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Development and validation of new analytical method for the simultaneous estimation of amitriptyline and perphenazine in bulk and pharmaceutical dosage form by RP-HPLC

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ABSTRACT

A new, simple, precise, accurate and reproducible RP-HPLC method for simultaneous estimation of Amitriptyline and Perphenazine in bulk and pharmaceutical formulations was developed. Separation of Amitriptyline and Perphenazine was successfully achieved on Inertsil ODS (250x4.6mm) 5μm column in an isocratic mode utilizing Methanol: ACN: Water (50:30:20) at a flow rate of 1.0 ml/min and eluents were monitored at 253nm with a retention time of 2.440 and 5.503 minutes for Amitriptyline and Perphenazine respectively. The method was validated and it was found to be linear. The values of the correlation coefficient were found to 0.992 for Amitriptyline and 0.9992 for Perphenazine respectively. The LOD for Perphenazine and Amitriptyline were found to be and 33.8μg/ml and 4.2 μg/ml. The LOQ for Perphenazine and Amitriptyline were found to be 20.88μg/ml and 12.12μg/ml respectively. The percentage recoveries for Amitriptyline and Perphenazine were found to be within the limit indicates that the proposed method is highly accurate. The method was extensively validated according to ICH guidelines.

Keywords: Amitriptyline, Perphenazine, RP-HPLC

INTRODUCTION

Analytical methods

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Often a time lag exists from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias[1]. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities (resulting in their withdrawal from the market), development of patient resistance and introduction of better drugs by competitors. Under these conditions, 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[2].

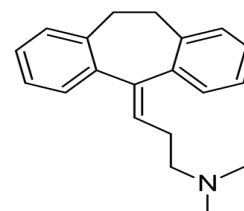

Figure 1: Amitriptyline

Analytical methods should be used within good manufacturing practice (GMP) and good laboratory practice (GLP) environments, and must be developed using the protocols set out in the International

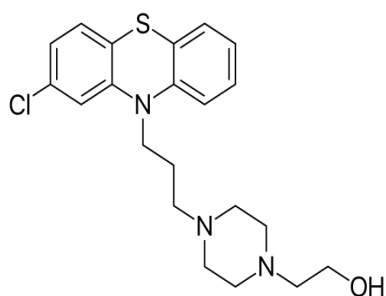Conference on Harmonization (ICH) guidelines (Q2A and Q2B). [3, 4]

Figure 2: Perphenazine

Method development is a continuous process that progresses in parallel with the evolution of the drug product. The goal and purpose of the method should reflect the phase of drug development. During early drug development, the methods may focus on API behaviour[5]. They should be suitable to support preclinical safety evaluations, pre-formulation studies, and prototype product stability studies. As drug development progresses, the analytical methods are refined and expanded, based on increased API and drug product knowledge. The methods should be robust and uncomplicated, while still meeting the appropriate regulatory guidelines. Scouting experiments are frequently performed during method development to establish the performance limits of the method, prior to formal validation experiments[6-9]. These may include forced degradation studies, which are an integral part of development of a stability-indicating method. API is typically subjected to degradation by acid, base, peroxide, heat, and light. This allows for a determination of the capability of the method to separate and quantify degradation products, while providing insight into the main mechanisms of degradation. Once a stability-indicating method is in place, the formulated drug product can then be subjected to heat and light in order to evaluate potential degradation of the API in the presence of formulation excipients[10]

Amitriptyline hydrochloride[11] is a dibenzocycloheptene-derivative tricyclic antidepressant (TCA). TCAs are structurally similar to phenothiazine. They contain a tricyclic ring system with an alkyl amine substituent on the central ring. In non-depressed individuals, amitriptyline does not affect mood or arousal, but may cause sedation. In depressed individuals, amitriptyline exerts a positive effect on mood. TCAs are potent inhibitors of serotonin and norepinephrine reuptake. Tertiary amine TCAs, such as amitriptyline are more potent inhibitors of serotonin reuptake than secondary amine TCAs, such as nortriptyline.

Perphenazine[12] is an antipsychotic phenothiazine derivative with actions and uses similar to those of chlorpromazine. This compound belongs to the class of organic compounds known as phenothiazines.

These are polycyclic aromatic compounds containing a phenothiazine moiety, which is a linear tricyclic system that consists of a two benzene rings joined by a para-thiazine ring. It acts by binding to the dopamine D1 and dopamine D2 receptors and inhibits their activity. The mechanism of the anti-emetic effect is due predominantly to blockage of the dopamine D2 neurotransmitter receptors in the chemoreceptor trigger zone and vomiting centre. Perphenazine also binds the alpha adrenergic receptor. This receptor's action is mediated by association with G proteins that activate a phosphatidylinositol-calcium second messenger system.

MATERIALS AND METHOD

Apparatus

The instrument used for the study was Shimadzu (LC20) HPLC, Separation module 2695, UV detector with Spin chrome software version 2.

Reagents and Materials

The solvents used were Methanol, Acetonitrile, Potassium dihydrogen ortho phosphate, Dipotassium hydrogen phosphate, Tri Ethyl Amine and HPLC Water.

Selection of detection wavelength

The sensitivity of method that uses UV- Vis detector depends upon the proper selection of wavelength. An ideal wavelength is that gives maximum absorbance and good response for both the drugs to be detected.

Standard solutions of Amitriptyline and Perphenazine were scanned in the UV range (200-400nm) and the spectrums obtained were overlaid and the overlain spectrum was recorded. From the overlain spectrum, 253 nm was selected as the detection wavelength for the present study.

Selection of mobile phase

Initially the mobile phase tried was Methanol and water, Methanol, Acetonitrile and water in various proportions. Finally, the mobile phase was optimized to Methanol: ACN: Water (50:30:20) v/v respectively.

Chromatographic trials for Simultaneous Estimation of Amitriptyline and Perphenazine by RP- HPLC.

Trial 1: Chromatographic conditions
Mobile phase : Methanol:ACN:Water
Column : Analytical(Hyperchrom) ODS
pH : 5.0
Ratio : 50:10:40
Column : Inertsil ODS 3V (250×4.6× 5µ)
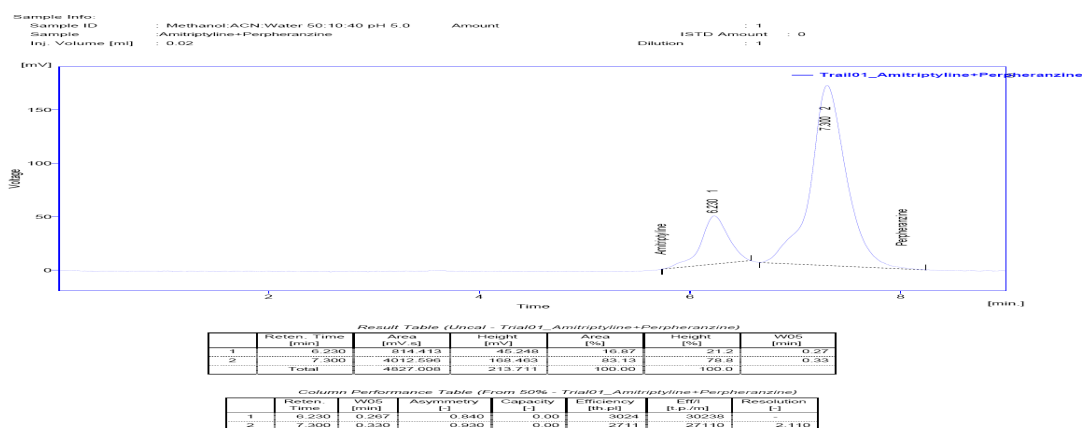Wavelength : 253 nm
Flow rate : 1ml/min

Sample Info:
Sample ID   : Methanol:ACN:Water 50:10:40 pH 6.0   Amount   : 1
Sample   :Amitriptyline+Perpheranzine   ISTD Amount   : 0
Inj. Volume [ml] : 0.02   Dilution   : 1

Result Table (Uncal - Trial01_Amitriptyline+Perpheranzine)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	6.230	814.413	45.248	16.87	21.2	0.27
2	7.300	4012.596	168.463	83.13	78.8	0.33
	Total	4827.008	213.711	100.00	100.0	

Column Performance Table (From 50% - Trial01_Amitriptyline+Perpheranzine)

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	6.230	0.267	0.840	0.00	3024	30238	
2	7.300	0.330	0.930	0.00	2711	27110	2.110

Figure 3: Chromatogram of Amitriptyline and Perphenazine by using mobile phase

Sample Info:
Sample ID   : Methonol+ACN+Phosphate buffer(50:30:20)PH4.5   Amount   : 1
Sample   :Amitriptyline+Perpheranzine   ISTD Amount   : 0
Inj. Volume [ml] : 0.02   Dilution   : 1

Result Table (Uncal - Trial02_Amitriptyline+Perpheranzine)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	3.233	2788.355	293.760	38.00	39.7	0.13
2	3.867	4550.231	446.637	62.00	60.3	0.15
	Total	7338.586	740.397	100.00	100.0	

Column Performance Table (From 50% - Trial02_Amitriptyline+Perpheranzine)

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	3.233	0.133	2.200	0.00	3258	32579	
2	3.867	0.147	2.448	0.00	3851	38505	2.662

Figure 4: Chromatogram of Amitriptyline and Perphenazine by using Mobile phase

Sample Info:
Sample ID   : Phosphate buffer+ACN+Methanol(30:30:40) pH4.0   Amount   : 1
Sample   :Amitriptyline+Perpheranzine   ISTD Amount   : 0
Inj. Volume [ml] : 0.02   Dilution   : 1

Result Table (Uncal - Trial03_Amitriptyline+Perpheranzine)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	4.307	1123.225	71.016	27.13	30.2	0.24
2	5.903	3016.940	164.089	72.87	69.8	0.28
	Total	4140.165	235.105	100.00	100.0	

Column Performance Table (From 50% - Trial03_Amitriptyline+Perpheranzine)

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	4.307	0.240	2.548	0.00	1784	17839	
2	5.903	0.277	2.208	0.00	2522	25223	3.637

Figure 5: Chromatogram of Amitriptyline and Perphenazine by using mobile phase

Preparation of mixed standard solution

Weigh accurately 10 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 μg/ml of Amitriptyline and Perphenazine is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Observation: The Efficiency was not satisfactory for Perphenazine and peak response of Amitriptyline was very less. Hence it was not taken for optimization.
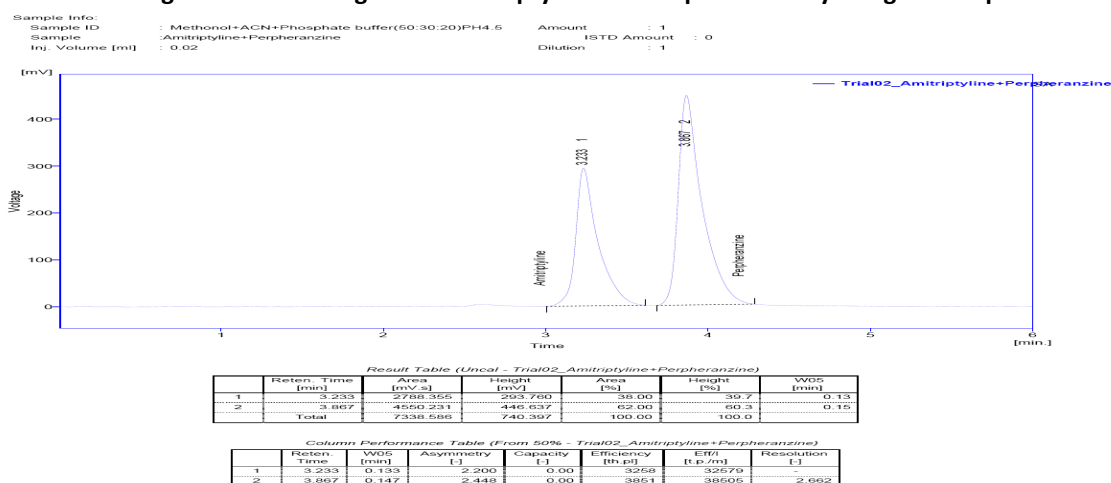
Figure 6: Chromatogram of Amitriptyline and Perphenazine by using mobile phase

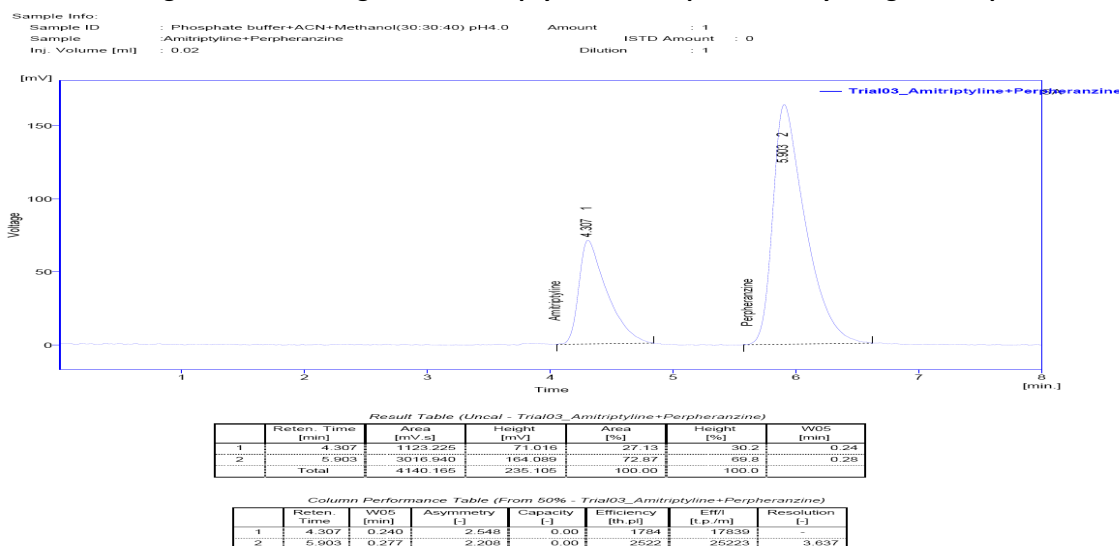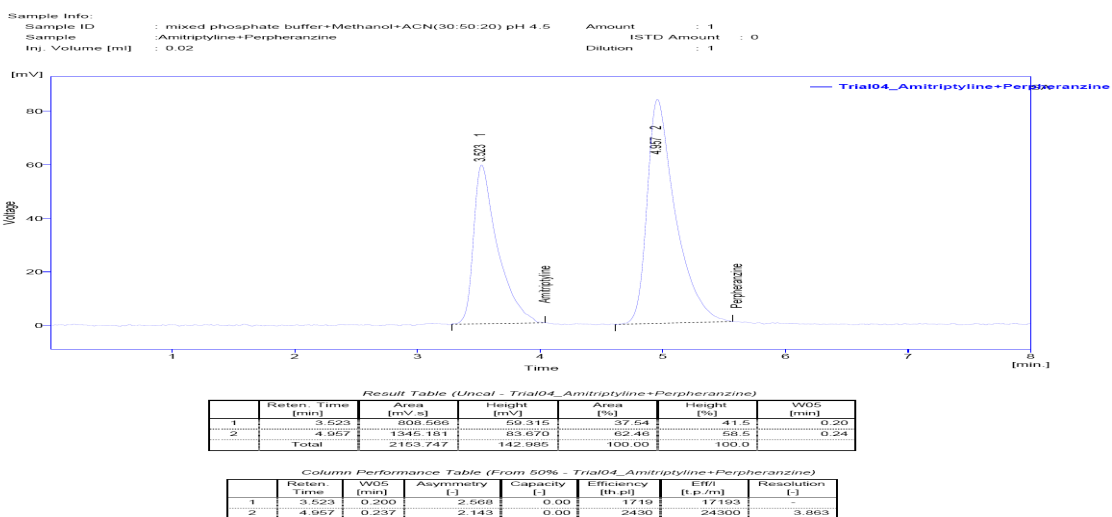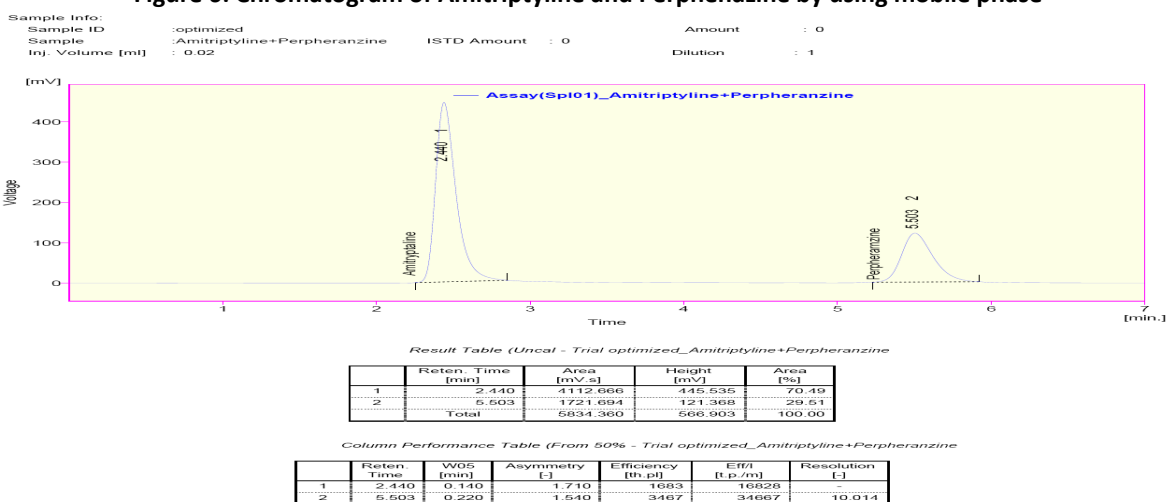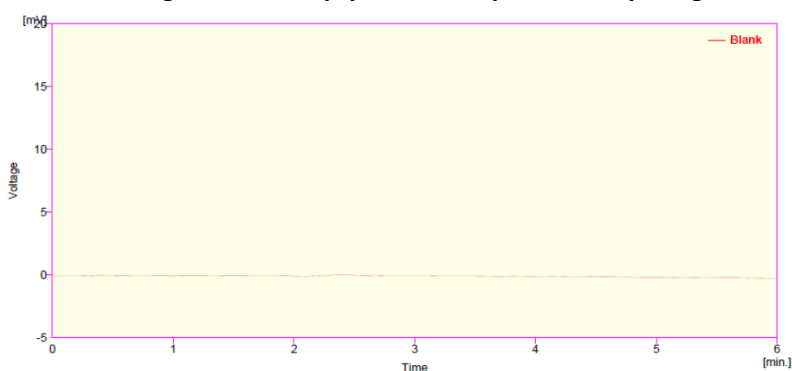**Figure 7: Chromatogram of Amitriptyline and Perphenazine by using mobile phase**

Figure 8: Chromatogram of Blank

Trial- 2: Chromatographic conditions

Mobile phase	: Methanol: ACN: Phosphate buffer
pH	: 4.5
Ratio	: 50:30:20
Column	: Inertsil ODS 3V (250×4.6 ×5µ)
Wavelength	: 253nm
Flow rate	: 1ml/min

Preparation of mixed standard solution

Weigh accurately 10 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Amitriptyline and Perphenazine is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.
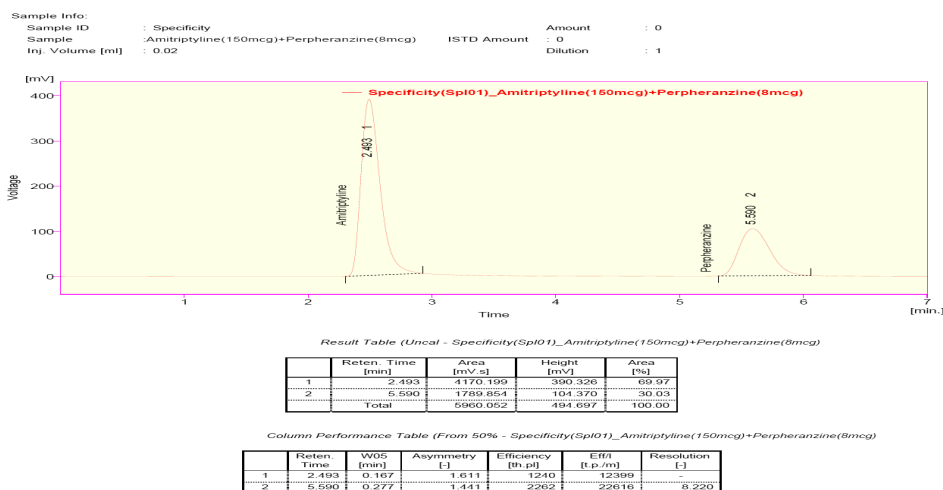
Sample Info:
Sample ID : Specificity   Amount : 0
Sample :Amitriptyline(150mcg)+Perpheranzine(8mcg)   ISTD Amount : 0
Inj. Volume [ml] : 0.02   Dilution : 1

Result Table (Uncal - Specificity(Spl01)_Amitriptyline(150mcg)+Perpheranzine(8mcg))

	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

Column Performance Table (From 50% - Specificity(Spl01)_Amitriptyline(150mcg)+Perpheranzine(8mcg))

	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

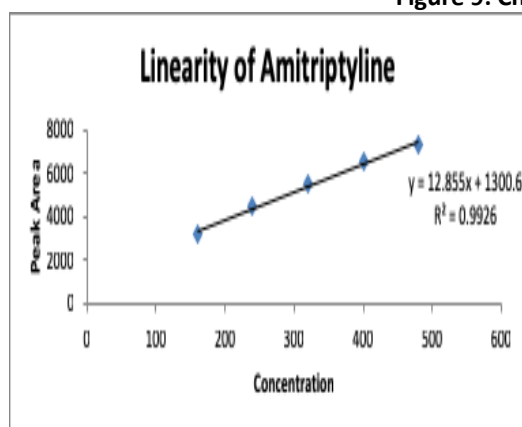**Figure 9: Chromatogram of Sample**

Figure 10: Calibration graph of Amitriptyline
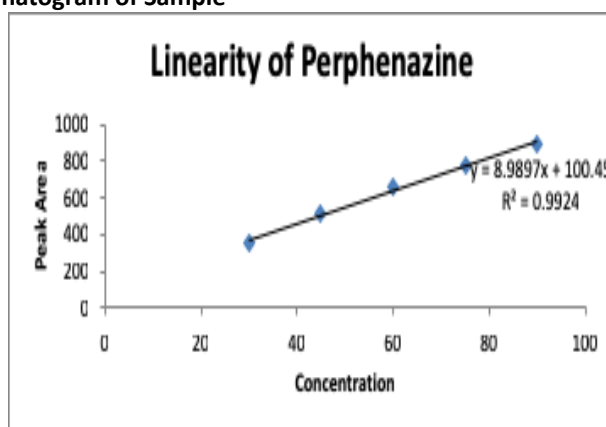
Figure 11: Calibration graph of Perphenazine

Table 1: Calibration data of Perphenazine

S.No.	Conc (µg/ml)	Area
1	30	347.912
2	45	520.885
3	60	657.488
4	75	780.529
5	90	892.314

Table 2: Calibration data of Amitriptyline

S.No.	Conc (µg/ml)	Area
1	160	3236.788
2	240	4409.861
3	320	5560.106
4	400	6560.326
5	480	7303.508

Observation: Efficiency of both the drugs was good. The run time is very more. The peaks of Amitriptyline and Perphenazine showed tailing. Hence it was not taken for optimization.

Trial- 3: Chromatographic conditions
Mobile phase   :Phosphate buffer : ACN : Methanol
pH   : 4.0
Ratio   : 30:30:40
Column   : Inertsil ODS 3V, (250×4.6× 5µ)
Wavelength   : 253nm
Flow rate   : 1ml/min

Preparation of mixed standard solution

Weigh accurately 10 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 µg/ml of Amitriptyline and Perphenazine is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Observation: Asymmetry factor for Perphenazine does not meet the system suitability requirements.

Table 3: Showing accuracy results for Accuracy amitriptyline

Recovery level	Accuracy amitriptyline					Average % Recovery
	Amount taken (µg/ml)	Area	Average area	Amount recovered (mcg/ml)	%Re-covery	
80%	240	4662.113	4671.027	254.21	99.30	
	240	4682.749				
	240	4668.220				
100%	320	5595.271	5589.477	321.69	100.53	
	320	5588.966				100.023
	320	5584.193				
120%	400	6296.468	6313.257	384.94	100.24	
	400	6324.060				
	400	6319.242				

Table 4: Showing accuracy results for Perphenazine

Recovery level	Accuracy perphenazine					Average % Recovery
	Amount taken (µg/ml)	Area	Average area	Amount recovered (mcg/ml)	% Re-covery	
80%	45	558.057	560.812	48.45	100.94	
	45	563.349				
	45	561.030				
100%	60	657.465	657.722	60.02	100.04	
	60	659.972				100.61
	60	655.729				
120%	75	751.964	755.928	72.64	100.88	
	75	755.857				
	75	759.963				

Table 5: 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
	1.0ml/min	2.447	1.710	5.513	1.540
	1.2 ml/min	2.053	1.704	4.607	1.558
Wave-length	240nm	2.450	1.742	5.507	1.571
	258nm	2.443	1.656	5.510	1.600
	253nm	2.447	1.710	5.500	1.540

Table 6: Results for method precision of amitriptyline and perphenazine

S.No.	Amitriptyline		Perpheranzine	
	Rt	Area	Rt	Area
1	2.487	4181.754	5.567	1759.963
2	2.450	4143.434	5.533	1782.254
3	2.477	4162.886	5.560	1776.727
4	2.493	4199.596	5.590	1773.634
5	2.493	4161.196	5.603	1762.717
6	2.460	4190.188	5.570	1756.825
Avg	2.476667	4173.176	5.5705	1768.687
Stdev	0.018052	20.95232	0.024354	10.25314
% RSD	0.728874	0.502071	0.43719	0.579703

Table 7: Results for Ruggedness

Amitriptyline	%Assay	Perpheranzine	%Assay
Analyst 01	100%	Analyst 01	100%
Anaylst 02	99.54%	Anaylst 02	99.93%

Table 8: LOD

Drug name	LOD(μg)
Amitriptyline	33.8
Perphenazine	4.2

Table 9: LOQ

Drug name	LOQ(μg)
Amitriptyline	20.88
Perphenazine	12.12

The run time is 8 minutes and hence it was not taken for optimization.

Trial- 4: Chromatographic conditions

Mobile phase: Mixed phosphate buffer: Methanol : ACN
pH   : 4.5
Ratio   : 30:50:20
Column   : Inertsil ODS (250×4.6× 5μ)
Wavelength   : 253 nm
Flow rate   : 1ml/min

Preparation of mixed standard solution

Weigh accurately 10 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 μg/ml of Amitriptyline and Perphenazine is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Observation: Peak Asymmetry factor for Amitriptyline and Perphenazine does not meet the system suitability requirements. The run time is very more hence it was not taken for optimization.

Trial- 5: Chromatographic conditions (Optimized Method)

Mobile phase   : METHANOL: ACN: WATER
 Ratio   : 50:30:20
Column   : Inertsil ODS (250×4.6× 5μ)
Wavelength   : 253 nm
Flow rate   : 1ml/min

Preparation of mixed standard solution

Weigh accurately 10 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 μg/ml of Amitriptyline and Perphenazine is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

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 for both Amitriptyline and Perphenazine Resolution between two peaks >1.5. Hence this method was for optimized.

Procedure

Preparation of mixed standard solution

weigh accurately 10 mg of Amitriptyline and Perphenazine in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. From above stock solution 10 μg/ml of Amitriptyline and Perphenazine is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Tablet sample

10 tablets (each tablet contains Amitriptyline 150 mg and Perphenazine 8 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 10 μg/ml of Amitriptyline and Perphenazine was made by adding 1 ml of stock solution to 10 ml of mobile phase.

Assay

Preparation of samples for Assay: Standard solution

Weigh accurately 8 mg of Amitriptyline and 1.5 mg of Perphenazine in 25 ml of volumetric flask and

dissolve in 10ml of mobile phase and make up the volume with mobile phase. This solution contains 320 μg/ml of Amitriptyline and 60 μg/ml of Perphenazine. This solution is used for recording chromatogram.

Tablet sample

10 tablets (each tablet contains Amitriptyline 150 mg and Perphenazine 8 mg) were weighed and taken into a mortar and crushed to fine powder and uniformly mixed. Tablet stock solution of Amitriptyline and Perphenazine (μg/ml) was prepared by dissolving weight equivalent to 800 mg of Amitriptyline and 150mg of Perphenazine dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 25 ml with mobile phase. This solution contains 320 μg/ml of Amitriptyline and 60 μg/ml of Perphenazine. This solution is used for recording chromatogram.

Calculation

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

Where,

AS: Average peak area due to standard preparation
AT: Peak area due to assay preparation
WS: Weight of Amitriptyline/Perphenazine in mg
WT: Weight of sample in assay preparation
DT: Dilution of assay preparation

RESULTS AND DISCUSSION

Method Validation Parameters

Specificity

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. Weigh accurately 16mg of Amitriptyline and 3 mg of Perphenazine in 50 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase. This solution contains 160-480 μg/ml of Amitriptyline and 30-90 μg/ml of Perphenazine Acceptance criteria: Correlation coefficient should be not less than 0.999.

Range

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 160-480 ppm and 30-90 ppm for Amitriptyline and Perphenazine respectively

Accuracy

Accuracy of the method was determined by recovery experiments. There are mainly 2types of recovery studies are there.

Standard addition method: To the formulation, the reference standard of the respective drug of known concentration was added, analyzed by HPLC and compared with the standard drug concentration.

Percentage method: For these assay method samples are prepared in three concentrations of 80%, 100%, and 120% respectively.

Acceptance criteria: The mean % recovery of the Amitriptyline and Perphenazine at each level should be not less than 95.0% and not more than 105.0%.

Assay procedure

10mL of the standard and sample solutions of Amitriptyline and Perphenazine were injected into the HPLC system and the chromatograms were recorded. Amount of drug present in the Tablets were calculated using the peak areas.

Precision

Method precision also called as repeatability/Intra-day precision indicates whether a method gives consistent results for a single batch. Method precision was demonstrated by preparing six test solutions at 100% concentration as per the test procedure & recording the chromatograms of six test solutions.

The % RSD of peak areas of six samples was calculated. The method precision was performed on Amitriptyline and Perphenazine formulation.

Acceptance criteria

The % RSD for the area of sample injections results should not be more than 2.

Selection of solvent

Solutions of Amitriptyline and Perphenazine were prepared by dissolving in mobile phase and UV spectrum of each was recorded by scanning between 200-400 nm.

VALIDATION OF THE METHOD

LINEARITY

Amitriptyline and Perphenazine: Serial dilutions of Amitriptyline and Perphenazine (160-480 ppm and

30-90 ppm) were injected into the column and detected at a wavelength set at 253 nm. The calibration curve was obtained by plotting the concentration vs. peak area and the correlation coefficient was found to be 0.992 and 0.992 respectively.

SUMMARY AND CONCLUSION

A new method was established for simultaneous estimation of Amitriptyline and Perphenazine by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Amitriptyline and Perphenazine by using C18 column (4.6×250mm) 5µ, flow rate was 1ml/min, mobile phase ratio was (50:30:20 v/v) Methanol: ACN: Water, detection wavelength was 253 nm. Precision and recovery studies were also found to be with the range. The proposed HPLC method was found to be simple, specific, precise, accurate, rapid and economical for simultaneous estimation of Amitriptyline and Perphenazine in pharmaceutical dosage form. The developed method was validated in terms of accuracy, precision, linearity, robustness and ruggedness, and results will be validated statistically according to ICH guidelines. The Sample recoveries in all formulations were in good agreement with their respective label claims.

Hence the suggested RP-HPLC method can be used for routine analysis of Amitriptyline and Perphenazine in API and Pharmaceutical dosage form

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