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CHIRAL LIQUID CHROMATOGRAPHIC METHOD DEVELOPMENT AND VALIDATION FOR SEPERATION OF PHENIRAMINE ENANTIOMERS

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Abstract:

Pheniramine is an antihistamine with anticholinergic properties used to treat allergic conditions such as hay fever or urticaria. It has relatively strong sedative effects, and may sometimes be used off-label as an over-the-counter sleeping pill in a similar manner to other sedating antihistamines such as diphenhydramine. Pheniramine is also commonly found in eyedrops used for the treatment of allergic conjunctivitis. According to literature survey there is no method reported for the seperation of Pheniramine enantiomers by RP-HPLC in pharmaceutical dosage forms. This present research work mainly focus to develop and validate a new RP-HPLC method for separation of Pheniramine enantiomers in pharmaceutical dosage forms in accordance with the ICH guidelines. A new simple, precise and accurate HPLC method was developed and validated for the seperation of Pheniramine 1 and Pheniramine 2 in pharmaceutical dosage form. In this method, Chiral pack column (150x4.6, 5µm) was selected as the stationary phase. Water and acetonitrile were taken in the ratio 90:10%v/v and used as mobile phase at a flow rate of 1.0 ml/min. The retention times of Pheniramine 1 and Pheniramine 2 were found to be 6.6 min & 9.1 min respectively. Hence, the developed method can be successfully employed for enantiomeric separation of Pheniramine maleate in drug testing laboratories and pharmaceutical industries.

Key words: *Pheiramine maleate, Chiral column, RP-HPLC, Acetonitrile, Enantiomers*

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INTRODUCTION:

Pheniramine (INN, trade name Avil, among others) is an antihistamine with anticholinergic properties used to treat allergic conditions such as hay fever or urticaria. It has relatively strong sedative effects, and may sometimes be used off-label as an over-the-counter sleeping pill in a similar manner to other sedating antihistamines such as diphenhydramine. Pheniramine is also commonly found in eyedrops used for the treatment of allergic conjunctivitis.

Pheniramine is generally sold in combination with other medications, rather than as a stand-alone drug, although some formulations are available containing Pheniramine by itself.

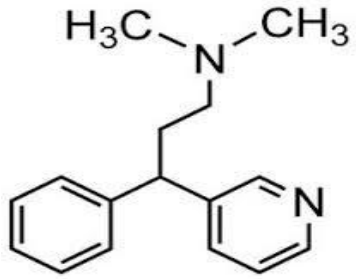

Fig 1: Chemical Structure of Pheniramine

The product of the above drug is being marketed under the brand name of Avil in India.Since there were no methods available for the separation of the Pheneramine enantiomers in the product when we started our work. We attempted the same and successfully developed and validated a RP-HPLC method for this purpose.The work done on this method is incorporated in this chapter. The analytical methods reported so far are reviewed in the following literature survey.

Literature Survey

Sanchaniya et al [1] developed a reversed phase high performance liquid chromatographic method for the quantitative determination of Chlorpheniramine maleate, Ibuprofen and Phenylephrine hydrochloride in combined pharmaceutical dosage forms. Taomin Huang et al [2] reported a stability indicating reverse phase liquid chromatographic method for the simultaneous determination of Pheniramine maleate and Naphazoline hydrochloride in bulk drugs and pharmaceutical formulations. Raghu et al[3] developed simple titrimetric methods for the determination of Pheniramine maleate (PAM) in pure form and in its dosage forms. Raghu et al [4] developed two spectrophotometric methods for the determination of Pheniramine maleate (PAM) in pure and dosage forms. Raghu et al [5] developed three extraction free spectrophotometric methods for the quantitation of Pheniramine maleate (PAM), an anti-allergic drug, in pure form and in its formulations. Wadher et al [6] reported spectrophotometric method for the estimation of Chlorpheniramine maleate (CPM) and Phenylephrine hydrochloride (PE) in bulk and combined capsule dosage forms. Taomin et al [7] developed a stability-indicating reverse phase liquid chromatographic method and validated for the simultaneous determination of Pheniramine maleate and Naphazoline hydrochloride in bulk drugs and pharmaceutical formulations. Redasani et al [8]developed a reversed-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous determination of quaternary mixture consisting of Chlorpheniramine maleate (CPM), Phenylephrine hydrochloride (PE), Paracetamol (PCM) and Caffeine in pharmaceutical preparation.

Aim and Objective

Various UV and HPLC methods were reported in the literature for the estimation of Pheniramine maleate in pharmaceutical dosage forms. According to literature survey there is no method reported for the seperation of Pheniramine enantiomers by RP-HPLC in pharmaceutical dosage forms. So we planned to develop and validate a new RP-HPLC method for separation of Pheniramine enantiomers in pharmaceutical dosage forms in accordance with the ICH guidelines.

The main aim and objective of the present study is

- To develop a new reverse phase high performance liquid chromatographic method for the seperation of Pheniramine enantiomers in pharmaceutical dosage form.
- To validate the developed method for the following parameters
  - System suitability
  - Specificity
  - Linearity
  - Accuracy
  - Precision
  - Limit of detection
  - Limit of quantification
  - Robustness
  - Solution stability
- To perform the assay of commercial product.

EXPERIMENTAL PROCEDURE:

Instrumentation: Chromatography was performed with Alliance Waters 2695 HPLC provided with high speed auto sampler, column oven, degasser and & 2996 PDA detector to provide a compact and with class Empower-2 software. Reagents and chemicals: The reference samples of Pheniramine was provided as gift samples from Spectrum pharma research solutions, Hyderabad. HPLC grade acetonitrile, HPLC grade methanol and all other chemicals were obtained

from Merck chemical division, Mumbai. HPLC grade water obtained from Milli-Q water purification system was used throughout the study. Commercial formulations; AVIL (Lable Claim: Pheniramine maleate 25 mg) were purchased from the local pharmacy. Preparation of Standard Stock Solution: Standard stock solutions were prepared by dissolving 25mg of Pheniramine into a clean and dry 25ml volumetric flasks, to that 20ml of diluent was added, sonicated for 5 minutes and volume was made up to 25 ml with diluent to get stock solution with a concentration of 1mg/ml of Pheniramine. Preparation of diluent solution: Water and acetonitrile in the ratio of 50:50%v/v. Preparation of Working Standard Solutions: Aliquot of 0.25, 0.5, 0.75, 1, 1.25 & 1.5 ml were pipette out from stock solution into 10 ml volumetric flask and volume was made up to 10 ml with diluent. This gives the solutions of 25, 50, 75, 100, 125 and 150µg/ml for Phenylephrine. Sample preparation: 20 tablets were weighed and calculated the average weight of each tablet. Then the weight equalent to one tablet powder was transferred into a 25ml volumetric flask, 20ml of diluent added and sonicated for 30 min, further the volume made up with diluent and filtered. From the filtered stock solution, 1ml was pipetted out into a 10 ml volumetric flask and made up to 10ml with diluent gives 100µg/ml solution. Chromatographic condition: The chromatographic separation was carried out under isocratic conditions. Chromatographic separation was achieved by injecting a volume of 10µl of standard into Chiral pack (150x4.6mm, 5µm) column. The mobile phase of composition 900 ml of water and 100ml of acetonitrile were allowed to flow through the column at a flow rate of 1.0 ml/min for a period of 12min at 30^0C column temperature. Detection of the component was carried out at a wavelength of 210 nm. The retention time of the components were found to be 6.5min and 9.0min for Pheniramine1 & Pheniramine 2 respectively.

Method Validation:
System Suitability Tests: Data from six injections of 10µl of the working standard solutions of Pheniramine (100µg/ml) was used for the evaluation of the system suitability parameters-like tailing factor, the number of theoretical plates, retention time and resolution factor.

Specificity:
The specificity of the method was performed by injecting blank solution, placebo solution and standard solutions of Pheniramine separately.

Linearity:
By taking appropriate aliquots of the standard Pheniramine solutions with the mobile phase, six working solutions ranging between 25-150 µg/ml were prepared. Each experiment linearity point was performed in triplicate according to optimized chromatographic conditions. The peak areas of the chromatograms were plotted against the concentration of Pheniramine 1 and Pheniramine 2 to obtain the calibration curve.

Accuracy:
Previously analyzed samples of Pheniramine (100µg/ml) to which known amounts of standard Pheniramine corresponding to 50%, 100% and 150% of target concentration were added. The accuracy was expressed as the percentage of analyte recovered by the proposed method.

Precision:
The repeatability and intermediate precision were determined by analyzing the samples of Pheniramine (100µg/ml).

Limit of detection and the limit of quantification:
Limit of detection (LOD) and limit of quantification (LOQ) of Pheniramine 1 and Pheniramine 2 were determined by calibration curve method. Solution of Pheniramine was prepared in linearity range and injected in triplicate. Average peak area of three analyses was plotted against concentration. LOD and LOQ were calculated by using following equations.
LOD = (3.3 ×Syx)/b, LOQ= (10.0×Syx)/b
Where Syx is residual variance due to regression; b is slope.

Robustness:
The robustness of the method was performed by deliberately changing the chromatographic conditions. The parameters included slight variation in mobile phase organic solution composition (5 and 15), flow rate (0.9, 1.1 ml/min) and column temperature (25, 35°C).

Stability:
The sample solutions were injected at 0 hr (comparison sample) and after 24 hr (stability sample) by keeping at ambient room temperature. Stability was determined by determining %RSD for sample and standard solutions.

RESULTS AND DISCUSSION:

Method Development:
Initially reverse phase liquid chromatography separation was attempted by using various ratios of methanol and water, acetonitrile and water as mobile phases, in which both the enantiomers did not responded properly, and the resolution was also poor.

Further systematic trials were performed to optimize the mobile phase and the organic content of mobile phase was also investigated further to optimize the separation of both enantiomers. Thereafter, water: acetonitrile were taken in ratio of 90:10%v/v and a flow rate of 1.0 ml/min was employed. Chiral Pack (150x4.6mm, 5µm) was selected as the stationary phase to improve resolution and the tailing of both peaks were reduced considerably and brought close to 1. To analyze Pheniramine detection was tried at various wavelengths from 205nm to 280nm. Pheniramine showed maximum absorption at 210nm of wavelength and the same was selected as the detection wavelength for PDA detector. The retention times were found to about 6.6min and 9.1 min for Pheniramine 1 and Pheniramine 2 respectively. The chromatograms obtained for blank injection, placebo injection and optimized method were shown in the Fig.2, 3 and 4 respectively and optimized chromatographic conditions were shown in Table 1.

Table 1: Optimized chromatographic conditions

S. No.	Parameter	Condition
1	Mobile phase	Water: Acetonitrile 90:10%v/v
2	Diluent	Water: Acetonitrile 50:50%v/v
3	Column, make	Chiral Pack (150x4.6mm, 5µm)
4	Column temperature	30^0C
5	Wave length	210nm
6	Injection volume	10µl
7	Flow rate	1.0ml/min
8	Run time	13min
9	Retention time (Pheniramine 1)	6.6 min
10	Retention time (Pheniramine 2)	9.1 min

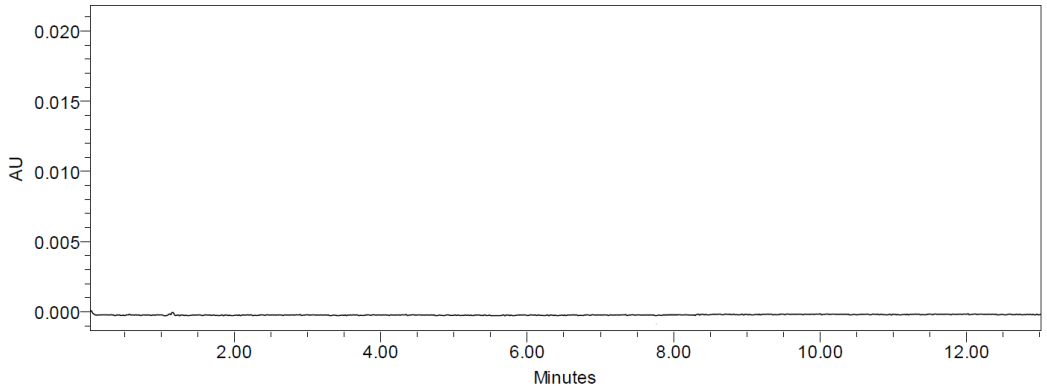

Fig 2: Chromatogram of Blank

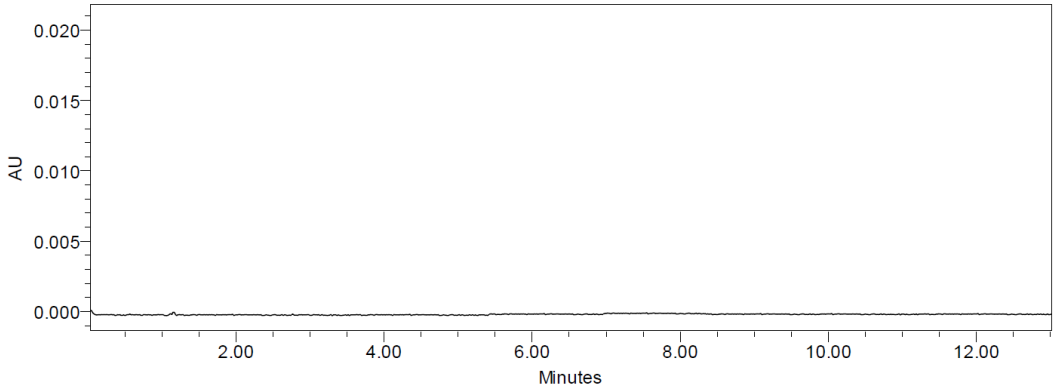

Fig 3: Chromatogram of Placebo

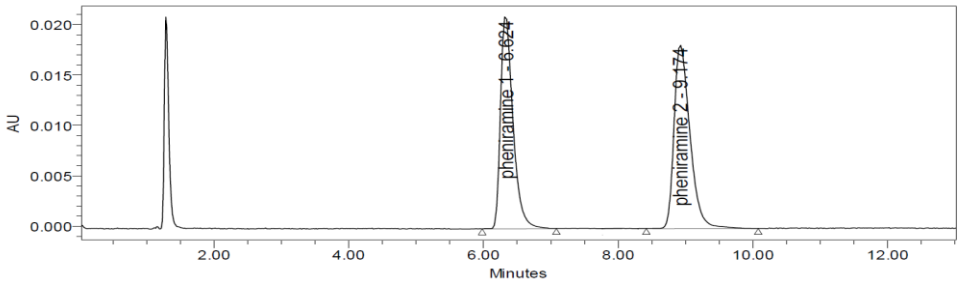

Fig 4: Chromatogram of Pheniramine 1 and Pheniramine 2 Standards

Method Validation:

System Suitability: System suitability parameters such as number of theoretical plates, peak tailing, retention time and resolution factor were determined. The total run time required for the method is only 13 minutes for eluting both Pheniramine 1 and Pheniramine 2. The results obtained were shown in Table 10.2. The number of theoretical plates was found to be > 2000, USP tailing was < 2 and USP resolution is above 2. The % RSD of areas for Pheniramine 1 and Pheniramine 2 were 1.3 and 0.9 respectively.

Specificity:

The specificity of the method was performed by injecting blank solution, placebo solution and standard solutions separately. The chromatogram of the drug was compared with blank and placebo chromatogram to verify the interference. No interefering peak was observed at the retention time of Pheniramine 1 and Pheniramine 2. Hence, the method is specific for the determination of Pheniramine 1 and Pheniramine 2.

Linearity:

Pheniramine 1 and Pheniramine 2 showed a linearity of response between 25-150 µg/ml. These were represented by a linear regression equation as follows: y(Pheniramine 1) = 4266.3x + 1690.1 (r^2=0.9998), y(Pheniramine 2) = 4004.5x + 2462.6 (r^2=0.9999) and regression line was established by least squares method and correlation coefficient (r^2) for Pheniramine 1 and Pheniramine 2 is found to be greater than 0.98. Hence, the curves established were linear. The results were shown in the table 3 and fig. 5-12.

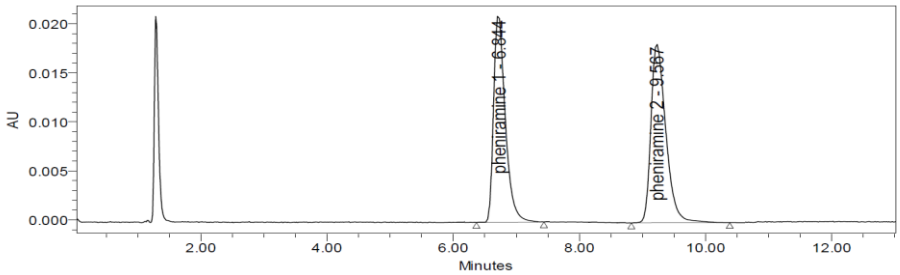

Fig. 5: Linearity 25% chromatogram of Pheniramine 1 and Pheniramine 2

Table 2: System Suitability of Pheniramine 1 and Pheniramine 2

	Pheniramine 1			Pheniramine 2		
S.No	**Area**	**USP Plate Count**	**USP Tailing**	**Area**	**USP Plate Count**	**USP Tailing**
1	426248	2793	1.35	408382	2069	1.56
2	420647	2786	1.39	411120	2082	1.63
3	413105	2855	1.36	407458	2122	1.46
4	418687	2800	1.45	408421	2109	1.71
5	426709	2762	1.40	404458	2098	1.69
6	426214	2762	1.36	401256	2145	1.65
Mean	421935			406849		
Std. Dev.	5475.04			3476.55		
% RSD	1.3			0.9		

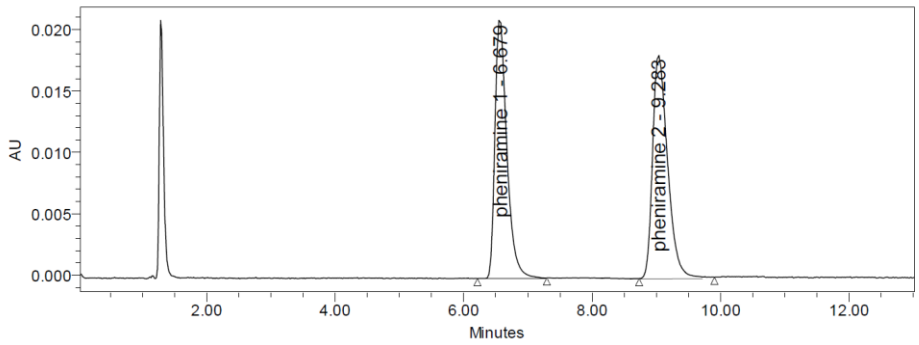

Fig.6: Linearity 50% chromatogram of Pheniramine 1 and Pheniramine 2

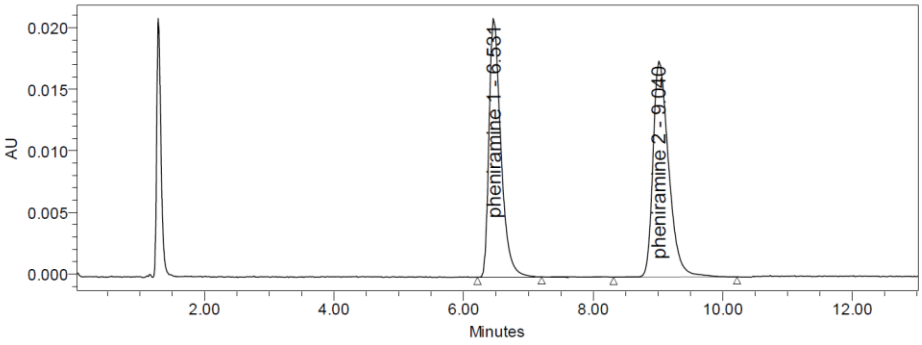

Fig.7: Linearity 75% chromatogram of Pheniramine 1 and Pheniramine 2

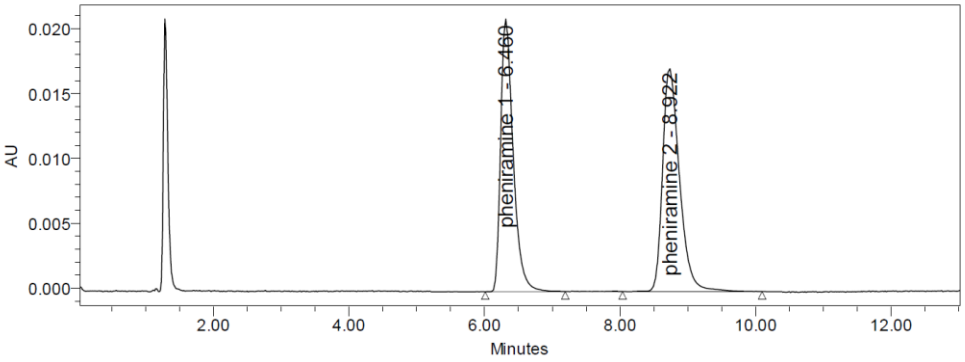

Fig.8: Linearity 100% chromatogram of Pheniramine 1 and Pheniramine 2

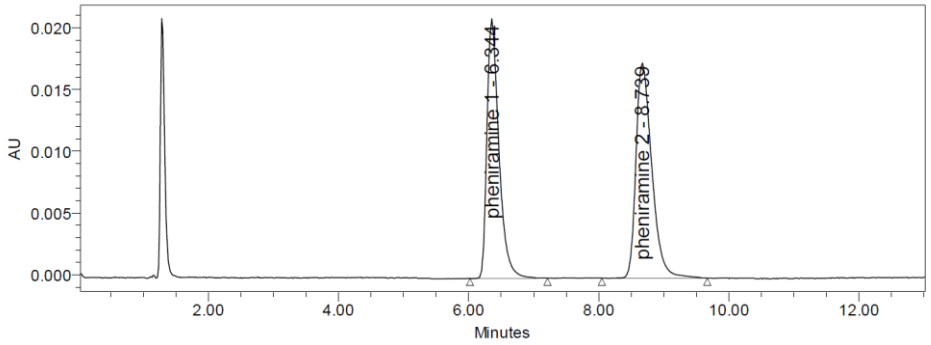

Fig.9: Linearity 125% chromatogram of Pheniramine 1 and Pheniramine 2

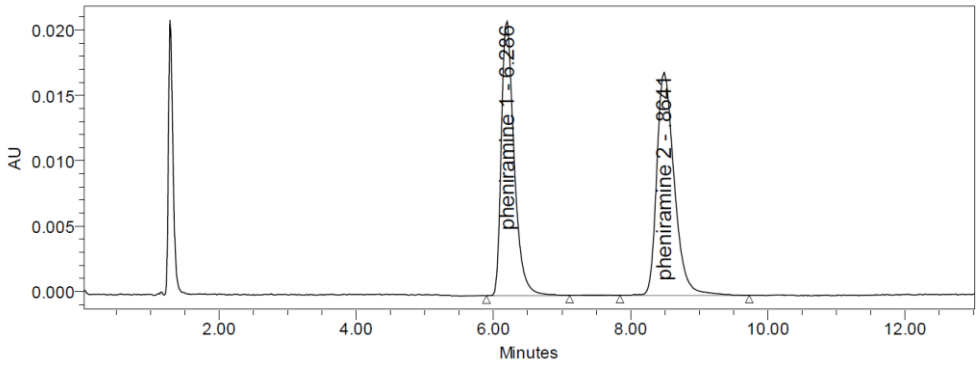

Fig.10: Linearity 150% chromatogram of Pheniramine 1 and Pheniramine 2

Table 3: Linearity data of Pheniramine 1 and Pheniramine 2

Pheniramine 1		Pheniramine 2	
Conc. (µg/ml)	**Peak area Average(n=3)**	**Conc. (µg/ml)**	**Peak area Average(n=3)**
25	107186	25	104674
50	219126	50	200686
75	322772	75	305096
100	424566	100	405693
125	538631	125	502883
150	639362	150	600580

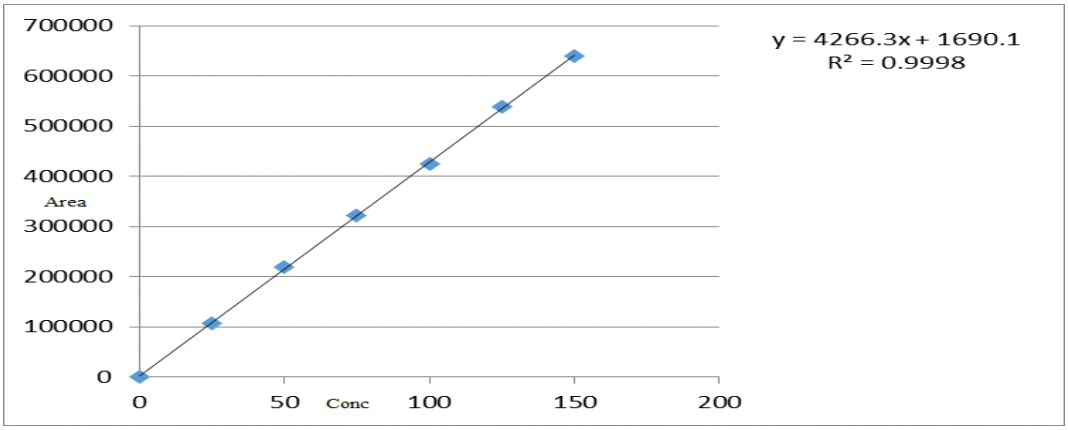

Fig.11: Calibration Curve for Pheniramine 1

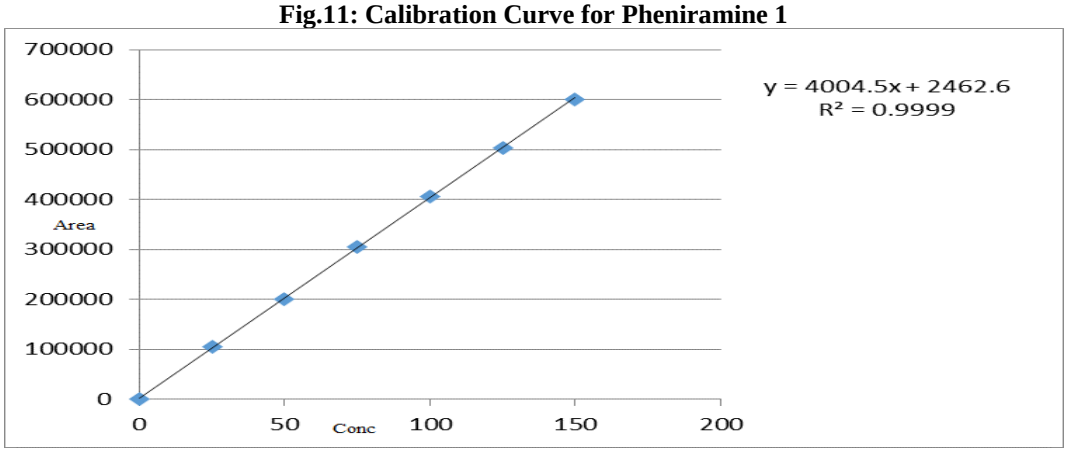

Fig.12: Calibration Curve for Pheniramine 2

Accuracy:

To the pre analyzed sample solution, a definite concentration of standard drug (50%, 100% & 150 % level) was added and recovery was studied. The % mean recovery for Pheniramine 1 and Pheniramine 2 are 99.38% and 99.35%, respectively and these results are within acceptable limit of 98-102. The % RSD for Pheniramine 1 and Pheniramine 2 are 0.9 and 1.2 respectively and %RSD for Pheniramine 1 and Pheniramine 2 is within limit of ≤2. Hence, the proposed method is accurate and the results are summarized in Table-4 and Figure 13-15.

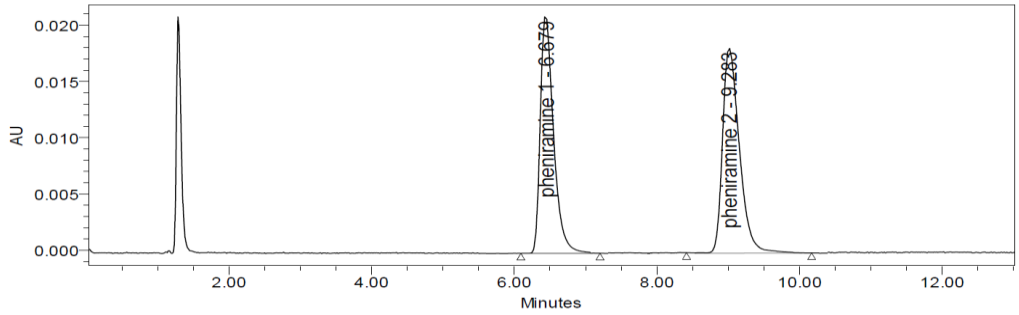

Fig. 13: Accuracy 50% chromatogram of Pheniramine 1 and Pheniramine 2

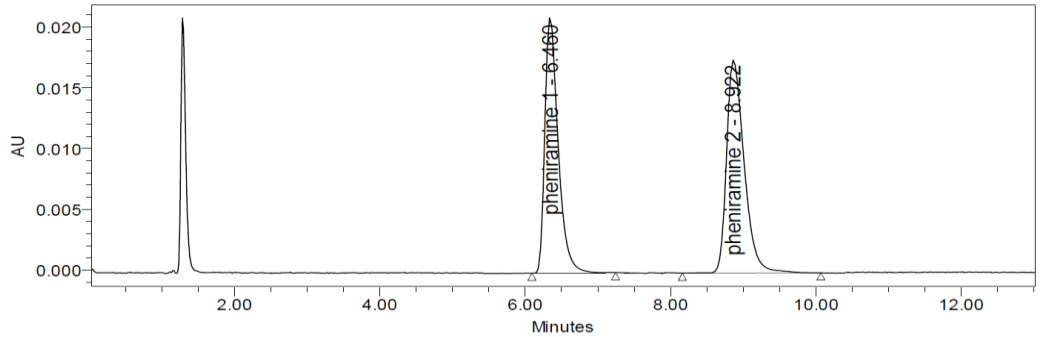

Fig. 14: Accuracy 100% chromatogram of Pheniramine 1 and Pheniramine 2

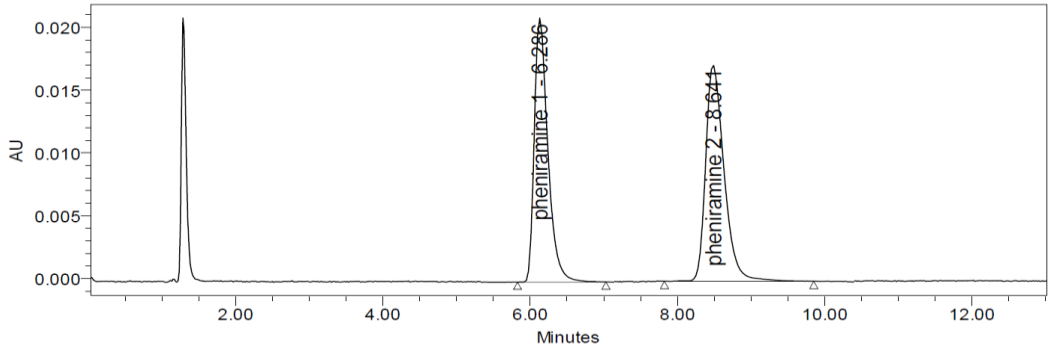

Fig.15: Accuracy 150% chromatogram of Pheniramine 1 and Pheniramine 2

Table 4: Results of Recovery Experiments of Pheniramine 1 and Pheniramine 2

Preanalysed amount (µg/ml)	Spiked Amount (µg/ml)	% Recovered	
		Pheniramine 1	Pheniramine 2
100	50	99.61	98.15
100	100	98.36	99.37
100	150	100.17	100.54
	MEAN	99.38	99.35
	SD	0.93	1.20
	%RSD	0.9	1.2

Precision:
The repeatability and intermediate precision data were summarized in Table 5 and 6, respectively and were assessed by the use of standard solutions of Pheniramine 1 and Pheniramine 2.

Repeatability:
Six replicates injections in same concentration of Pheniramine 1 and Pheniramine 2 were analyzed in the same day for repeatability and the % RSD for Pheniramine 1 and Pheniramine 2 found to be 0.6 and 0.6 respectively and % RSD for Pheniramine 1 and Pheniramine 2 found to be within acceptable limit of ≤2 and hence, method is reproducible.The results were shown in the Table 5.
Intermediate Precision: Six replicates injections in same concentration were analyzed on two different days with different analyst and column for verifying the variation in the precision and the % RSD for Pheniramine 1 and Pheniramine 2 is found to be 1.3 and 0.5 respectively and it is within acceptable limit of ≤2. Hence, the method is reproducible on different days with different analyst and column. This indicates that the method is precise. The results were shown in the Table 6.

Table 5: Results of Repeatability of Pheniramine 1 and Pheniramine 2

	Pheniramine 1			Pheniramine 2		
S.NO	**Area**	**USP Plate Count**	**USP Tailing**	**Area**	**USP Plate Count**	**USP Tailing**
1	422543	2614	1.31	402744	2178	1.64
2	420693	2745	1.29	408699	2125	1.68
3	420141	2698	1.24	406813	2098	1.71
4	426489	2678	1.32	402899	2134	1.65
5	424463	2714	1.24	403735	2015	1.68
6	425644	2745	1.29	406628	2112	1.69
Mean	423329			405253		
Std. Dev.	2621.47			2463.33		
% RSD	0.6			0.6		

Table 6: Results of Precision of Pheniramine 1 and Pheniramine 2

	Pheniramine 1			Pheniramine 2		
S.No	**Area**	**USP Plate Count**	**USP Tailing**	**Area**	**USP Plate Count**	**USP Tailing**
1	426248	2672	1.20	408382	2210	1.75
2	420647	2548	1.24	411120	2109	1.69
3	413105	2547	1.19	407458	2216	1.85
4	418687	2619	1.22	408421	2152	1.65
5	426709	2524	1.26	404458	2168	1.74
6	425112	2608	1.22	406451	2185	1.68
Mean	421751			407715		
Std. Dev.	5319.05			2227.52		
% RSD	1.3			0.5		

Robustness:

To evaluate the robustness of the developed HPLC method, few chromatographic conditions were deliberately altered.The robustness was established by changing the flow rate, column temperature and composition of the mobile phase within allowable limits from actual chromatographic conditions. It was observed that there were no marked changes in mean R_t and RSD is within limit of ≤2 .The tailing factor, resolution factor and no. of theoretical plates were found to be acceptable limits for both Pheniramine 1 and Pheniramine 2. Hence, the method is reliable with variations in the analytical conditions and the results are shown in Table No.7 and Figure No. 16-21.

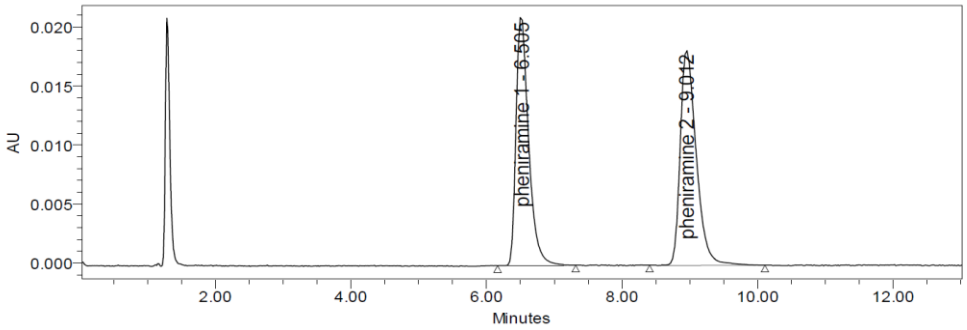

Fig.16:.Robustness (Flow Minus: 0.9ml/min) chromatogram of Pheniramine 1 and Pheniramine 2.

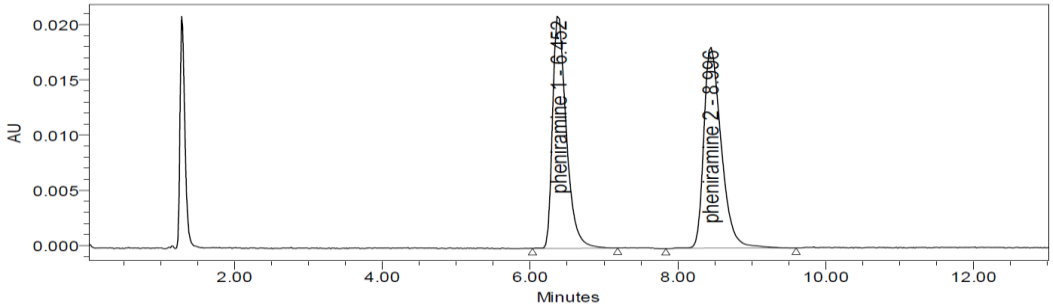

Fig.17: Robustness (Flow Plus: 1.1ml/min) chromatogram of Pheniramine 1 and Pheniramine 2.

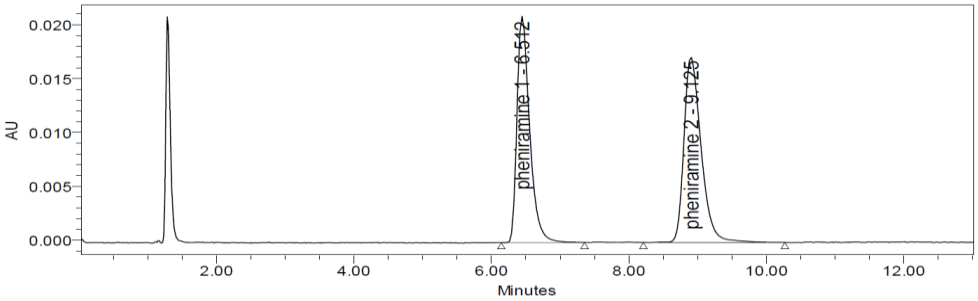

Fig. 18: Robustness (Mobile Phase Minus: 5%) chromatogram of Pheniramine 1 and Pheniramine 2.

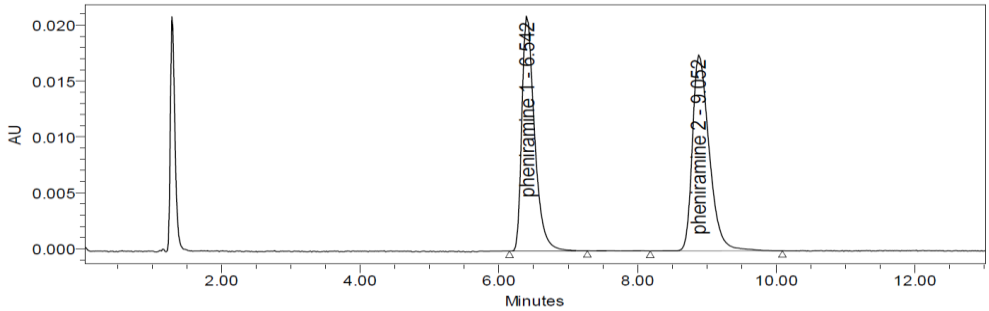

Fig. 19: Robustness (Mobile Phase Plus: 15%) chromatogram of Pheniramine 1 and Pheniramine 2

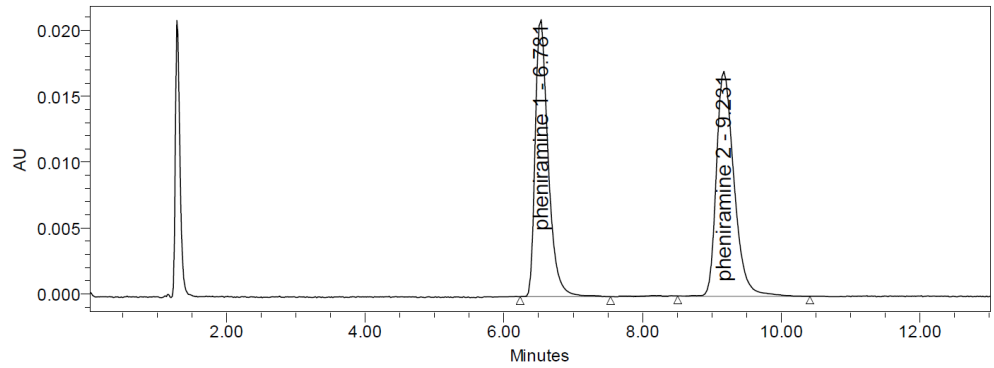

Fig.20: Robustness (Temperature Minus: 25°C) chromatogram of Pheniramine 1 and Pheniramine 2

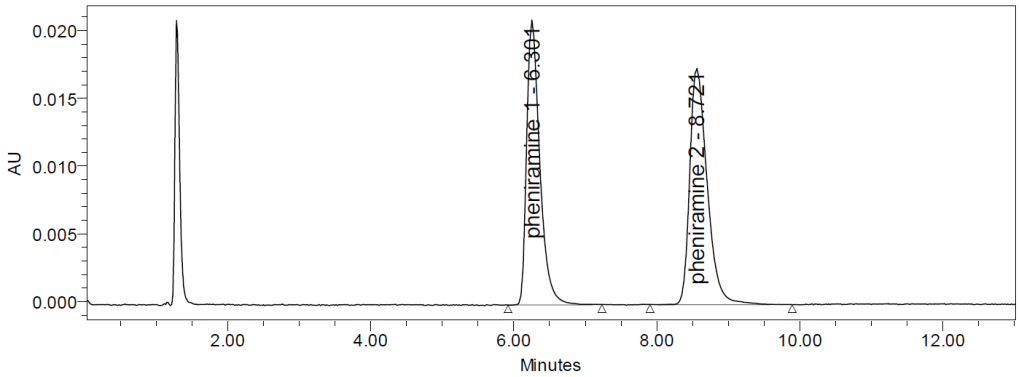

Fig.21: Robustness (Temperature Plus: 35°C) chromatogram of Pheniramine 1 and Pheniramine 2.

Table-7(a): Robustness – Flow Minus (n=6)

S.No.	Parameter	Pheniramine 1	Pheniramine 2
1.	% RSD of area	0.9	0.4
2.	Tailing Factor	1.19	1.54
3.	Plate count	2846	2087

Table-7(b): Robustness- Flow Plus (n=6)

S.No.	Parameter	Pheniramine 1	Pheniramine 2
1.	% RSD of area	0.2	0.1
2.	Tailing Factor	1.15	1.52
3.	Plate count	2869	2028

Table-7(c): Robustness - Mobile Phase Minus (n=6)

S.No.	Parameter	Pheniramine 1	Pheniramine 2
1.	% RSD of area	1.2	0.1
2.	Tailing Factor	1.16	1.61
3.	Plate count	2872	2198

Table-7(d): Robustness – Mobile Phase Plus (n=6)

S.No.	Parameter	Pheniramine 1	Pheniramine 2
1.	% RSD of area	0.03	0.6
2.	Tailing Factor	1.18	1.60
3.	Plate count	2958	2087

Table- 7(e): Robustness- Temperature Minus (n=6)

S.No.	Parameter	Pheniramine 1	Pheniramine 2
1.	% RSD of area	0.04	0.4
2.	Tailing Factor	1.26	1.59
3.	Plate count	2956	2097

Table-7(f): Robustness – Temperature Plus (n=6)

S.No.	Parameter	Pheniramine 1	Pheniramine 2
1.	% RSD of area	0.2	0.2
2.	Tailing Factor	1.15	1.58
3.	Plate count	2861	2090

Stability of sample solution: The sample solution injected after 24 hrs by keeping at ambient room temperature 30^0C did not show any appreciable change. The deviation in the assay is not more than 2 and the results are shown in Table-8.

Table 8: Stability data of Pheniramine 1 and Pheniramine 2

Drug	%Assay at 0 hr*	%Assay at 24hr*	Deviation
Pheniramine 1	100.13	99.96	0.12
Pheniramine 2	99.92	99.67	0.18

* n=6 for each parameter

LOD and LOQ:

LOD and LOQ for Pheniramine 1 were 0.03 and 0.11 µg/mL, respectively and for Pheniramine 2 were 0.14 and 0.44 µg/ml respectively. The lowest values of LOD and LOQ as obtained by the proposed method indicate that the method is sensitive and the results are shown in Table-9.

Table 9: LOD and LOQ data of Pheniramine 1 and Pheniramine 2

Pheniramine 1				Pheniramine 2		
S.NO	SLOPE	Y-INTERCEPT		S.NO	SLOPE	Y-INTERCEPT
1	4266	1681		1	4006	2261
2	4263	1771		2	3999	2580
3	4263	1722		3	4003	2296
AVG	4264	1725		AVG	4003	2379
SD		45.06		SD		174.95
LOD		0.03		LOD		0.14
LOQ		0.11		LOQ		0.44

Assay:

The percentage assay of labeled claim of Pheniramine 1 and Pheniramine 2 present in the AVIL were 100.13±0.62 % and 99.92±0.61%, respectively. RSD values for both Pheniramine 1 and Pheniramine 2 are within limit of ≤2 and the results were shown in Figure No. 22 and Table 10.

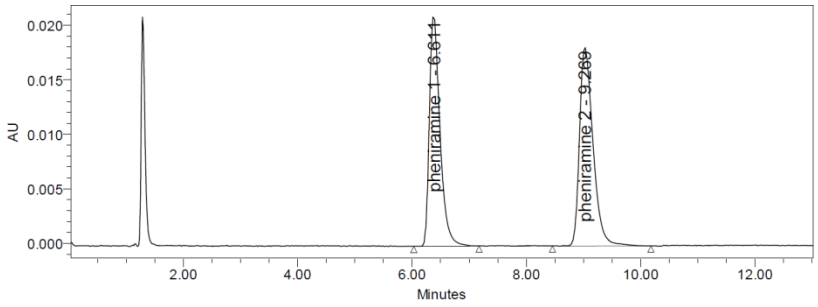

Fig.22. Assay chromatogram of Pheniramine 1 and Pheniramine 2

Table 10: Assay of Pharmaceutical dosage form

S. No.	Drug Name	Amount injected (µg/mL)	Amount found (µg/mL)	% Assay ± SD*
1	Pheniramine 1	100	100.13	100.13±0.62
2	Pheniramine 2	100	99.92	99.92±0.61

* n=6 for each parameter; Lable Claim: Pheniramine maleate 25mg

CONCLUSION:

A new simple, precise and accurate HPLC method was developed and validated for the seperation of Pheniramine 1 and Pheniramine 2 in pharmaceutical dosage form. In this method, Chiral pack column (150x4.6, 5µm) was selected as the stationary phase. Water and acetonitrile were taken in the ratio 90:10%v/v and used as mobile phase at a flow rate of 1.0 ml/min. The retention times of Pheniramine 1 and Pheniramine 2 were found to be 6.6 min & 9.1 min respectively. This HPLC method for the determination of Pheniramine 1 and Pheniramine 2 was validated as per the ICH guidelines. In this method, the numbers of theoretical plates were more than 2000, tailing factor was satisfactory and RSD of peak area is less than 2, this indicates that the optimized method met the system suitability parameters. The regression coefficient value was 0.999 for Pheniramine 1 and Pheniramine 2 and the response was linear. The percentage mean recovery of Pheniramine 1 and Pheniramine 2 were found to be 99.38% and 99.35% respectively and it showed that the proposed method is accurate. RSD values of repeatability and intermediate precision were ≤2 and the method is precise. The lowest values of LOD and LOQ as obtained by the proposed HPLC method indicate that the method is sensitive. The solution stability studies of method indicate that the Pheniramine 1 and Pheniramine 2 enantiomers were stable up to 24 hours. In robustness chromatographic conditions were changed as flow minus: 0.9 ml/min; flow plus: 1.1ml/min; temperature minus: 25^0C; temperature plus: 35^0C; mobile phase minus: organic phase 5%v/v; mobile phase plus: organic phase 15%v/v. These changes didn't show any variation in results and it showed the reliability of the method. Hence, the developed method can be successfully employed for enantiomeric separation of Pheniramine maleate in drug testing laboratories and pharmaceutical industries.

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