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RESEARCH ARTICLE

STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF DALFAMPRIDINE IN ITS BULK AND FORMULATION.

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Key words:-
Dalfampridine, RP-HPLC, Validation, Forced degradation, ICH guidelines.

Abstract

A stability indicating reverse phase high performance liquid chromatographic method has been developed and validated for estimation of Dalfampridine in its bulk and formulation. Method development was carried out on Inertsil-C_{18} column, (250×4.6mm, particle size 5µ, maintained at ambient temperature). The chromatographic separation was achieved using a mobile phase containing acetonitrile and potassium dihydrogen phosphate buffer (p^H was adjusted to 4 with orthophosphoric acid) in the ratio of 70:30 v/v at flow rate of 1.0 ml/min using detection at 298 nm. In the linear study, linearity was observed from 20-80 µg/ml with correlation coefficient of 0.999. The LOD and LOQ for the method were found to be 0.71µg/ml and 2.16µg/ml respectively. The statistical analysis shows that the method was found to be accurate, reliable, simple and reproducible. The values of relative standard deviation for precision did not exceed 2%. The chromatographic retention time of proposed method was 2.433 min. The percentage purity of Dalfampridine was found to be within the limit. For stability study, the drug was exposed to the stress conditions such as acid, alkaline, oxidation, thermal by using 0.1 M HCl, 0.1 M NaOH, 30% H_2O_2, 100º C. Degradation behaviour shows that the major degradation was observed at acidic condition (80.11%), followed by thermal (70.25%), alkaline (67.12%) and oxidation (63.42%). The proposed method was successfully applied for the quantitative determination of Dalfampridine in bulk and formulation.

Introduction:-

Dalfampridine is a potassium channel blocker that to improve conduction and propagation of the action potential along the demyelinated axons (Perreault and P, Avoli M. 1991, Sahraian M.A et al., 2011). Further it facilitates neuromuscular and synaptic transmission by relieving conduction blocks in demyelinated axons (Wu ZZ et al 2009). Clinically Dalfampridine is a neurofunctional modifier that helps improve walking speed in patients with multiple sclerosis (MS). The IUPAC name of Dalfampridine is 4-Aminopyridine. The structure of Dalfampridine is given Fig.1. Dalfampridine is a white crystalline powder with molecular weight 94.11g/mol. At ambient conditions Dalfampridine is soluble in methanol, acetone, acetonitrile, water, tetrahydrofuran, isopropanol, N, N-dimethylformamide, dimethyl sulfoxide and ethanol (Beckett AH and Stenlake 1988).

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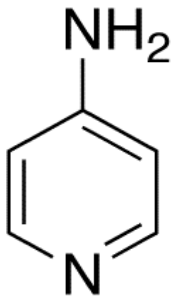

Figure 1:- Structure of Dalfampridine.

Literature survey reveals that few analytical methods were reported for the determination of Dalfampridine in bulk and formulation by UV (EL- Fatatry H.M et al., 2013, C.H. Madhumathi et al., 2014 and Vivekkumar k. R et al., 2014). The main aim of the present research work is to develop a simple, accurate, precise and rapid stability indicating RP-HPLC method development and validation for estimation of Dalfampridine in its bulk and formulation. The method was validated as per International Conference on Harmonization (ICH) guidelines.

Meterials and methods:-

Materials:-

Dalfampridine standard and Ampyra (10mg) commercially available tablet dosage form was obtained as free sample from Aurobindo pharma limited, Hyderabad, A.P, India. All chemicals were analytical grade.

Instruments:-

Method development was carried out on HPLC-WATERS Model 2690 series compact system containing Inertsil C18 column, (250×4.6mm,5µ). The mobile phase consists of a mixture of acetonitrile and potassium dihydrogen phosphate buffer (pH adjusted to 4 using orthophosphoric acid) in the ratio 70:30 v/v. The mobile phase was set at a flow rate 1.0ml/min. The column was maintained at ambient temperature. Detector was programmed at 298nm detection of Dalfampridine. In addition, an electronic balance (Sartorious), digital p^H meter (Poloman), a sonicator (Fast clean), UV-Visible spectrophotometer (Agilent resolutions) were used in this study.

Preparation of (KH_2PO_4 0.1M) buffer (IP, 1996):-

Accurately weighed 13.6085gms of potassium dihydrogen phosphate in to a beaker containing 1000 ml of distilled water and dissolved completely. Then p^H was adjusted 4 with orthophosphoric acid and then filtered through 0.45µm membrane filter.

Preparation of mobile phase:-

Acetonitrile and 0.1 M potassium dihydrogen phosphate were mixed in the ratio of 70:30 v/v and sonicated to degas.

Selection of Detection wavelength:-

Standard solution for Dalfampridine drug was scanned in UV-Vis region at 200-800 nm and the spectra was recorded. Maximum absorption of wavelength for Dalfampridine was observed at 298nm.

Preparation of stock solution and working standard solution for Dalfampridine:-

Accurately weighed and transferred 10 mg of Dalfampridine working standard into 10 ml of clean dry volumetric flask and mobile phase was added and sonicated to dissolve it completely and made up to the mark with same solvent to get 1000 µg/ml. Pipette out 0.4 ml was taken from this and diluted to 10 ml with mobile phase to prepare 40 µg/ml solution. From the above stock solution 20µg/ml to 80µg/ml concentration solutions were prepared, sonicated and filtered through 0.45µ membrane filter.

Method Validation (ICH guidelines, 2005):-

Linearity:-

A Series of solutions were prepared using Dalfampridine working standard at concentration levels from 20µg/ml to 80µg/ml of target concentration and the peak area response of solution was measured and calibration plot is shown in results Fig 3.

Specificity:-
Solutions of standard and sample were prepared as per the test method are injected into chromatographic system. In order to prove the method is specific and selective, the standard peak of the drug and the sample peak were compared retention time against the blank chromatogram and readings were recorded.

Accuracy:-
Accuracy of the developed method was determined based on the recovery studies. Recovery studies were carried out by adding equivalent amount of Dalfampridine into each volumetric flask. For each spike level to get the concentration of Dalfampridine equivalent to 50%, 100% and 150% of the labeled amount as per the test method results are shown in Table 2.

Precision:-
Precision was measured in terms of repeatability of application and measurement. The study was carried out by injecting six replicates of the standard at a concentration 40µg/ml of Dalfampridine. The % RSD for the area of six replicate injections was found to be within the specified limit results are shown in Table 3, 4, 5.

Limit of detection and Limit of Quantification (LOD and LOQ):-
From the linearity data the limit of detection and quantification was calculated.

Robustness:-
Effect of variation of flow rate:-
A study was conducted to determine the effect of variation in flow rate. Standard solution was prepared as per the test method and injected into the HPLC system by various flow rates like 0.8ml/min,1.0ml/min and 1.2ml/min results are shown in Table 6.

Ruggedness:-
System to system variability:-
System to system variability study was conducted on different HPLC systems, under similar conditions at different times in two different systems. Six samples were prepared and each was analyzed as per test method results are shown in Table 7.

System suitability:-
A Standard solution was prepared by using Dalfampridine working standard as per test method and was injected five times into the HPLC system. Then calculate the % RSD and results are shown in Table 8.

Assay of Dalfampridine:-
Twenty tablets containing Dalfampridine marketed formulation were taken and powdered. The powder equivalent to 10 mg of the active ingredient was accurately weighed and taken in a 100ml volumetric flask containing mobile phase and sonicated for 15 minutes and the solution was made up to volume with mobile phase and filtered through 0.45micron membrane. Further diluent take 1ml of above Solution in 10ml volumetric flask made up to volume with mobile phase results.

Forced degradation studies (ICH guidelines, 2003):_
Forced degradation studies were carried out as per ICH Q1A (R2) guidelines and the parameters such as acid degradation, alkali degradation, oxidative degradation, thermal degradation and results are shown in Table 9.

Acid degradation:-
Forced degradation in acid media was performed by adding stock solution (1 mg/ml) of Dalfampridine to 10 ml of 0.1 M HCl into a100 ml standard flask and refluxing the mixture at 60°C for approximately three hours. The solution was then left to reach room temperature, neutralized and diluted to 100 ml with the mobile phase so as to get a final concentration of 10µg/ml.

Alkaline degradation:-
Forced degradation in alkaline media was performed by adding stock solution (1 mg/ml) of Dalfampridine to 10 ml of 0.1 M NaOH into a 100 ml standard flask and refluxing the mixture at 60°C for approximately three hours. The

solution was then left to reach room temperature, neutralized and diluted to 100 ml with the mobile phase, so as to get a final concentration of 10µg/ml.

Oxidative degradation:-
To study the effect of oxidizing conditions, an aliquot of stock solution (1mg/ml) of Dalfampridine was added to 10 ml of 30% H_2O_2 solution and the mixture was refluxed at 60°C for approximately three hours. The solution was left to reach room temperature and transferred in to a 100 ml volumetric flask and made up the volume with the mobile phase, so as to get a final concentration of 10µg/ml.

Thermal degradation:-
To study the effect of temperature accurately weighed 25 mg of Dalfampridine and transferred into 25ml volumetric flask and stored at 100°C in a hot air oven for six hours. It was then dissolved in mobile phase and the volume was made up to the mark with mobile phase. The above solution was further diluted with the mobile phase, to give a solution of final concentration equivalent to 10µg/ml of Dalfampridine.

Results and Discussion:-

Optimized chromatographic method:-
Chromatographic conditions for estimation of Dalfampridine were optimised.
Table 1-: Optimized chromatographic conditions.

PARAMETERS	CONDITIONS
Column	Inertsil - C_{18} column (250x4.6 mm, 5µ)
Flow rate (ml/min)	1.0ml/min
Detector wavelength (nm)	298nm
Column temperature	Ambient
Injection volume	20µl
Run time (min)	5min
Mobile phase	Acetonitrile and Potassium dihydrogen phosphate buffer (70:30v/v)

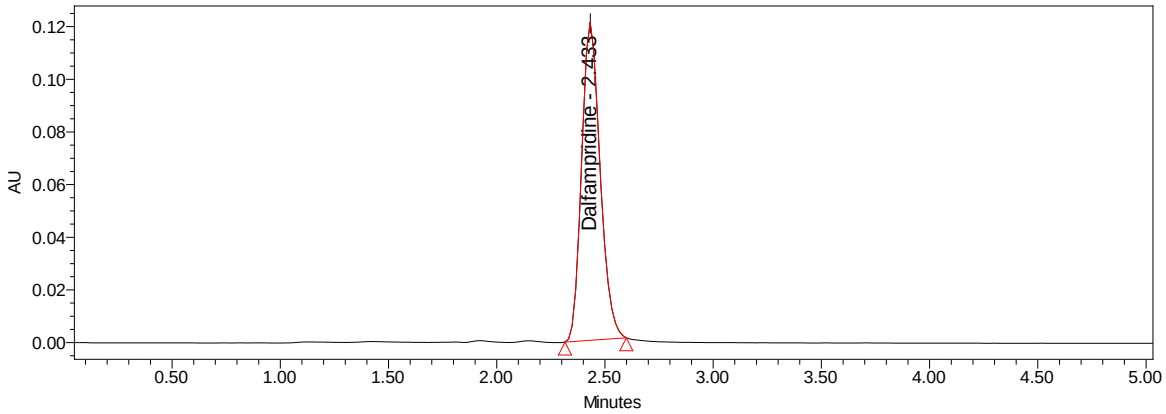

Fig 2:- Optimized Chromatogram of Dalfampridine.

The drug peak eluted at 2.433 min at the mobile phase composition of acetonitrile and potassium dihydrogen phosphate buffer in the ratio of 70:30 and a sharp symmetric peak was observed**.**

Linearity:-

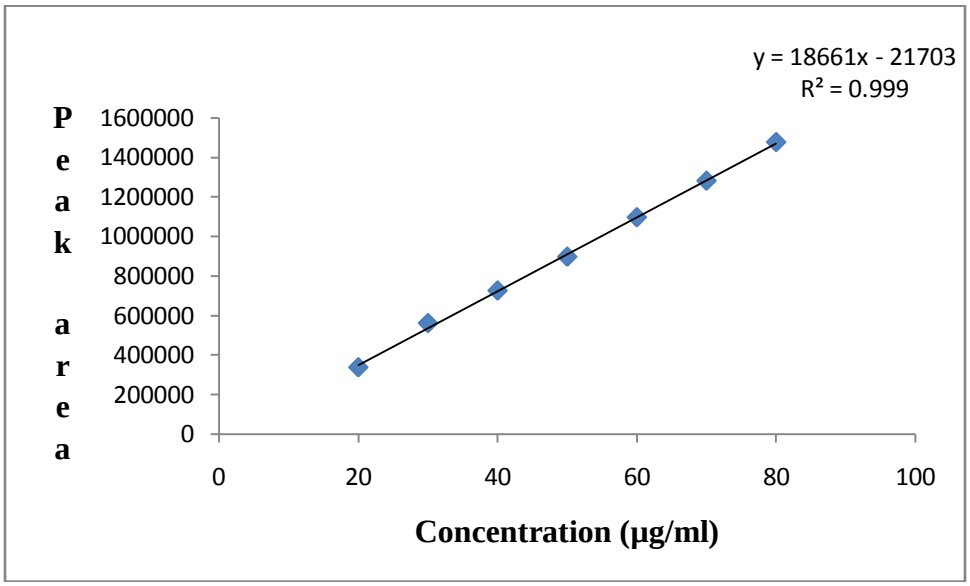

Fig 3:- Linearity plot of Dalfampridine.

The perfect linear graph was observed between peak area and concentration with the range from 20-80 µg/ml. The calibration curve was linear with slope, y-intercept, Correlation Coefficient was found to be 18661, 21703 and 0.999 respectively.

Specificity:-

Specificity studies of Dalfampridine was performed for blank, standard and sample. The results showed that there were no peak observed in blank with respective to drug peak, which reviled that the selected method is specific.

Accuracy (Recovery):-

Table 2:- Accuracy studies for the Dalfampridine.

Concentration % of spiked level	Amount added (µg/ml)	Amount found (µg/ml) (n=3)			Statistical Analysis of % Recovery		
		1	2	3	Mean of %Recovery	SD of % Recovery	% RSD
50%	20	19.68	19.74	19.85	98.76	0.4041	0.40
100%	40	39.96	38.92	38.84	98.1	1.5620	1.59
150%	60	59.84	59.52	59.79	99.5	0.2857	0.28

Accuracy of the developed analytical method was estimated by performing recovery studies. By adding known concentrations of the standard to the sample solution recovery of this method was analysed.

Precision:-

Repeatability:-

System precision:-

Table 3:- Repeatability studies (System precision) for the Dalfampridine.

Concentration (µg/ml)	No. of Injections	Peak Area of Dalfampridine	Statistical Analysis		
			Mean	SD	% RSD
40	1	726629	727601.2	2007.317	0.27
	2	724883			
	3	726175			
	4	729942			
	5	728516			
	6	729462			

Repeatability studies was determined in system precision. Six replicates at concentration level of 40µg/ml of Dalfampridine was spiked and the mean, standard deviation and %RSD was calculated and found to be within the limits.

Method precision:-

Table 4:- Repeatability Studies (Method precision) for the Dalfampridine.

Concentration (µg/ml)	No. of Injections	Peak Area of Dalfampridine	Statistical Analysis		
			Mean	SD	% RSD
40	1	728391	726701.8	1484.421	0.20
	2	724779			
	3	726154			
	4	725581			
	5	726911			
	6	728395			

Six replicates at concentration level of 40µg/ml of Dalfampridine was spiked and the mean, standard deviation and %RSD was calculated and found to be within the limit. The results showed that percentage RSD %RSD of the analytes was determined and it was found to be <2%.

Intermediate precision:-

Table 5:- Intermediate precision studies (Analyst 1) and (Analyst 2) for Dalfampridine.

Concentration (µg/ml)	No .of Injections	Peak Area of Dalfampridine (Analyst 1)	Peak Area of Dalfampridine (Analyst 2)	Statistical Analysis		
				Mean	SD	% RSD
40	1	725924	723775	725030.9	466.1248	0.06
	2	723189	725911			
	3	724673	726645			
	4	723881	721593			
	5	728137	724689			
	6	726359	725595			
	Mean peak Area	725360.5	724701.3			

Intermediate precision studies for Dalfampridine was performed by two analysts. Six replicates at concentration level of 40µg/ml of Dalfampridine was spiked and the mean, standard deviation and %RSD was calculated and found to be within the limit. The results showed that %RSD of the analytes was determined and was found to be <2%.

Limit of detection and limit of Quantification (LOD and LOQ):-

From the linearity plot the LOD and LOQ was found to be 0.71µg/ml and 2.16µg/ml respectively.

Robustness:-

Table 6:- Robustness studies for Effect of variation of flow rate for the Dalfampridine.

No. of Injections	Flow 0.8 ml/min		Flow 1.0 ml/min		Flow 1.2 ml/min	
	Std Area	Tailing factor	Std Area	Tailing factor	Std Area	Tailing factor
1	726079	1.106	725901	1.110	729381	1.123
2	725895	1.110	727442	1.112	726537	1.125
3	729313	1.112	723715	1.110	726221	1.124
4	726056	1.118	726419	1.111	724038	1.124
5	726921	1.117	726883	1.112	726189	1.123
Mean peak area	726852.8	1.1126	726072	1.111	726473.2	1.238

Mean , SD and % RSD of 0.8 ml/min and l.0ml/min			Mean, SD , % RSD of 1.0 ml/min and 1.2 ml/min		
Mean	SD	%RSD	Mean	SD	% RSD
726462.4	552.109	0.07	726272.6	283.6912	0.03

Robustness studies for effect of variation of flow rate of Dalfampridine was determined at different flow rates of 0.8ml/min, 1.0ml/min, 1.2ml/min. The % RSD was found to be within the limit <2%.

Ruggedness:-

Table 7:- System to system variability studies for the Dalfampridine.

No. of Injections	Peak Area of Dalfampridine System-1	Peak area of Dalfampridine System-2	Statistical Analysis		
			Mean	SD	%RSD
1	723937	721977	726631.3	908.9858	0.12
2	729382	723848			
3	724355	728911			
4	729677	729476			
5	727949	725585			
6	728344	726134			

System to system variability studies for Dalfampridine was performed by two systems. Six replicates at concentration level of 40 µg/ml of Dalfampridine was spiked and the mean, standard deviation and % RSD was calculated and found to be within the limit.

System suitability:-

Table 8:- System suitability studies for the Dalfampridine.

No. of Injections	Rt	Peak Area	USP Plate count	USP Tailing
1	2.434	721975	10168	1.106
2	2.433	725443	10214	1.109
3	2.435	723689	10200	1.110
4	2.436	729246	10198	1.107
5	2.436	728158	10210	1.108
Mean	2.4348	725702.2	10198	1.108
SD	0.001304	3024.965	18.05547	0.001581
%RSD	0.05	0.41	0.17	0.14

System suitability was carried out spiking five replicates at concentration level of 40 µg/ml of Dalfampridine and this method was evaluated for the calculation mean, standard deviation and % RSD.

Assay:-

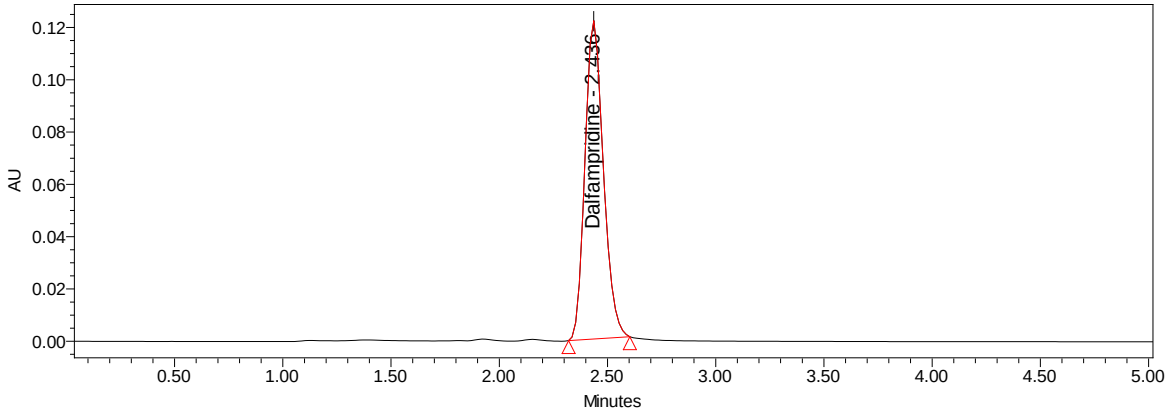

Fig.4:- Representative chromatogram for assay studies.

Forced Degradation studies:-

Table 9:- Forced Degradation Studies for Dalfampridine.

Mode of Degradation	Condition	Peak Area	% Degradation as compared with Control
Control sample	No treatment	725941	-
Acid	0.1 M HCl	144318	80.11
Alakaline	0.1 M NaOH	238678	67.12
Oxidative	30% H_2O_2	265539	63.42
Thermal	100°C	215962	70.25

Conclusion:-

Stability indicating RP-HPLC method was developed for estimation of Dalfampridine in its bulk and formulation. The developed RP-HPLC method for estimation of Dalfampridine was found to be simple, precise, accurate and reproducible.

The developed method was validated as per the ICH guidelines and the results obtained were well within the limits. This current investigation was intended to develop a method for the estimation of Dalfampridine and its application to forced degradation studies. Percent recovery and estimated concentration of active ingredient in pharmaceutical formulations showed that the amount of drug present is consistent with the label claim. Hence the proposed method was found to be satisfactory and applied for analysis of Dalfampridine.

The results of forced degradation studies of Dalfampridine shows that the major route of degradation is in acidic condition and less degradation in oxidative condition compared to other stress conditions such as alkaline and thermal conditions.

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Conflict of interest statement:-

The authors hereby declare that there are no conflicts of interest in the proposed article.

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