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Development and Validation of an Improved RP-HPLC Method for the Quantitative Determination of Flunarizine in Bulk and Tablet Dosage Form

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ABSTRACT

An accurate, simple, sensitive, selective, economic reversed phase high performance liquid chromatographic (RP-HPLC) method has been developed and validated for determination of flunarizine in its bulk form and pharmaceutical preparations. The chromatographic separation was achieved using Welchrom C_{18} isocratic column, (250 mm × 4.6 mm i.d., particle size 5 µm, maintained at ambient temperature), Shimadzu LC-20AT Prominence Liquid Chromatograph. The mobile phase containing a mixture of Methanol: Acetonitrile: Water 50:30:20 v/v, with apparent pH of 4.6 and a flow rate of 1.0 ml/min. The UV detection was set at 245 nm. The method was linear, regression coefficient (0.9998) at a concentration range of 2 - 10 µg/ml. The limit of detection and limit of quantitation for the method were 0.101629µg/ml and 0.307968µg/ml, respectively. The intra- and inter-assay precisions were satisfactory; the values of relative standard deviations did not exceed 2%. The accuracy of the method was proved; the mean recovery of flunarizine was 99.07% to 100.35%. The chromatographic retention time of proposed method was 6.023 min. Validation studies were performed according to ICH Guidelines revealed that the proposed method is specific, rapid, reliable and reproducible. The proposed method was successfully applied for the determination of flunarizine in its bulk form and pharmaceutical tablets with phenomenal accuracy and precisions. The label claim percentages were 100.47 ± 0.79 %. The proposed method is selective and involved simple procedures and this method is practical and valuable for routine application in quality control laboratories for determination of flunarizine.

Keywords: RP-HPLC, Flunarizine, Pharmaceutical analysis, Method validation, ICH guidelines.

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INTRODUCTION

Flunarizine (FLN) is chemically 1-[bis(4-fluorophenyl)methyl]-4-[(2E)-3-phenylprop-2-en-1-yl] piperazine (Fig. 1). It is one of the latest calcium antagonists with 'proven' anti-migraine effect [1]. Flunarizine prevents cell damage due to calcium over load by selectively blocking the entry of calcium into the cells of tissues. It has been found to inhibit the contractions of vascular smooth muscle and protected blood cells from membrane rigidity and protects brain cells from the effects of hypoxia. Migraine [2] is the most common headache diagnosis in neurological services in Asia and is among the top 10 most disabling disorders worldwide. However, it still remains under diagnosed and under treated. Many patients require management of individual migraine episodes as well as prophylactic treatment to prevent future episodes, treatment of vertigo, occlusive peripheral vascular disease and as an adjuvant in the therapy of epilepsy. The drugs of first choice are beta-blockers, flunarizine, topiramate, valproate and amitriptyline.

Literature survey reveals few analytical methods were reported for the determination of FLN in bulk and pharmaceutical preparations and in biological fluids by spectrophotometry [3-4], spectrofluorimetry [5], High Performance Liquid Chromatography [6-10], LC-Tandem MS [11], LC-ESI-MS [12]. Indeed the reported HPLC methods so far in the literature suffers from the low sensitivity and are considered to be uneconomical, time consuming and have poor symmetry. For this reason there is a need for the development of a novel, simple, rapid, efficient RP-HPLC analytical method with reproducibility for determination of FLN in bulk and pharmaceutical dosage forms. The present study illustrates development and validation of a novel, simple, rapid and efficient RP-HPLC analytical method with reproducibility for determination of FLN in bulk and pharmaceutical tablet dosage form. In fact the established method was validated with respect to specificity, linearity, precision, accuracy, robustness, LOD and LOQ according to ICH guidelines (ICH, 1997) [13].

MATERIALS AND METHODS

Chemicals and Reagents:

The reference sample of FLN standard was kindly supplied as gift sample by Hetero Drugs Ltd., Hyderabad, Andhra Pradesh, India. All the chemicals were analytical grade. Acetonitrile (HPLC grade) and triethylamine (HPLC grade) were purchased from Merck Pharmaceuticals Private Ltd., Mumbai, India. Methanol and water used were of HPLC grade and purchased from Merck Specialties Private Ltd., Mumbai, India. Commercial tablets of FLN formulation was procured from local market. MIGARID tablets containing 10 mg of FLN are manufactured by Cipla Ltd., Mumbai, India.

Instruments and Chromatographic Conditions:

Chromatographic separations were achieved by using Shimadzu LC-20AT Prominence Liquid Chromatograph comprising a LC-20AT VP pump, Shimadzu SPD-20A Prominence UV-Vis

detector and Welchrom C_{18} column (4.6 mm i.d. X 250 mm, 5 micron particle size). 20 µL of sample was injected into the HPLC system. The HPLC system was equipped with "Spinchrom" data acquisition software. Separations were performed on the reversed phase column using a mixture of methanol, acetonitrile and water (pH adjusted to 4.6 using o-phosphoric acid) in ratio of 50:30:20, v/v as mobile phase. Triethylamine was used as column modifier. The mobile phase was delivered at a flow rate of 1 mL/min. Eluate was monitored at 245 nm. In addition, an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), a sonicator (spectra lab, model UCB 40), UV-Visible Spectrophotometer (Systronics model 2203) were used in this study.

Preparation of Reagents and Standards

Mobile phase:

The mobile phase was prepared by mixing of methanol, acetonitrile and water (all of HPLC grade) in the ratio of 50:30:20, v/v. Then pH is adjusted to 4.6 with 0.1N *o*-phosphoric acid and 0.5ml triethylamine is added as column modifier. It is filtered through 0.45 µm nylon membrane filter and then sonicated for degassing.

Stock and Working Standard Solutions:

Accurately weigh and transfer about 10 mg of FLN, dissolve in a 100ml volumetric flask with mobile phase. This is stock standard solution of FLN with concentration of 100 µg/mL. Prepare five working standard solutions for calibration by adding defined volumes of the stock standard solution and diluting with mobile phase. The concentrations of FLN are 2.0, 4.0, 6.0, 8.0, 10.0 µg/mL, respectively.

Tablet Sample Preparation:

Weigh accurately not less than 20 tablets and determine average weight. Crush the tablets of FLN (MIGARID) into fine powder. Weigh equivalent to 10 mg of FLN into 100 mL volumetric flask. Add 70 mL mobile phase and sonicate until dissolution is complete. Make up the volume to 100 mL. Pipette out 1.0 mL of solution into a 10 mL volumetric flask and dilute with mobile phase upto the mark. Mix well. The resulting solution was filtered using 0.2 µm filter and degassed by sonication.

Selection of Detection Wavelength:

The UV spectrum of diluted solutions of various concentrations of FLN in mobile phase was recorded using UV spectrophotometer. The wavelength of maximum absorbance was observed at 245 nm. This wavelength was used for detection of FLN.

Calibration Curve for Flunarizine:

Replicates of each calibration standard solutions (2, 4, 6, 8, 10 µg/mL) were injected using a 20 µl fixed loop system and the chromatograms were recorded. Calibration curves were constructed by plotting concentration of FLN on X-axis and peak areas of standard FLN on Y-axis and regression equations were computed for FLN.

VALIDATION OF THE PROPOSED METHOD

The developed method of analysis was validated as per the ICH for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness and system suitability, limit of detection (LOD) and limit of quantitation (LOQ).

System Suitability:

The chromatographic systems used for analysis must pass the system suitability limits before sample analysis can commence. Set up the chromatographic system, allow the HPLC system to stabilize for 40 min. Inject blank preparation (single injection) and standard preparation (six replicates) and record the chromatograms to evaluate the system suitability parameters like resolution (NLT 2.0), tailing factor (NMT 1.5), theoretical plate count (NLT 3000) and % RSD for peak area of six replicate injections of FLN standard (%RSD NMT 2.0). If system suitability parameters are met, then inject sample (MIGARID) preparation in duplicate and record the chromatograms.

Specificity:

Specificity is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The specificity of the proposed method was demonstrated by studying the effect of various excipients and other additives usually present in the formulations of FLN in the determinations under optimum conditions. The blank, standard, placebo, placebo spiked with analyte and test preparations were analyzed as per the method to examine the interference of blank and placebo with FLN peaks. The common excipients such as lactose anhydrous, microcrystalline cellulose, purified talc and magnesium stearate have been added to the placebo solution and injected and tested. Furthermore the well-shaped peaks also indicate the specificity of the method. The chromatogram for placebo indicating the specificity of developed method is presented in Fig. 2.

Linearity:

Linearity for FLN was checked by preparing standard solutions at five different concentration levels of each of FLN ranging from 2-10 µg/ml of FLN. Method of least square analysis was carried out for getting the slope, intercept and correlation coefficient, regression data values. A calibration curve was plotted between concentration and peak area response and statistical analysis of the calibration curve was performed.

Precision:

Intra-day precision was determined by replicate applications and measurements of peak area for FLN for six times on the same day. Inter-day precision was obtained from % RSD values obtained by repeating the assay six times on two different days. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0.

Accuracy/Recovery:

The accuracy of the method was tested by triplicate samples at 3 different concentrations equivalent to 80%, 100% and 120% of the active ingredient, by adding a known amount of FLN standard to a sample with pre-determined amount of FLN. The recovered amount of FLN, %RSD of recovery, % recovery of each concentration is calculated to determine the accuracy.

Robustness:

In order to measure the extent of method robustness, the most critical parameters were interchanged while keeping the other parameters unchanged and in parallel, the chromatographic profile was observed and recorded. The chromatographic parameters were interchanged within the range of 1-10% of the optimum recommended conditions. The studied parameters were: the composition of mobile phase, flow rate, detection wavelength. The result indicated that the small change in the conditions did not significantly affect the determination of FLN.

LOD and LOQ:

Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of Detection and Limit of Quantitation were calculated using following formula LOD= 3.3σ/S and LOQ= 10σ/S, where SD=standard deviation of response (peak area) and S= slope of the calibration curve.

RESULTS AND DISCUSSION

The present study was aimed to develop a rapid, accurate and precise HPLC method for the determination of FLN in pharmaceutical dosage forms. Actual chromatographic conditions were established after number of preliminary experiments for selecting the proper column and mobile phase system. Different columns like C_8 and C_{18} columns and mobile phase systems were tested and selection of proper system depended on its ability to give good peak shape. Acceptable peak symmetry was achieved using a column of C_{18} column (250mm X 4.6mm i.d, 5 µm particle size) and mobile phase composed of methanol, acetonitrile and HPLC grade water in a ratio of 50:30:20, v/v, with pH adjusted to 4.6 using o-phosphoric acid and triethylamine as

column modifier at a flow rate of 1mL/min. The retention time for FLN was found to be 6.023 min. UV spectra of FLN showed that the drug absorbed maximum at 245 nm, so this wavelength was selected as the detection wavelength. System suitability parameters and optimized chromatographic conditions are shown in Table 1. The calibration curve for FLN was found to be linear over the range of 2-10 µg/mL. The data of regression analysis of the calibration curve is shown in Table 2 and Table 3. A good linear relationship (R^2=0.9997) was observed between the concentrations of FLN and the corresponding peak areas. The regression equation of the calibration curve was found to be Y= 97.852X – 3.7742 where Y is the peak area and X is the concentration of FLN. The developed method was applied to the assay of FLN tablets. The experimental results are given in Table 4. The results were very close to labeled value of commercial tablets. The representative standard and sample chromatograms of FLN are shown in Fig. 3 and Fig. 4 respectively. The representative chromatograms of the standard FLN concentrations are shown in Fig. 5 to Fig. 9. The linearity graph is shown in Fig. 10. The specificity was studied for the examination of the presence of interfering components, while the comparison of chromatograms there was no interference from placebo (Fig. 2) with sample peak. They do not disturb the elution or quantification of FLN; furthermore the well-shaped peaks also indicate the specificity of the method. Therefore, it was concluded that the method is specific. The specificity results are summarized in Table 5. Precision was studied to find out intra-day and inter-day variations in the test methods of FLN for the three times on the same day and different day. The %RSD for intra-day and inter-day precision variations studied at 10µg/mL obtained were 0.5947 and 0.7768 respectively showed a low Coefficient of Variation. This reveals that the proposed method is quite precise and reproducible and the precision results for intra-day and inter-day are shown in Table 6 and Table 7 respectively. The % recoveries of the drug solutions were studied at 3 different concentration levels. The % individual recovery and the % RSD values at each level were within the acceptance limits (99.07 - 100.35). The results are presented in Table 8. Generally the mean percentage recovery of FLN at each level was not less than 99% and not more than 101%. Robustness was done by deliberate changes in the chromatographic conditions like mobile phase flow rate, temperature, mobile phase composition etc., deliberate changes in developed method had not much affected the peak tailing, theoretical peaks and % assay which indicates that the present method is robust. As a matter of fact, the robustness results are presented in Table 9. The limit of detection (LOD) and limit of quantitation (LOQ) was calculated based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at levels approximating the LOD and LOQ. The limit of detection (LOD) was 0.101629µg/mL and the limit of quantitation (LOQ) was 0.307968µg/mL which shows that this method is very sensitive. The results are presented in Table 10.

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Table.No:1 Optimized Chromatographic Conditions and System Suitability Parameters of Proposed RP-HPLC Method for Flunarizine

Parameter	Chromatographic Conditions
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph
Column	WELCHROM C_{18} Column (4.6 mm i.d. X 250 mm, 5 µm particle size)
Detector	SHIMADZU SPD-20A prominence UV-Vis detector
Diluents	Methanol: Acetonitrile: Water (50:30:20, v/v, pH-4.6 using o-phosphoric acid)
Mobile phase	Methanol: Acetonitrile: Water (50:30:20, v/v, pH-4.6 using o-phosphoric acid)
Column modifier	Triethylamine (0.5 mL)
Flow rate	1 mL/min.
Detection wave length	UV at 245 nm.
Run time	8 minutes
Column back pressure	156 kgf
Temperature	Ambient temperature(25^{o}C)
Volume of injection loop	20 µL
Retention time (t_R)	6.023 min
Theoretical plates [th.pl] (Efficiency)	12,541
Theoretical plates per meter [t.p/m]	250,824
Tailing factor (asymmetry factor)	1.089

Table.No:2 Linear Regression Data of the Proposed HPLC Method of Flunarizine

Parameter	Method
Detection wavelength(λ_{max})	UV at 245 nm
Linearity range (µg/mL)	2-10 µg/mL
Regression equation (Y = a + bX)	Y= -3.774 + 97.852X
Slope(b)	97.852
Intercept(a)	-3.774
Standard error of slope (S_b)	0.497667
Standard error of intercept (S_a)	3.013524
Standard error of estimation (S_e)	4.163782
Regression coefficient (R^2)	0.9998
% Relative standard deviation* i.e., Coefficient of variation(CV)	0.802582
Percentage range of errors* (Confidence limits) 0.005significance level 0.001 significance level	 1.258124 1.973458

*Average of 6 determinations; Acceptance criteria < 2.0.

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Table.No:3 Calibration Data of the Proposed HPLC Method for Estimation of Flunarizine

S.No	Concentration, µg/mL.	Retention Time, (t_R) min.	Peak Area, mV.s.
1.	0	-	0
2.	2	6.023	189.929
3.	4	6.023	382.288
4.	6	6.023	583.638
5.	8	6.023	783.598
6.	10	6.027	973.452
Slope		97.851671	
Intercept		-3.774190	
Correlation Coefficient [CC] (r)		0.9999	
Squared CC (R^2)		0.9998	
Residual sum of squares		69.348337	

Table.No:4 Assay Results of Flunarizine Formulation

S.No	Formulations	Labelled Amount	Amount Found*	% Assay ±SD*
1	MIGARID tablets (Cipla Ltd., Mumbai, India)	10 mg/tablet	10.09 mg/tablet	100.472 ± 0.799%

*Average of 6 determinations; SD is standard deviation.

Table.No:5 Specificity Study for Flunarizine

Name of the Solution	Retention Time, (t_R) min.
Mobile phase	No peaks
Placebo	No peaks
Flunarizine, 10 µg/mL	6.023 min.

Table.No:6 Results of Precision Study (Intra-day) for Flunarizine

Sample	Concentration (µg/mL)	Injection no.	Peak Area (mV.s)	%RSD#
Flunarizine	10	1	992.884	0.5947
		2	976.568	
		3	984.924	
		4	978.912	
		5	981.826	
		6	979.362	

Acceptance criteria < 2.0.

Table.No:7 Results of Precision Study (Inter-day) for Flunarizine

Sample	Concentration (µg/mL)	Injection no.	Peak Area (mV.s)	%RSD#
Flunarizine	10	1	980.458	0.7768
		2	968.324	
		3	982.964	
		4	970.562	
		5	962.746	
		6	972.862	

Acceptance criteria < 2.0.

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Table.No:8 Recovery Data of the Proposed RP-HPLC Method for Flunarizine

Recovery level	Amount added (mg)	Total amount (mg)	Amount found (mg)	Amount recovered (mg)	% recovery	Mean % Recovery ± SD	%RSD[#]
80%	7.92	18.01	18	7.91	99.87	99.79± 0.753	0.755
	8.01	18.1	18.02	7.93	99.00		
	7.97	18.06	18.1	8.01	100.50		
100%	10.03	20.12	20.07	9.98	99.50	99.89± 0.458	0.458
	9.86	19.95	19.93	9.84	99.79		
	9.98	20.07	20.11	10.02	100.40		
120%	11.9	21.99	21.91	11.82	99.32	99.77± 0.704	0.705
	12.06	22.15	22.08	11.99	99.41		
	11.85	21.94	22.01	11.92	100.59		

[#]Acceptance criteria < 2.0.

Table.No:9 Robustness Results of Flunarizine

S.No	Parameter [a]	Optimized	Used	Retention Time (t_R), min	Plate Count[$]	Peak Asymmetry[#]	Remark
1.	Flow rate (±0.2 mL/min)	1.0 mL/min	0.8 mL/min	6.384	12937	1.098	*Robust
			1.2 mL/min	5.872	12479	1.084	*Robust
2.	Detection wavelength (±5 nm)	245 nm	240 nm	6.026	12542	1.096	Robust
			250 nm	6.123	12558	1.092	Robust
3.	Mobile phase composition (±5 %)	50:50, v/v	55:45, v/v	6.428	12875	1.094	*Robust
			45:55, v/v	5.898	12363	1.085	*Robust

Acceptance criteria (Limits):
[#]Peak Asymmetry < 1.5, [$] Plate count > 3000, * Significant change in Retention time

Table.No:10 Limit of Detection (Lod) and Limit of Quantitation (Loq)

Limit of Detection(LOD)	0.101629 µg/mL
Limit of Quantitation(LOQ)	0.307968 µg/mL

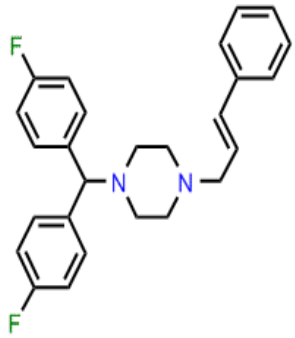

Figure.No:1 Structure of Flunarizine

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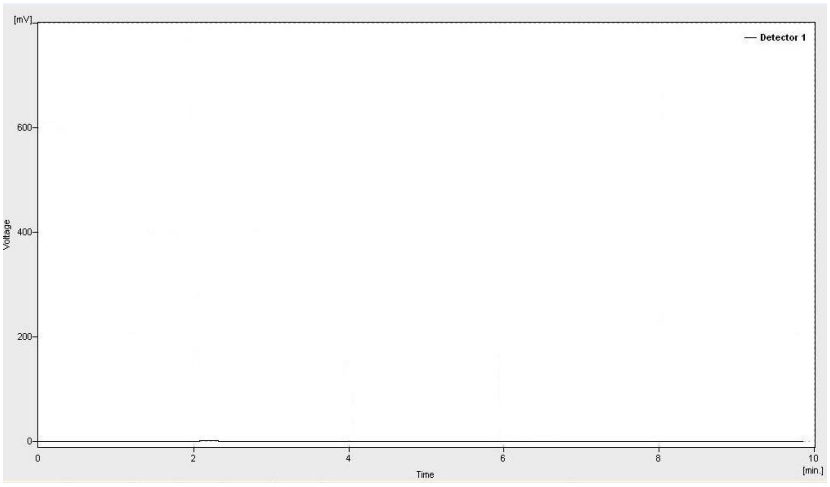

Figure.No:2 Chromatogram of Placebo

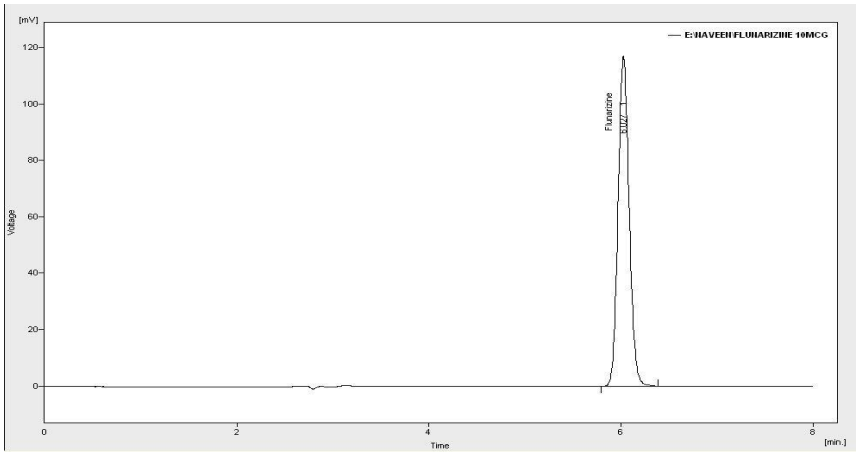

Figure.No:3 A Typical Chromatogram of Flunarizine Standard

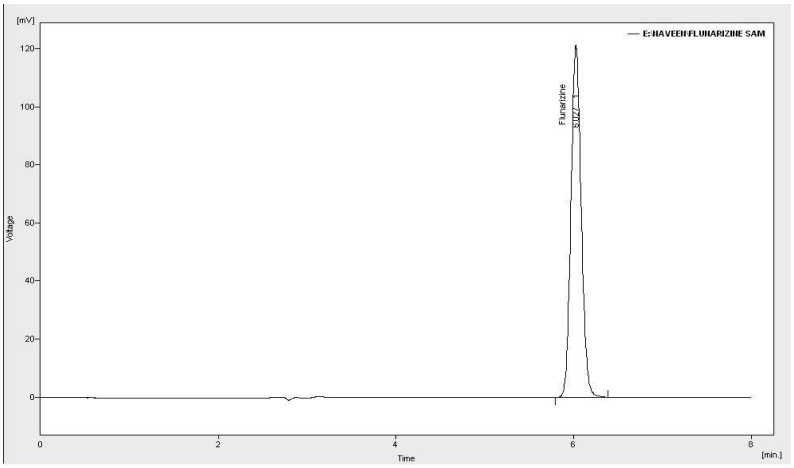

Figure.No:4 Chromatogram of Market Formulation (Migarid 10 Mg Tablets) of Flunarizine

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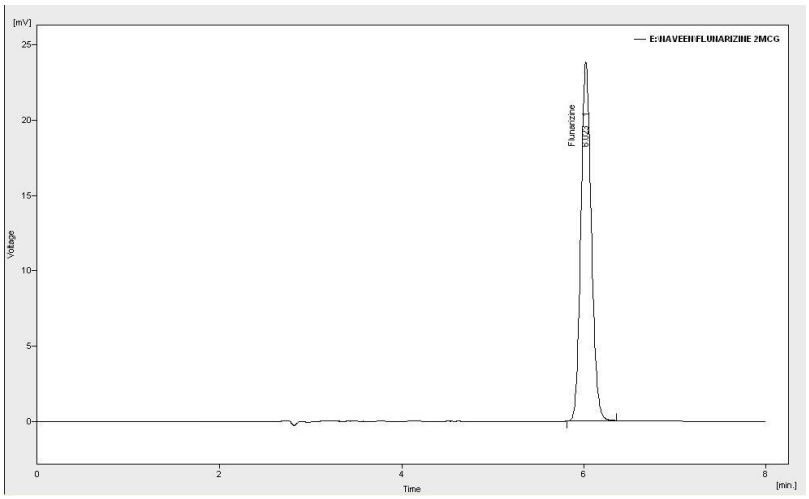

Figure.No:5 Standard chromatogram of Flunarizine (2 µg/ml)

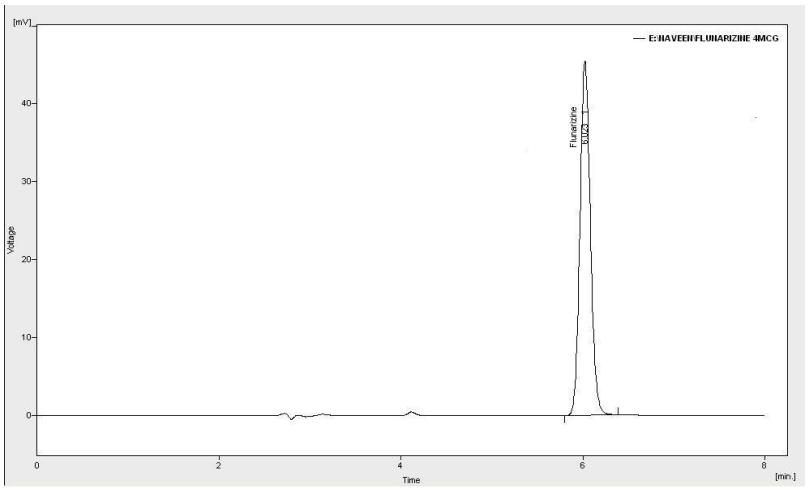

Figure.No:6 Standard chromatogram of Flunarizine (4 µg/ml)

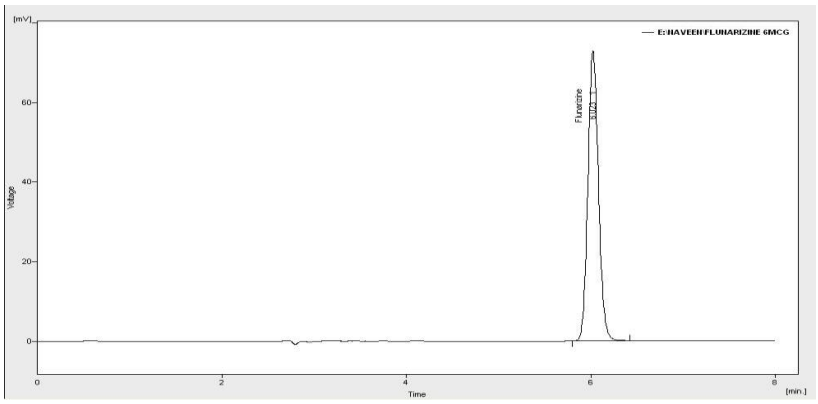

Figure.No:7 Standard chromatogram of Flunarizine (6 µg/ml)

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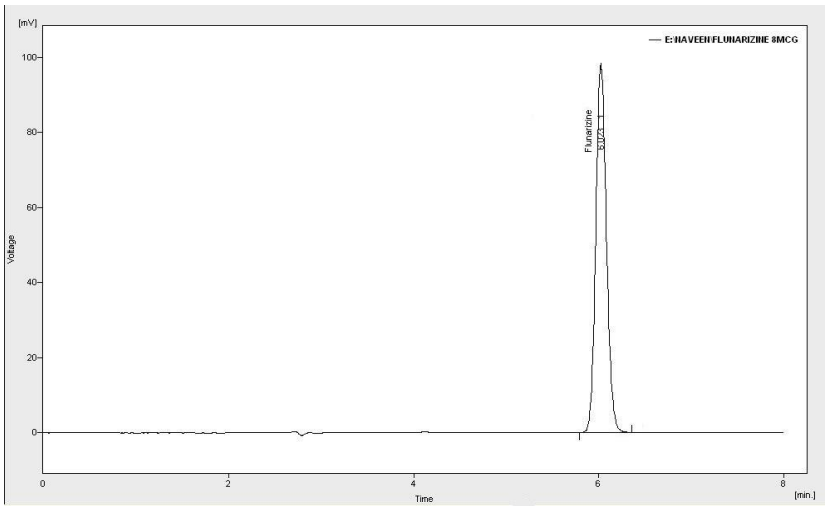

Figure.No:8 Standard chromatogram of Flunarizine (8 µg/ml)

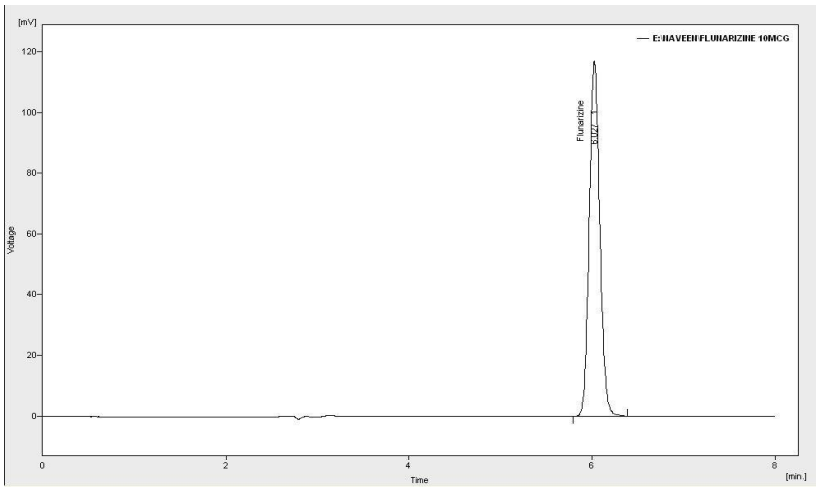

Figure.No:9 Standard chromatogram of Flunarizine (10 µg/ml)

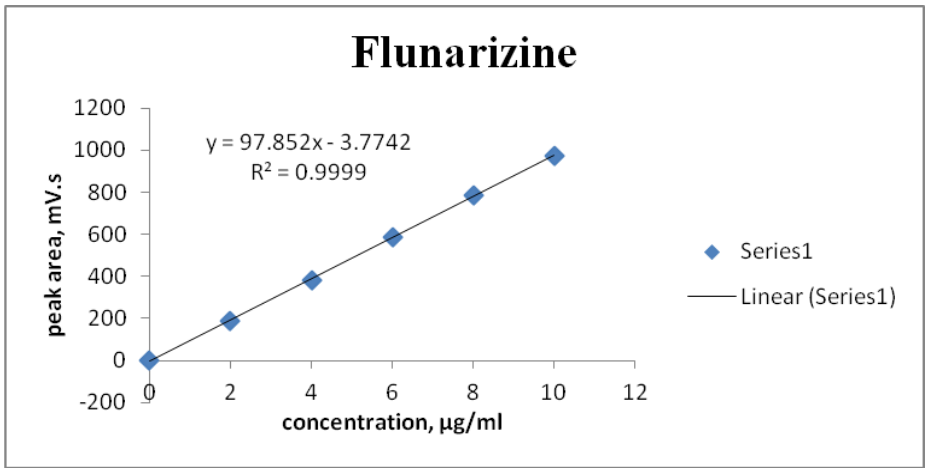

Figure.No:10 Calibration plot of Flunarizine

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CONCLUSION

A new simple sensitive, specific and accurate RP-HPLC method was explored for the quantitative determination of FLN in bulk and pharmaceutical tablet dosage forms. Statistical analysis of the results shows that the proposed method had good precision and accuracy. The drug solutions employed in the study were stable upto 48 hours. The tailing factor, numbers of theoretical plates are within the acceptable limits. From the previous discussion, the proposed procedures are simple, rapid over chromatographic methods in literature and it does not need sample preparation with sophisticated techniques or instruments. From the economic point of view, the analytical reagents are inexpensive and available in all laboratories. The validation of the proposed method according to the ICH guidelines proved the applicability and phenomenal value of this method for routine application in quality control laboratories for the analysis of FLN in their pure powder and dosage forms without excipient interference.

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