An Elsevier Indexed Journal ISSN-2230-7346

Journal of Global Trends in Pharmaceutical Sciences

ANALYTICAL METHOD DEVELOPMENT AND STABILITY STUDIES FOR ESTIMATION OF AMIFAMPRIDINE IN BULK AND TABLETS USING RP-HPLC

Dr. P. Harshalatha

Department of Chemistry, SVGM Government College, Kalyandurg, Anantapur, A.P.

*Corresponding author E-mail: harsha.pankaj@yahoo.in

ARTICLE INFO

Key Words

Amifampridine; Lambert Eaton myasthenia syndrome; Firdapse; Di Potassium Hydrogen Ortho phosphate; Ortho Phospharic Acid and Acetonitrile

ABSTRACT

A simple and reproducible method of isocratic reverse phase liquid chromatography (RP-LC) was developed for the quantitative determination of amifampridine in bulk drug and tablets, used to treat some symptoms found in Lambert Eaton myasthenia syndrome (LEMS). The proposed RP-HPLC method uses Waters C-18 analytical column (250 mm × 4.6 mm inner diameter, 5 µm) maintained at ambient temperature using Di Potassium Hydrogen Ortho phosphate pH 6.4 regulated with OPA : Acetonitrile (70:30v/v), effluent flow rate (1.0 ml / min) and UV detection at 290 nm for amifampridine analysis. The method was validated according to the ICH guidelines in terms of specificity, linearity, precision and accuracy. The retention time for amifampridine was 4.05 min. The recovery determinations allowed the calculation of a confidence interval of 99.85-100.88% with a relative standard deviation value of 0.91%. LOD and LOQ were estimated at 1.78 and 5.93 µg/ml respectively. The validated method was successfully applied to the determination of amifampridine in tablets (Firdapse10 mg, Biomarin). Amifampridine was exposed to conditions of acid, basic, oxidative, heat and UV stress and the stressed samples were analyzed with the proposed method. The chromatographic peak purity results indicated the absence of elution peaks with the main amifampridine peak, which demonstrated the specificity of the test method for estimating amifampridine in the presence of degradation products. This method has advantages that include a short period of time, a simple and quick sample preparation that uses this method used for routine amifampridine analysis in quality control laboratories.

INTRODUCTION

Lambert-Eaton myasthenic syndrome is a rare disease whose clinical expressions are myasthenic features in the form of variable muscle weakness and the fatigability[1-3] .This pathology is due to disorders of neuromuscular junctions that cause in blocking acetylcholine (ACn) release. Amifampridine (AFP), 3, 4-Diaminopyridine (3, 4-DAP), shown its chemical structure in figure 1, is usually prescribed to treat this disorder: it releases acetylcholine (ACn), and it is more efficient and less toxic than other treatments. [4-8] So far there are few reported analytical methods to estimate amifampridine. Lamiable developed a HPLC method for the determination of amifampridine in rat cerebrospinal fluid and serum.[9]. Another high-performance liquid chromatography method was reported for determining amifampridine in stomach contents of horses and was not applicable for biological fluids.[10] We present here, for the first time for reversed-phase HPLC, we developed an economical and time saving HPLC method with UV detection

for amifampridine determination, avoiding long sample preparation steps and using acetonitrile in a mobile phase composition, which retained adequate sensitivity for routine analysis in a pharmaceutical analytical laboratory. To determine the stability indicating nature of the method, the forced degradation of amifampridine was performed under various stress conditions (base, acid, oxidative and thermal) and the proposed method analyzed the samples subjected to stress. The proposed LC method was able to separate the drug from the degradation products generated during the forced degradation studies. The method developed was validated according to the guidelines of ICH.[11, 12] The linearity of response, precision, accuracy and robustness of the described method was checked.

EXPERIMENTAL INVESTIGATION

Chemicals and solvents

The pure amifampridine drug was procured as a gift sample from Zydus Pharma, India. The amifampridine tablets were purchased at Mukesh pharmacy, Hyderabad, India. HPLC grade Acetonitrile, Di Potassium Hydrogen Ortho phosphate, Ortho phosphoric acid and water were obtained from SD Fine Chem, Mumbai, India. All other chemicals used were of AR grade.

HPLC–PDA instrumentation and chromatographic conditions: The HPLC system was an LC Waters (Waters, Milford, MA, USA) composed of a quaternary gradient system (600 Controller), in line degasser (Waters, model AF), photodiode array detector (Water, 2998 model) and auto sampler (Waters, model 717 plus). The data was processed using Empower Pro software (Waters, Milford, MA, USA). The chromatographic separation assay was performed with a Waters C-18 analytical column (250 mm × 4.6 mm ×5 µm, Waters, Dublin, Ireland) maintained at room temperature. The mobile phase consists of Di Potassium Hydrogen Ortho phosphate pH 6.4 regulated with OPA: Acetonitrile (70:30v/v). The mobile phase was pumped at a flow rate of 1.0 ml min^{-1}. The detection wavelength was 290 nm. The mobile phase was used as a diluent for the preparation of amifampridine working standards.

Preparation of Standard Solution: 10 mg of amifampridine was carefully weighed and transferred into a 10 ml volumetric flask, added 5 ml of mobile phase and sonicated for 5 mins to dissolve and diluted to volume with the same. Further diluted 1.25 ml of the above solution to 10 ml with diluent to get the final concentration of 125 µg/ml. The Solution was filtered through 0.45 µm membrane filter before injection and 20 µl was injected into chromatograph.

Sample Preparation (Assay of pharmaceutical preparation): Twenty tablets were weighed and average weight was determined. Then all the tablets were ground to fine powder. A quantity of powder equivalent to 10 mg of amifampridine was weighed and transferred to 10 ml volumetric flask. Added 5 ml of mobile phase and sonicated for 5 mins to dissolve and made up to the mark with the same. The solution was filtered if necessary. 1.25 ml of a clear filtered solution was pipette out in to a 10 ml volumetric flask and make up to the mark with mobile phase.

Method validation: The proposed method was validated in accordance with the guidelines established by the International Harmonization Conference for the validation of analytical procedures.[12] The parameters used to validate the method of analysis were: system suitability, specificity, linearity, precision and accuracy, limit of detection (LOD), limit of quantification (LOQ) and robustness.

System suitability: The system suitability test is an integral part of the validation of the liquid chromatography method performed to verify and guarantee the continuous performance of a chromatography system. The system repeatability was estimated from 6 repeated injections of standard working solution at 100% of the test concentration (125 µg / ml of amifampridine). The system suitability parameters were calculated according to the recommendation of ICH.[12]

Specificity: Specificity is the ability to unequivocally evaluate the analyte in the presence of components that can be presumed to be present (impurities, degrading, matrix, etc.).[12] Specificity has been demonstrated by determining amifampridine in the presence of degradation products generated by forced

decomposition. The stress conditions applied in the study were: basic hydrolysis (3 ml of 0.5 N NaOH, kept a side for 6 hours and neutralize with 0.5 N HCl), acid hydrolysis (3 ml of 0.5 N HCl, heat at 85 °C for 8 hours, Cool and neutralize with 0.5 N NaOH) and oxidative degradation (5 ml of 3% H_2O_2, Kept a side for 1 day) and thermal degradation (Heat at 80 °C for 5 hours, kept a side for 1 day). The blank solutions have been treated in the same way.

Linearity: For the evaluation of linearity, the calibration curve was obtained at 6 concentration levels of standard amifampridine solutions (31.23–187.5 µg / ml). The solutions (20 µl) were injected in triplicate in a chromatographic system with the chromatographic conditions previously provided. For the evaluation of linearity, the peak area and concentrations were subjected to a least squares regression analysis to calculate the calibration equation and for the determination of coefficient.

Precision: The precision of the analytical procedure (intra-assay precision) was studied by analyzing six sample solutions obtained by multiple sampling of the same homogeneous sample under the prescribed conditions (at 100% of the test concentration of amifampridine (125 µg/ml)) on the same day, by the same analyst and using the same equipment. The intermediate precision of the analytical procedure was investigated by analyzing sample solutions on three consecutive days. The precision of the analytical procedure was expressed as the relative standard deviation of a series of measurements.

Limit of detection and limit of quantification

The detection limit (LOD) and the limit of quantification (LOQ) of the proposed method were determined by consecutively injecting low concentrations of the standard solutions using the proposed RP-HPLC method. LOD and LOQ were calculated according to ICH guidelines [12].

Accuracy: To study the accuracy of the proposed analytical method, recovery tests were performed. To discover whether excipients interfered with the analyte, equivalent amounts at 50, 100 and 150% of amifampridine from the tablet formulation were evaluated and the resulting mixtures were analyzed with the proposed methods.

Robustness: The ability of the proposed method to remain unaffected by small (deliberate) variations in parameters was evaluated in order to determine method robustness. Changes were made to the following method parameters: flow rate (±0.2 ml min–1), organic solvent concentration (± 5%), pH of the mobile phase (± 0.2), and temperature (± 2 °C).

RESULTS AND DISCUSSION

Method Development: Various mobile phases have been studied in the development of an HPLC method for amifampridine analysis. These include: methanol - water, 50:50 (V/V), acetonitrile - water, 30:70 (V / V), methanol - orthophosphoric acid buffer (pH 4.5–6.5), 50:50 (V / V), Methanol buffer - phosphate (pH 3.0–6.5), 25:75 (V / V) and acetonitrile - phosphoric acid buffer (pH 3.2-4.5) 60:40 v / v. The suitability of the mobile phase was decided based on the sensitivity of the assay, the suitability for stability studies, the ease of preparation and the use of readily available solvents. Therefore, the mobile phase consisting of Di Potassium Hydrogen Ortho phosphate pH 6.4 adjusted with OPA: Acetonitrile (70:30v/v), has been found to be optimal for isocratic determination of AFP in pharmaceutical products. The wavelength was selected by scanning the standard amifampridine solution at more than 200-400 nm and the wavelength of 290 nm was chosen for the detection of amifampridine. Amifampridine has been identified as a function of retention time compared to the amifampridine standard. Furthermore, amifampridine was identified by adding the standard to the sample prior to analysis, which resulted in an increase in the sample peak area that was proportional to the amount added. The mean amifampridine retention time was approximately 4.053 minutes at a flow rate of 1.0 ml min-1. Amifampridine was rapidly determined as a single sharp peak. No interference was observed from other degradation products.

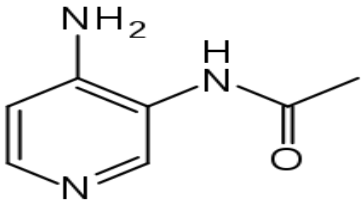

Figure 1: Chemical structure of Amifampridine

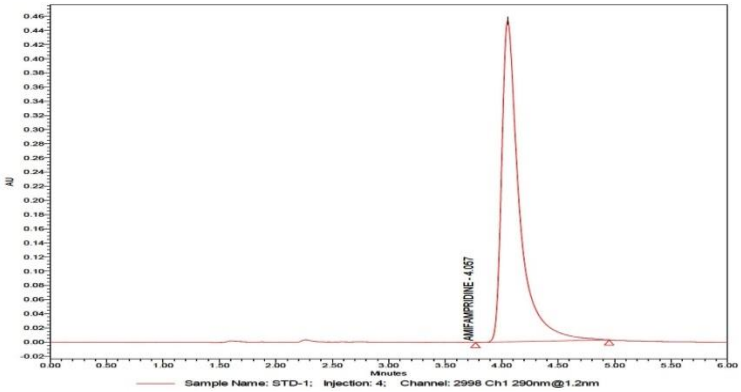

Figure 2: Standard chromatogram of Amifampridine

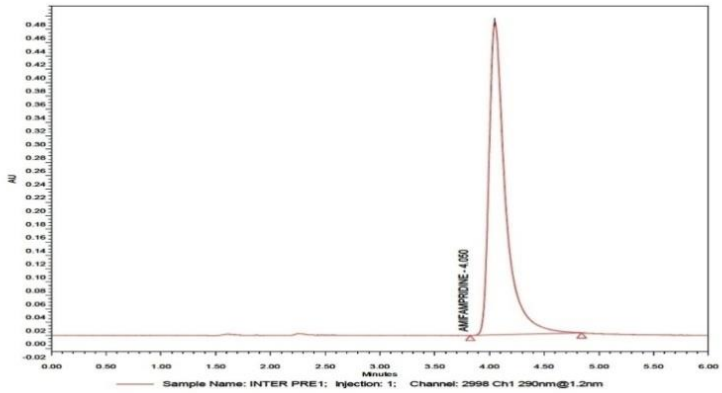

Figure 3: Sample chromatogram of Amifampridine

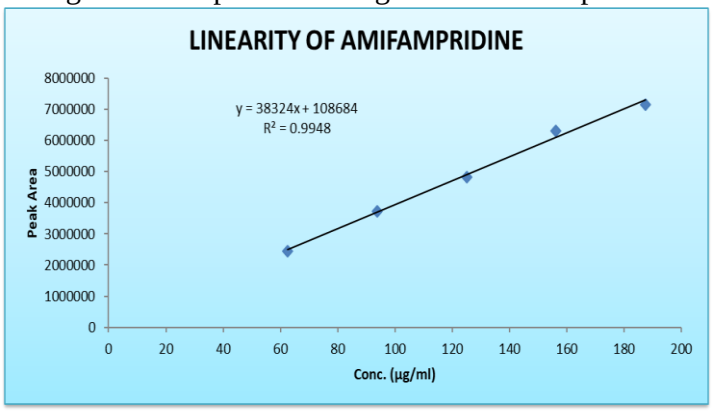

Figure 4: Linearity Plot of Amifampridine

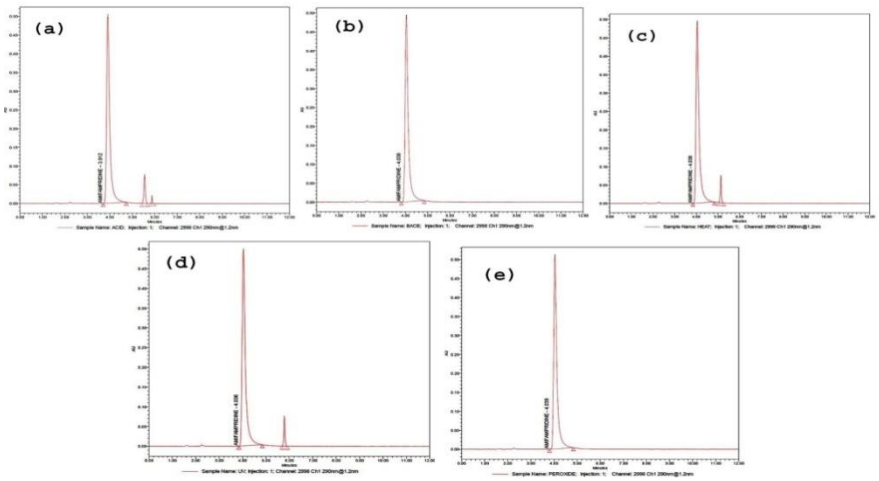

Figure 5: Stress Chromatograms of acid degradation (a), basic degradation (b), Thermal degradation (c), UV degradation (d) and Peroxide degradation (e) of Amifampridine

Table 1: Results of system suitability

S. No	STD Area	Theoretical Plates	Tailing Factor	RT
1	4564033	3815	1.77	4.057
2	4567981	4246	1.77	4.053
3	4611907	4094	1.76	4.053
4	4600642	3887	1.75	4.05
5	4619105	3947	1.71	4.05
6	4597419	4008	1.70	4.049
Avg	4593515	---	1.74	4.05
SD	22717	---	0.03	0.003
%RSD	0.495	---	1.765	0.073

Table 2: Forced degradation studies Results

Nature of the Sample	Sample Weight	Sample Area	% Assay	% of Degradation
Acid	219.45	4031787	87.77	12.23
Base	219.45	4198846	91.41	8.59
Peroxide	219.45	4182358	91.05	8.95
Heat	219.45	4093507	89.11	10.89
UV	219.45	4261195	92.77	7.23

Table 3: Precision Results

S. No	Sample Weight	Inter day		Intra day	
		Sample Area	% Assay	Sample Area	% Assay
1	219.45	4590773	99.94	4625313	100.69
2	219.45	4557664	99.22	4619827	100.57
3	219.45	4611459	100.39	4514342	98.28
4	219.45	4512328	98.23	4608856	100.33
5	219.45	4620138	100.58	4603370	100.21
6	219.45	4614715	100.46	4597885	100.10
Avarage Assay:			99.80		100.03
STD			0.92		0.89
% RSD			0.92		0.89

Table 4: Accuracy Results

Sample No.	Spiked Level	Sample Weight (mg)	Sample Area	µg/ml added	µg/ml found	% Recovery	% Mean Recovery
1	50	109.725	2315877	6.24	6.30	100.92	100.88
2	50	109.725	2321689	6.24	6.32	101.17	
3	50	109.725	2325228	6.24	6.33	101.32	
4	50	109.725	2314892	6.24	6.30	100.87	
5	50	109.725	2305698	6.24	6.27	100.47	
6	50	109.725	2306820	6.24	6.28	100.52	
7	100	219.45	4566049	12.49	12.43	99.48	99.85
8	100	219.45	4587118	12.49	12.48	99.94	
9	100	219.45	4595347	12.49	12.50	100.12	
10	150	329.175	6897461	18.73	18.77	100.19	100.66
11	150	329.175	6908035	18.73	18.80	100.34	
12	150	329.175	6990677	18.73	19.02	101.54	
13	150	329.175	6975367	18.73	18.98	101.32	
14	150	329.175	6896341	18.73	18.77	100.17	
15	150	329.175	6910346	18.73	18.80	100.37	

Table 5: Robustness study Results

S.No	Parameter	Condition	Peak area	% Assay
1	pH	6.2	4571353	99.52
2		6.4	4614342	100.45
3		6.6	4624526	100.68
4	Mobile Phase composition	65:35:00	4598532	100.11
5		70:30:00	4614342	100.45
6		75:25:00	4615093	100.47
7	Column Temperature	28°C	4560067	99.27
8		30°C	4614342	100.45
9		32°C	4617191	100.52
10	Flow rate	0.8 ml/min	46099011	1003.57
11		1 ml/min	4614342	100.45
12		1.2 ml/min	4556436	99.19

Figure 2 and 3 shows the standard and sample chromatograms.

Method Validation

System suitability: The results of system suitability test were found within the acceptable range [12] indicating that the system was suitable for the intended analysis (Table 1).

Specificity: In the specificity study, standard amifampridine solutions and the sample solution were injected and a single peak was obtained for amifampridine, indicating that there was no interference from the excipients or from the mobile phase. Furthermore, a forced degradation study was conducted to demonstrate the specificity of the proposed method. This study also provides information on degradation pathways and degradation products that could be formed during storage. The result of the studies on forced degradation, with an approximate percentage of degradation and a relative retention time of the degradation products, is shown in Table 2. Figure 5 (a-e) shows the stress chromatograms of amifampridine. HPLC chromatograms of acid,

base, oxidative, UV and thermal degradation of amifampridine show that amifampridine peak is well separated from all the degradation products formed during the different stress conditions. Thus specificity study ensures that the developed analytical method is able to separate and quantify amifampridine in presence of different degradation products.

Linearity: In the present study, linearity was studied in the concentration range 31.23-187.5 µg/ml amifampridine and the following regression equation was found by plotting the peak area (y) expressed in mAU versus the amifampridine concentration (x) expressed in µg/ml: y = 38324x +108684(R^2 = 0.9948). The determination coefficient (r^2) demonstrates the excellent relationship between the peak area and concentration of amifampridine. The calibration curves of amifampridine API and with placebo were linear. The excipients had no influence and there was no matrix effect observed. Figure 4 shows the linearity curve of amifampridine.

Precision and accuracy: Precision was demonstrated by Interday and intraday variation studies. In the intraday and Interday studies the solutions were injected 6 times and %RSD was calculated resulting in less than 1%. Accuracy (%Recovery) of the proposed method was demonstrated by analyzing different concentrations covering the points in the calibration range. The average percentage recovery was found to be 100.46%. The precision and accuracy were shown in Table 3 and 4 respectively.

Limit of detection and limit of quantification: The detection limit (LOD) and quantification limit (LOQ) were 1.78µg/ml and 5.93µg/ml respectively. The proposed HPLC method for amifampridine determination was demonstrated to be sensitive for performing the stability indicating assay and the assay evaluation of the release of the product and of the stability studies and of the profile of Firdapse tablets.

Robustness: Based on the results obtained, it has been shown that the proposed HPLC analytical method is robust (Table 5).

Method Applications: The validated method was applied for the determination of amifampridine in commercially available Firdapse tablets. Figure 2 and 3 shows two typical HPLC chromatograms obtained later the test of the standard amifampridine reference solution and of the Firdapse tablets sampling solution, respectively. The results of the trials (n = 6) produced 99.92% (RSD = 0.91%) of the label declaration for amifampridine in Firdapse tablets, respectively (Table 3). The mean retention time of amifampridine was approximately 4.053 minutes. The test results indicate that the method is specific for the analysis of amifampridine without interference from the excipients used to formulate and produce these tablets.

CONCLUSION

In conclusion, a sensitive and selective stability indicating RP-HPLC method has been developed and validated for the analysis of amifampridine in API and tablets. Based on the peak purity results obtained from the analysis of samples degraded by force using the described method, it can be concluded that the absence of a co-eluent peak together with the main peak amifampridine indicated that the developed method is specific for the estimation of amifampridine in the presence of degradation products. Furthermore, the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility. Although no attempt has been made to identify degraded products, the proposed method can be used as a stability indicator method for the use of amifampridine dosage.

Acknowledgements:

First and foremost I take this opportunity to thank University Grants Commission, Southern Eastern Regional Office (SERO) Hyderabad for providing the financial assistance to accomplish this research project successfully. I also take this opportunity to express my gratitude to the Zydas Pharma, India, for gifting API of amifampridine.

REFERENCES:

1. Lundh H1, Nilsson O, Rosén I, Johansson S. Practical aspects of 3,4-diaminopyridine treatment of the Lambert-Eaton myasthenic syndrome. Acta Neurol Scand. 1993 Aug; 88(2):136-40.
2. Do, B., Goulay-Dufaÿ, S., Hoang, M.D.L. *et al.* HPLC Method for Determination of 3,4-Diaminopyridine in the Presence of Related Substances and Degradation Products Formed Under Stress Conditions. *Chroma* **63,** 599–603 (2006).
3. Lundh H, Nilsson O, Rose´ n I, HPLC Method for Determination of 3,4-Diaminopyridine in the Presence of Related Substances and Degradation Products Formed Under Stress Conditions, Chromatographia 63(11):599-603 · June 2006.
4. Matthew N Meriggioli, Donald B Sanders, Autoimmune myasthenia gravis: emerging clinical and biological heterogeneity, Lancet Neurol. 2009 May; 8(5): 475–490.
5. Hoch W, McConville J, Helms S, Newsom-Davis J, Melms A, Vincent A. Auto-antibodies to the receptor tyrosine kinase MuSK in patients with myasthenia gravis without acetylcholine receptor antibodies. Nat Med. 2001; 7:365–68.
6. Sanders DB, Juel VC, Harati Y, Smith AG, Peltier AC, Marburger T et. al., 3,4-diaminopyridine base effectively treats the weakness of Lambert-Eaton myasthenia. Muscle Nerve. 2018 Apr; 57(4):561-568.
7. Shieh P, Sharma K, Kohrman B, Oh SJ, Amifampridine Phosphate (Firdapse) Is Effective in a Confirmatory Phase 3 Clinical Trial in LEMS. J Clin Neuromuscul Dis. 2019 Mar; 20(3):111-119.
8. Mantegazza R, Amifampridine tablets for the treatment of Lambert-Eaton myasthenic syndrome. Expert Rev Clin Pharmacol. 2019 Nov;12(11):1013-1018.
9. D. Lamiable, H. Millart, High-performance liquid chromatographic determination of 4-aminopyridine and 3,4diaminopyridine in rat cerebrospinal fluid and serum. Journal of Chromatography, 272 (1983) 221-225.
10. John A. Perry, Mimi Chang. Sensitive Trace-First Liquid Chromatographic Determination of 4-Aminopyridine in 3,4- Diaminopyridine. Journal of Pharmaceutical Sciences. Volume 79, Issue 5, May 1990, Pages 437-439.
11. ICH, "Guideline on Analytical Method Validation," Proceeding of International Convention on Quality for the Pharmaceutical Industry, Toronto, Canada, 2002.
12. ICH Q1A: Stability Testing of New Drug Substances and Products. (International Conference on Harmonization of Technical Requirements for the Registration of Drugs for Human Use, Geneva, Switzerland, February 2003).