial test methods are provided by the FDA draft document, "Analytical procedures and method validation: Chemistry, Manufacturing and Controls Documentation". This recent document applies to the method development and validation process for products included in investigational new drug (IND), new drug application (NDA) and abbreviated new drug application (ANDA) submissions. Therefore, expectations from regulatory agencies for method development and validation are clear. The International Conference on Harmonization (ICH) as issued guidelines for analytical method validation. The recent FDA method validation draft guidance document as well as USP both refer to ICH guidelines. Analytical guidance documents recently published by the ICH are the following.

a. Stability testing, b. Validation of analytical procedures, c. Impurities in drug substances and products, d. Specification for new drug substance and product.

Method validation is a continuous process. The goal is to ensure confidence in the analytical data throughout product development. The steps of method development and method validation depend upon the type of method being developed.

However, the following steps are common to most types of projects: Method development plan definition, Background information gathering, Laboratory method development, Generation of test procedure, Method validation protocol definition, Laboratory method validation, Validated test method generation and Validation report.

A well-developed method should be easy to validate. A method should be developed with the goal to rapidly test preclinical samples, formulation prototypes and commercial samples. As the method development and validation processes advance, the information gathered is captured in the design and subsequent improvement of the method. Ideally the validation protocol should be written only following a thorough understanding of the method's capabilities and intended use. The validation protocol will list the acceptance criteria that the method can meet. Any failure to meet the criteria will require that a formal investigation be conducted.

The required validation parameters, also termed analytical performance characteristics depend upon the type of analytical method. Pharmaceutical analytical methods are categorized into general types.[6-10]

Identification tests, Potency assays, Impurity test: quantitative and Impurity test: limit

Validation requirements depend upon the type of test method including, Specificity: ability to measure desired analyte in a complex mixture, Accuracy: agreement between measured and real value, Linearity: proportionality of measured value to concentration, Precision: agreement between a series of measurements, Range: concentration interval where method is precise, accurate and linear, Detection limit: lowest amount of analyte that can be detected, Quantitation limit: lowest amount of analyte that can be measured, Robustness: reproducibility under normal but variable laboratory conditions.

Only specificity is needed for an identification test. However, the full range of specificity, accuracy, linearity range, limit of detection (LOD), limit of quantization (LOQ), precision, and robustness testing is needed.

Quality investigation plays a very important role in quality specification establishment of chemical drugs. The number of drugs introduced into the market every year. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. Hence, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

Basic criteria for new method development of drug analysis: The drug or drug combination may not be official in any pharmacopoeias, A proper analytical procedure for the drug may not be available in the literature due to patent regulations, Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients, Analytical methods for a drug in combination with other drugs may not be available, The existing analytical procedures may require expensive reagents and solvents, It may also involve cumbersome extraction and separation procedures and these may not be reliable.

Analytical method development provides the support to track the quality of the product from batch to batch. Method development involves considerable trial and error procedures. The

most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase. Single dosage forms with combination of drugs are widely used today due to their advantages and their simultaneous estimation of individual component is a challenging task.[11-15]

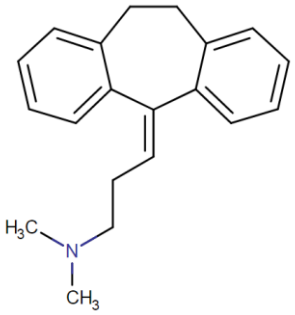
MATERIALS AND METHODS

Amitriptyline

Summary: Amitriptyline is a tricyclic antidepressant indicated in the treatment of depressive illness, either endogenous or psychotic, and to relieve depression associated anxiety.

Brand Names: *Elavil*

Structure:



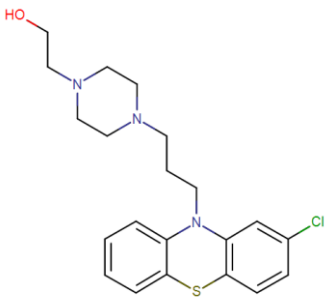
Chemical Formula: $C_{20}H_{23}N$

Perphenazine

Perphenazine is a phenothiazine used to treat schizophrenia as well as nausea and vomiting.

Background: An antipsychotic phenothiazine derivative with actions and uses similar to those of chlorpromazine.

Structure:



Chemical Formula: $C_{21}H_{26}ClN_3OS$

Instruments used

Table No. 1: Instruments used

UV-Visible Spectrophotometer	Nicolet evolution 100
UV-Visible Spectrophotometer software	Vision Pro
HPLC software	Spin chrome (LC SOLUTIONS)
HPLC	Shimadzu(LC 20 AT VP)
Ultra sonicator	Citizen, Digital Ultrasonic Cleaner
pH meter	Global digital
Electronic balance	Shimadzu
Syringe	Hamilton
HPLC Column	Inertsil ODS 3V(250x4.6mm) 5µm

Table No. 2: Reagents used

Water	HPLC Grade
Methanol	HPLC Grade
Acetonitrile	HPLC Grade

Table No. 3: Drugs used

Amitryptyline and Perphenazine drugs	Chandra labs, Hyd.
Amitryptyline and Perphenazine (150/8mg)	Obtained from local pharmacy

Preparation of Mobile Phase

A mixture 50 volumes of Triethylamine and 50 volumes of acetonitrile were prepared. The mobile phase was sonicated for 10min to remove gases and filtered through 0.45µ membrane filter for degassing of mobile phase.[16-20]

Solubility Studies

These studies are carried out at 25 ^{0} C.

AMITRIPTYLINE

Freely soluble in chloroform and ethanol and sparingly soluble in water.

PERPHENAZINE :

soluble in water, ACN, and slightly soluble in methanol,

Determination of Working Wavelength (λmax)

In simultaneous estimation of two drugs isobestic wavelength is used. Isobestic point is the wavelength where the molar absorptivity is the same for two substances that are interconvertible. So this wavelength is used in simultaneous estimation to estimate both drugs accurately.

Preparation of standard stock solution of AMITRIPTYLINE

10 mg of AMITRIPTYLINE was weighed and transferred in to 100ml volumetric flask and dissolved in methanol and then make up to the mark with methanol and prepare 10 µg /ml of solution by diluting 1ml to 10ml with methanol.

Preparation of standard stock solution of PERPHENAZINE

10mg of PERPHENAZINE was weighed in to 100ml volumetric flask and dissolved in Methanol and then dilute up to the mark with methanol and prepare 10 µg /ml of solution by diluting 1ml to 10ml with methanol.

Method development Amitryptaline and perpheranzine are relatively polar compounds. Preliminary attempts using reversed-phase HPLC using C_{18} column were not successful.

Method validation:

Accuracy:

For accuracy determination, three quality control samples were prepared i.e., 10 ppm, 25ppm and 50ppm of Amitryptaline and perphenazine injected in five replicate volumes of 20µL each. Accuracy is reported as the percent recovery of the known, added amount.

Acceptance criteria: The percentage recovery should be in the range of 85 to 115%.

Precision: Precision was determined by replicate processing. Precision was reported as Percent Relative Standard Deviation.10 ppm, 25ppm and 50ppm of Amitryptaline and perpheranzine was selected to determine precision of the method. The Percentage Relative Standard Deviation for the areas were calculated (should not be more than 15%).

Acceptance criteria: The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

Linearity: Linearity of the developed method was demonstrated with Amitryptaline and perpheranzine at six different concentrations from 1-100 ppm. Calibration QC standards were prepared fresh on the day of analysis by diluting the appropriate working solutions with mobile phase and injected into chromatographic system. The data were subjected to statistical analysis using a linear-regression model. The calibration curves were obtained by weighted linear regression (weighing factor $1/x^2$) using Microsoft Excel 2007 software. A graph was plotted with concentration versus peak area by covering six points.

Acceptance criteria: The plot for concentration versus peak area should be linear with a regression coefficient not less than 0.9990.

LOD and LOQ:

LOD and LOQ was calculated according to ICH guidelines. The LOD and LOQ are shown in table 4-15. The detection limit (LOD) and quantitation limit (LOQ) may be expressed as:

$$L.O.D. = 3.3(SD/S).$$

$$L.O.Q. = 10(SD/S)$$

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

System suitability: System suitability was demonstrated using 50ppm Amitryptaline and perpheranzine and 10µL volume of this solution was injected six times into the chromatographic system and the chromatogram was recorded. Results are shown in table 4-16.

System suitability was determined with the below mention parameters.

- ✓ Resolution.
- ✓ Capacity factor.
- ✓ Retention Time

Stability related impurity studies:

Acid hydrolysis: An accurately weighed 25 mg. of pure drugs were transferred to a clean & dry 25 ml of two separate volumetric flask. To which 0.1 N Hydrochloric acid was added & make up to the mark & kept for 24 hrs. from both the volumetric flask 0.3 ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of HCl (after all optimized conditions).

Basic hydrolysis: An accurately weighed 25 mg. of pure drugs were transferred to a clean & dry 25 ml of two separate volumetric flasks. To which 0.1 N Sodium hydroxide was added & make up to the mark & kept for 24 hrs. from both 0.3 ml was taken in to a 10 ml volumetric flask & make up to the mark with mobile phase, then injected into the HPLC system against a blank of NaOH (after all optimized conditions).

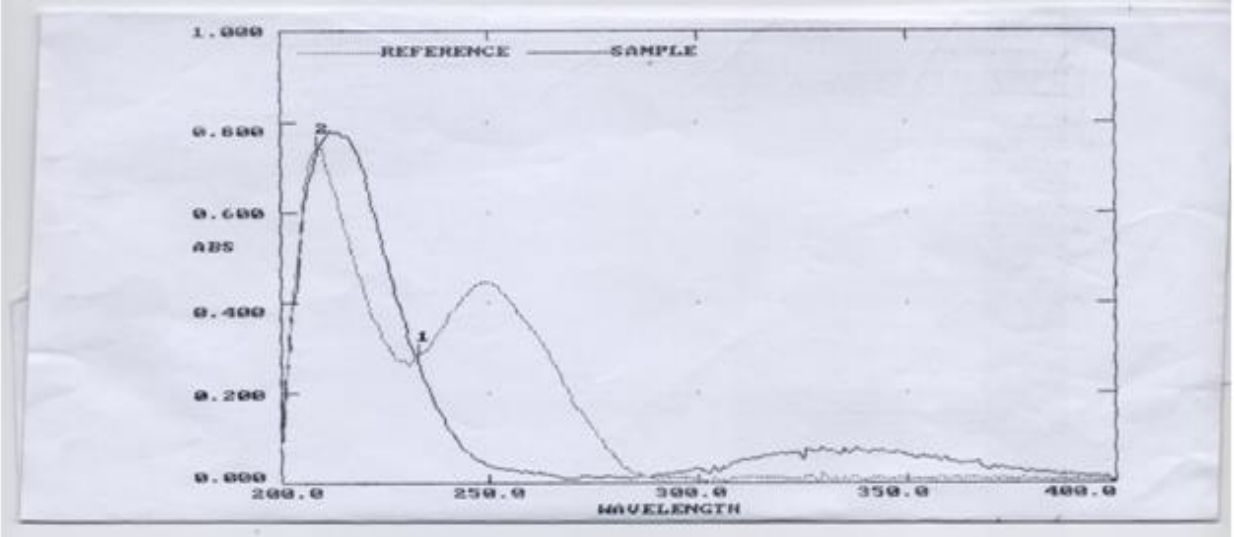
Thermal degradation: An accurately weighed 25 mg. of pure drugs were transferred to a clean & dry 25 ml of two separate volumetric flasks, make up to the mark with mobile phase. From this solution take 0.3 ml make up to the volume 10 ml & was maintained at 50^0C for 24 hrs then injected into the HPLC system against a blank of mobile phase (after all optimized conditions).

Photolytic Degradation: Approximately 10 mg. of pure drugs were taken in different clean & dry Petridis. It was kept in a UV cabinet at 254 nm wavelength for 24 hours without interruption. Accurately weighed 0.3 mg. of each UV exposed drugs were transferred to a clean & dry 10 ml. volumetric flask. First the UV exposed drug was dissolved in mobile phase & make up to the mark then injected into the HPLC system against a blank of mobile phase (after all optimized conditions).

Oxidation with (3%) H_2O_2: Accurately weighed 10 mg. of pure drug was taken in a clean & dry 100 ml volumetric flask. 30 ml of 3% H_2O_2 and a little methanol was added to it to make it soluble & then kept as such in dark for 24 hours. Final volume was made up to 100 ml using water to prepare 100 ppm solution. The above sample was injected into the HPLC system.[21-28]

RESULTS AND DISCUSSION

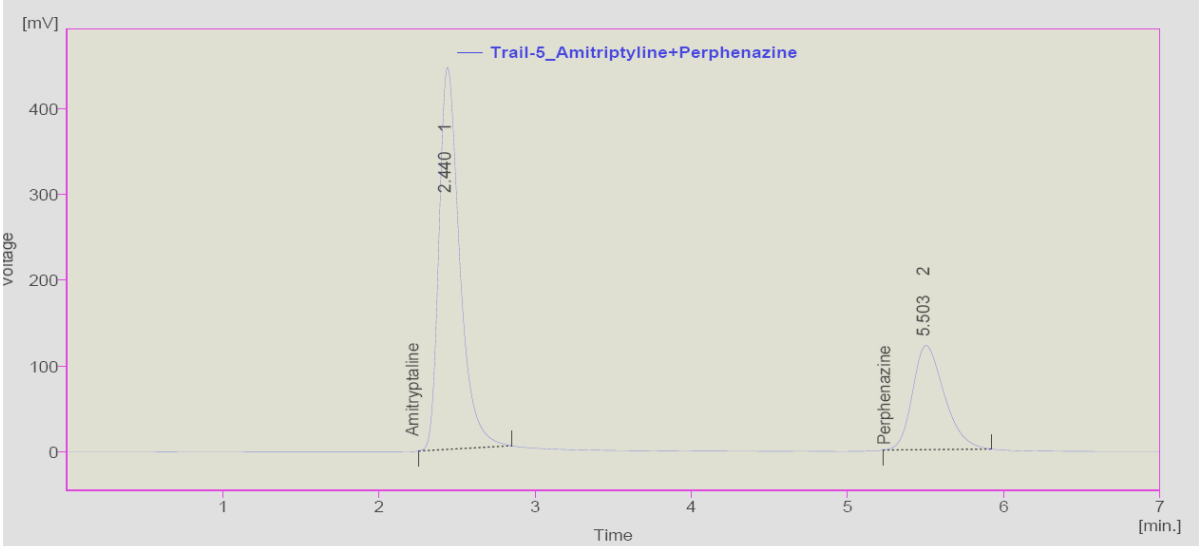
HPLC method development: The wavelength of maximum absorption (λ_{max}) of the drug, 10 µg/ml solution of the drugs in methanol were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against methanol as blank. The resulting spectra are shown in the fig. The isobestic point was found to be 247nm for the combination.



Observation: The Isobestic point was found to be 247 nm for Amitriptyline and Perphenazine in combination.

Optimized chromatographic conditions

Mobile phase	TRIETHYLAMINE:ACN(50:50)
Ph	3.5
Column	Inertsil ODS 3V column,C18(150x4.6 ID) 5µm
Flow rate	1.0 ml/min
Column temperature	Room temperature(20-25^{o}C)
Sample temperature	Room temperature(20-25^{o}C)
Wavelength	247
Injection volume	20 µl
Run time	6 min
Retention time	About 2.440 min for AMITRIPTYLINE and 5.503 min for PERPHENAZINE



Result Table (Uncal - Trial optimized_Amitriptyline+Perphenazine

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.440	4112.666	445.535	70.49
2	5.503	1721.694	121.368	29.51
	Total	5834.360	566.903	100.00

Column Performance Table (From 50% - Trial optimized_Amitriptyline+Perphenazine

	Reten. Time	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.440	0.140	1.710	1683	16828	-
2	5.503	0.220	1.540	3467	34667	10.014

Fig. No. 1: Chromatogram of Amitriptyline and Perphenazine by using mobile phase

Observation:

- All the system suitability requirements were met.
- The peak Asymmetry factor was less than 2 for both Amitriptyline and Perphenazine. The efficiency was more than 2000 Amitriptyline and Perphenazine.
- Resolution between two peaks >1.5.
- The details are given in the table, hence this method was for optimized.

Assay

Preparation of samples for Assay

Preparation of mixed standard solution: Weigh accurately 100 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 100ml of mobile phase and make

up the volume with mobile phase. From above stock solution 150 µg/ml of Amitriptyline and 8 µg/ml Perphenazine is prepared by diluting 2.3ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Tablet sample: 10 tablets (each tablet contains Perphenazine-8 mg Amitriptyline -150 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solutions of Amitriptyline and Perphenazine (µg/ml) were prepared by dissolving weight equivalent to 8 mg of PERPHENAZINE and 150 mg of AMITRIPTYLINE and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 50ml with mobile phase. Further dilutions are prepared in 5 replicates of 8µg/ml of PERPHENAZINE and 150µg/ml of AMITRIPTYLINE was made by adding 2.3 ml of stock solution to 10 ml of mobile phase.

Calculation: The amount of Amitriptyline and Perphenazine present in the formulation by using the formula given below, and results shown in above table:

$$\% \text{ Assay} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{AW}{LC} \times 100$$

Where,

AS: Average peak area due to standard preparation

AT: Peak area due to assay preparation

WS: Weight of Amitriptyline and Perphenazine in mg

WT: Weight of sample in assay preparation

DT: Dilution of assay preparation

Table No. 4: Assay Results

Amitriptyline			**Perphenazine**	
	Standard Area	**Sample Area**	**Standard Area**	**Sample Area**
Injection-1	4061.531	4112.666	1686.841	1721.694
Injection-2	4131.268	4137.227	1719.233	1719.908
Injection-3	4105.102	4133.868	1660.028	1686.784
Injection-4	4039.465	4136.435	1682.571	1702.908
Injection-5	4112.666	4116.338	1721.694	1710.467
Average Area	4090.006	4127.307	1694.073	1708.352
Standard deviation	11.82636		14.23762	
% RSD	0.286539		0.833412	
Assay (%purity)	100.912		100.8429	

Observation

The amount of Amitriptyline and Perphenazine present in the taken dosage form was found to be 100.912 % and 100.842% respectively.

Validation

1. System suitability

Standard solutions were prepared as per the test method and injected into the chromatographic system. The system suitability parameters like theoretical plates, resolution and asymmetric factor were evaluated.

Table No. 5: Results for system suitability of AMITRIPTYLINE

Injection	Retention time (min)	Peak area	Theoretical plates (TP)	Tailing factor (TF)
1	2.447	4061.531	1614	1.710
2	2.443	4131.268	1687	1.710
3	2.447	4105.102	1692	1.710
4	2.447	4039.465	1692	1.677
5	2.440	4112.666	1683	1.710
Mean	2.4448	4090.006	-	-
SD	0.003194	38.10884	-	-
%RSD	0.130634	0.931755	-	-

Table No. 6: Results for system suitability of PERPHENAZINE

Injection	Retention time (min)	Peak area	Theoretical plates	Tailing factor
1	5.500	1686.841	3360	1.520
2	5.490	1719.233	3557	1.560
3	5.507	1660.028	3579	1.531
4	5.513	1682.571	3587	1.500
5	5.503	1721.694	3467	1.540
Mean	5.5026	1694.073	-	-
SD	0.008562	26.17062	-	-
% RSD	0.155591	1.544834	-	-

Acceptance criteria

1. The % RSD for the retention times of Amitriptyline and Perphenazine Peaks from 6 replicate injections of each Standard solution should be not more than 2.0 %.

2. The number of theoretical plates (N) for the Amitriptyline and Perphenazine peaks is not less than 2000.

3. The Tailing factor (T) for the Amitriptyline and Perphenazine peak is not more than 2.0.

Observation:

The % RSD for the retention times and peak area of Amitriptyline and Perphenazine were found to be less than 2%. The plate count and tailing factor results were found to be satisfactory and are found to be within the limit.

Specificity by Direct comparison method: There is no interference of mobile phase, solvent and placebo with the analyte peak and also the peak purity of analyte peak which indicate that the method is specific for the analysis of analytes in their dosage form.

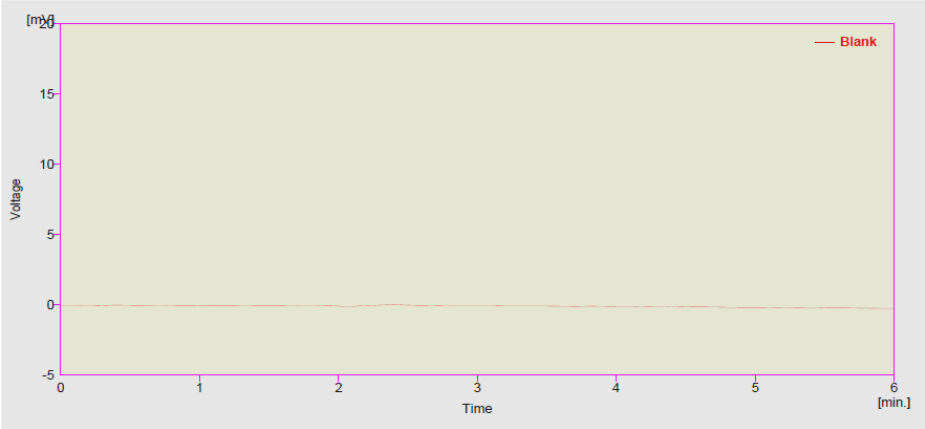
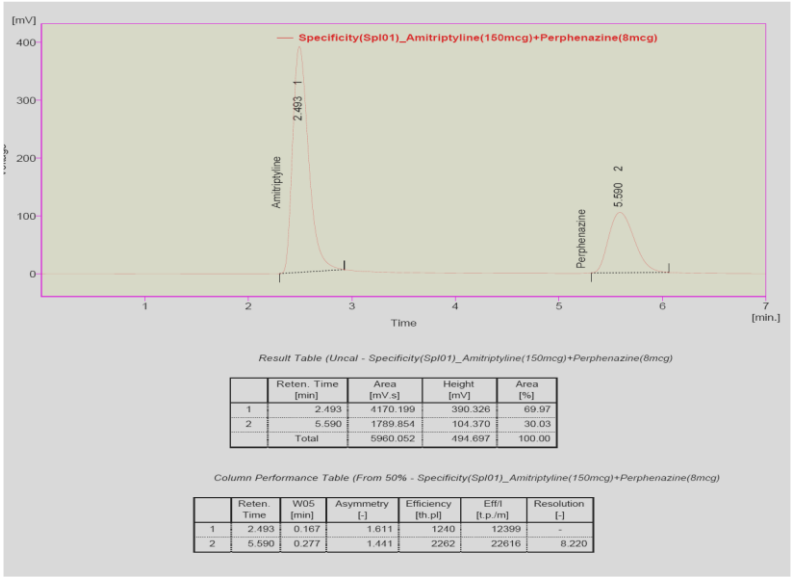


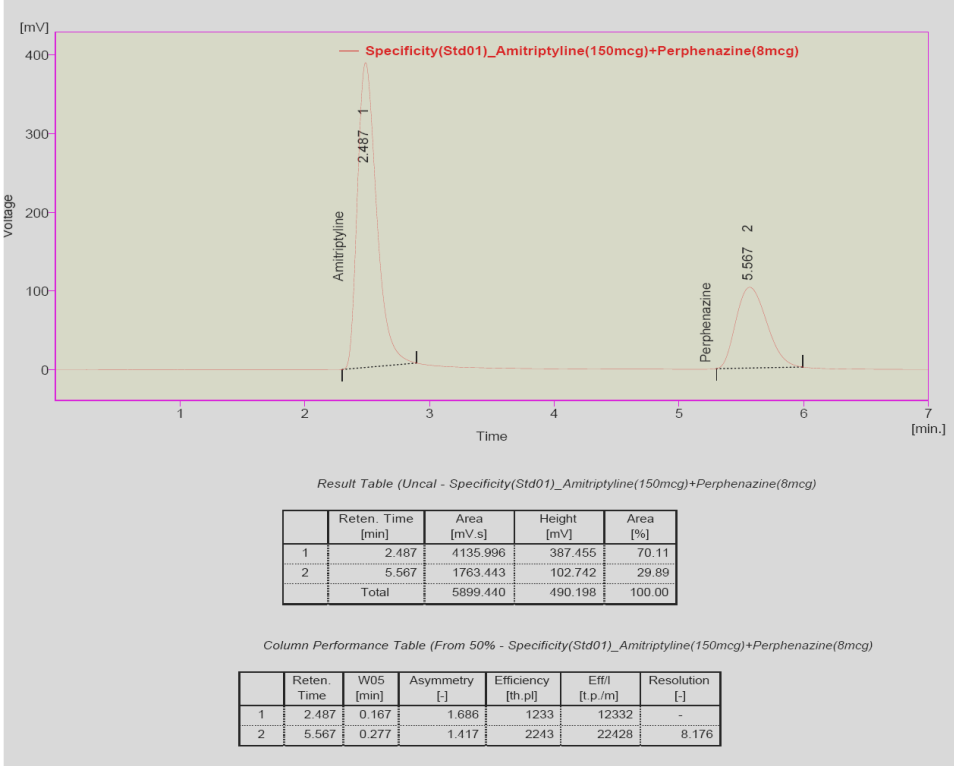
Fig. No. 2.1: Blank chromatogram for specificity by using mobile phase



	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.493	4170.199	390.326	69.97
2	5.590	1789.854	104.370	30.03
	Total	5960.052	494.697	100.00

	Reten. Time	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.493	0.167	1.611	1240	12399	-
2	5.590	0.277	1.441	2262	22616	8.220

Fig. No. 2.2: Chromatogram for specificity of Amitriptyline and Perphenazine sample



Result Table (Uncal - Specificity(Std01)_Amitriptyline(150mcg)+Perphenazine(8mcg)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]
1	2.487	4135.996	387.455	70.11
2	5.567	1763.443	102.742	29.89
	Total	5899.440	490.198	100.00

Column Performance Table (From 50% - Specificity(Std01)_Amitriptyline(150mcg)+Perphenazine(8mcg)

	Reten. Time	W05 [min]	Asymmetry [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.487	0.167	1.686	1233	12332	-
2	5.567	0.277	1.417	2243	22428	8.176

Fig. No. 2.3: Chromatogram for Specificity of Amitriptyline and Perphenazine standard

Observation: It is observed from the above data, diluents or excipients peaks are not interfering with the Amitriptyline and Perphenazine peaks.

Linearity and range

Preparation of standard stock solution: Standard stock solutions of Amitriptyline and Perphenazine (microgram/ml) were prepared by dissolving 100 mg of Amitriptyline and 100 mg of Perphenazine dissolved in sufficient mobile phase and dilute to 100 ml with mobile phase.

Further dilutions were given in the table.

Table No. 7: Linearity Preparations

Preparations	Volume from standard stock transferred in ml		Volume made up in ml (with mobile phase)	Concentration of solution(µg /ml)	
				AMITRIPTYLINE	PERPHENAZINE
Preparation 1	0.75	0.4	10	110	4
Preparation 2	1.125	0.6	10	130	6
Preparation 3	1.5	0.8	10	150	8
Preparation 4	1.875 1.0		10	170	10
Preparation 5	2.75 1.2		10	190	12

Table No. 8: Linearity of AMITRIPTYLINE

S. No.	Conc. (µg/ml)	Area
1	110	2045.19
2	130	3001.15
3	150	4044.02
4	170	4467
5	190	5404.99

Table No. 9: Linearity of PERPHENAZINE

S. No.	Conc.(µg/ml)	Area
1	4	1360.49
2	6	2059.435
3	8	2768.534
4	10	3478.472
5	12	4192.586

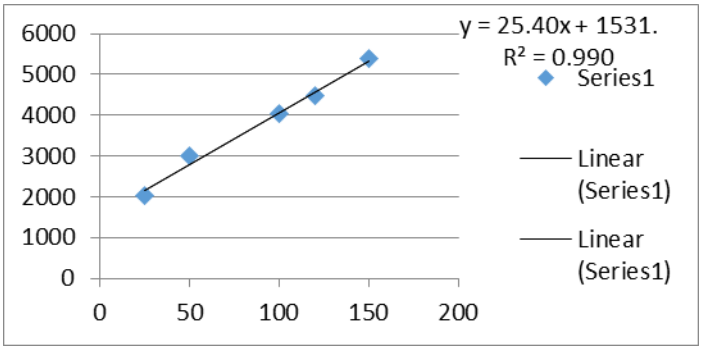


Fig. No. 3.1: Linearity graph of Amitriptyline

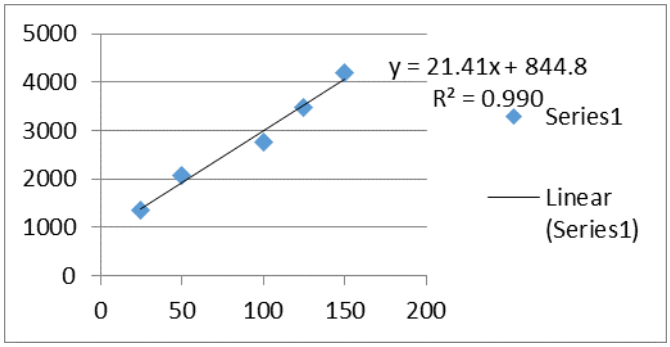


Fig. No. 3.2: Linearity graph of Perphenazine

Acceptance criteria: The relationship between the concentration should be linear in the specified range and the correlation should not be less than 0.99.

Observation

The correlation coefficient for linear curve obtained between concentration vs. Area for standard preparations of Amitriptyline and Perphenazine is 0.989 and 0.982.

The relationship and area is linear in the range examined since all points lie in a straight line and the correlation coefficient is well within limits.

Accuracy: Accuracy of the method was determined by Recovery studies. To the formulation (pre analyzed sample), the reference standards of the drugs were added at the level of 50%, 100%, 150%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for drug is shown in table. To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample solution at three different levels 50%, 100%, 150%.

Acceptance criteria: The % recovery of Amitriptyline and Perphenazine should lie between 98% and 102%.

Table No. 10: Recovery results for Amitriptyline

	Amount taken (mcg/ml)	Area	% Recovery	Average % Recovery
50%	150	4213.432	102.93	101.84
	150	4282.743		
	150	4257.137		
100%	160	4394.244	101.87	
	160	4472.693		
	160	4481.172		
150%	170	5819.409	100.74	
	170	5819.409		
	170	5875.644		

Table No. 11: Recovery results for Perphenazine

Recovery level	Accuracy Perphenazine			Average % Recovery
	Amount taken (mcg/ml)	Area	% Recovery	
50%	8	1777.947	101.97	101.61
	8	1858.465		
	8	1851.301		
100%	9	2820.602	102.64	
	9	3005.032		
	9	2868.945		
150%	10	3953.430	100.23	
	10	3953.430		
	10	3972.157		

Observation: The percentage mean recovery of Amitriptyline and Perphenazine is 101.84% and 101.61% respectively.

Precision

Method precision: Prepared sample preparations of Amitriptyline and Perphenazine as per test method and injected 6 times in to the column.

Acceptance criteria: The % Relative standard deviation of Assay preparations of Amitriptyline and Perphenazine should be not more than 2.0%.

Table No. 12: Results for Method precision of Amitriptyline and Perphenazine

Amitriptyline		
S. No.	**Rt**	**Area**
1	2.487	4181.754
2	2.450	4143.434
3	2.477	4162.886
4	2.493	4199.596
5	2.493	4161.196
6	2.460	4190.188
avg	2.476667	4173.176
stdev	0.018052	20.95232
% RSD	0.728874	0.502071

Perphenazine		
S. No.	**Rt**	**Area**
1	5.567	1759.963
2	5.533	1782.254
3	5.560	1776.727
4	5.590	1773.634
5	5.603	1762.717
6	5.570	1756.825
avg	5.5705	1768.687
stdev	0.024354	10.25314
% RSD	0.43719	0.579703

Observation: Test results for Amitriptyline and Perphenazine are showing that the % RSD of Assay results are within limits. The results were shown in Table.

Limit of Detection

$$LOD = \frac{3.3\sigma}{S}$$

Where, σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

Observation: The LOD for this method was found to be 4.0 µg/ml for Amitriptyline and 33.8µg/ml for Perphenazine.

Limit of Quantification

$$LOQ = \frac{10\sigma}{S}$$

Where,

σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

Observation: The LOQ for this method was found to be 12.12 µg/ml for Amitriptyline and 20.88µg/ml for Perphenazine.

Robustness

Chromatographic conditions variation: To demonstrate the robustness of the method, prepared solution as per test method and injected at different variable conditions like using different conditions like flow rate and wavelength. System suitability parameters were compared with that of method precision.

Acceptance criteria: The system suitability should pass as per the test method at variable conditions.

Table No. 13: Result of Robustness study

Parameter	Amitriptyline		Perphenazine	
	Retention time(min)	**Tailing factor**	**Retention time(min)**	**Tailing factor**
Flow Rate				
0.8 ml/min	3.033	1.784	6.830	1.583
1.2 ml/min	2.053	1.704	4.607	1.558
Wavelength				
245nm	2.450	1.742	5.507	1.571
249nm	2.447	1.710	5.500	1.540

Observation: From the observation it was found that the system suitability parameters were within limit at all variable conditions.

Ruggedness: The ruggedness of the method was studied by the determining the analyst to analyst variation by performing the Assay by two different analysts

Acceptance criteria: The % Relative standard deviation of Assay values between two analysts should be not more than 2.0%.

Table No. 14: Results for Ruggedness

Amitriptyline	**% Assay**	**Perphenazine**	**% Assay**
Analyst 01	100%	**Analyst 01**	100%
Analyst 02	99.54%	**Analyst 02**	97.93%

Observation

From the observation the between two analysts Assay values not greater than 2.0%, hence the method was rugged.

Stability related impurity studies:

Amitriptyline and Perphenazine stock solutions were subjected to acid and alkali hydrolysis, chemical oxidation and dry heat degradation. The degraded product peaks were well resolved from the pure drug peak with significant difference in their retention time values.

Table No. 15: Results of stability studies of Amitriptyline API

Stress condition	**Time**	**Assay of active substance**	**Assay of degraded products**	**Mass Balance (%)**
Acid Hydrolysis (0.1 M HCl)	24Hrs.	83.3	17.7	100
Basic Hydrolysis (0.I M NaOH)	24Hrs.	82.05	18.12	100.17
Thermal Degradation (50 ^{0}C)	24Hrs.	99.54	----------	99.54
UV (310 nm)	24Hrs.	99.73	----------	99.73
3 % Hydrogen peroxide	24Hrs.	65.12	34.16	99.28

Table No. 16: Results of stability studies of Perphenazine API

Stress condition	Time	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1 M HCl)	24Hrs.	65.02	35.12	100.14
Basic Hydrolysis (0.I M NaOH)	24Hrs.	84.32	15.17	99.49
Thermal Degradation (50 ^{0}C)	24Hrs.	98.75	----------	98.75
UV (310 nm)	24Hrs.	99.51	----------	99.51
3 % Hydrogen peroxide	24Hrs.	82.92	15.92	98.84

The results of the stress studies indicated the **specificity** of the method that has been developed. Amitriptyline and Perphenazine were almost stable in all stress conditions & areas reduced in acid, 3% H_2O_2 & basic stress conditions. We did not find any impurity peaks related to forced degradation or stability studies.

CONCLUSION

From the above experimental results and parameters it was concluded that, this newly developed method for the simultaneous estimation Amitryptaline and Perphenazine was found to be simple, precise, accurate and high resolution and shorter retention time makes this method more acceptable and cost effective and it can be effectively applied for routine analysis in research institutions, quality control department in meant in industries, approved testing laboratories studies in near future. The results obtained on the validation parameters met ICH and USP requirements.

Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

Even though no attempt has been made to identify the degraded products proposed method can be used as a stability indicating method for assay of Amitriptyline and Perphenazine commercial formulations.

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