DEVELOPMENT AND VALIDATION OF STABILITY INDICATING REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF RELATED SUBSTANCES IN FAMPRIIDINE DRUG SUBSTANCE AND TABLET DOSAGE FORMS

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INTRODUCTION

Fampridine (4-aminopyridine [AP]) is an organic compound with the chemical formula C4H4N−NH2. It has been used as a drug, to manage some of the symptoms of multiple sclerosis (MS) and is indicated for symptomatic improvement of walking in adults with several variations of the disease [1]. The potassium channel blockers AP and 3,4-diaminopyridine increase nerve conduction in demyelinated nerve fibers and have been proposed as a suggestive therapy for people who are suffering with MS [1]. Fampridine-SR is a sustained-release, orally administered potassium channel blocker acting in the central nervous system to enhance conduction in demyelinated axons and has been proposed as a suggestive therapy for people who are suffering with MS [1]. Fampridine-SR is a sustained-release, orally administered potassium channel blocker acting in the central nervous system to enhance conduction in demyelinated axons and several small trials have evaluated the safety and efficacy of Fampridine-SR in patients with MS to improve their walking ability [2].

Literature survey showed that few analytical methods were reported for the determination of Fampridine in bulk and its formulations. The literature survey showed that Jain et al. [9] published a paper on LC method for the determination of genotoxic impurities in Fampridine active pharmaceutical ingredient. As per the literature, no method was reported for the related compounds in Fampridine dosage forms. The objective of this study is to develop a stability indicating RP-HPLC method for determination of related substances in Fampridine drug substances and tablet dosage form.

Keywords: Fampridine, Related substances, Method validation, Reversed phase high performance liquid chromatographic.

MATERIALS AND METHODS

Materials

HPLC grade acetonitrile was procured from Qualigens, India. Potassium dihydrogen orthophosphate and orthophosphoric acid were purchased from Merck, India. All other chemicals and solvents used were of analytical grade of Rankem. Water used in the HPLC analysis was purified by the water purifier (Milli-Q Millipore). Reference standards of Fampridine and impurities are supplied by GSN Pharmaceuticals Private Limited, Hyderabad, India, as gift samples. Tablets of these drugs were purchased from local market.

Instrumentation

The HPLC system was composed of 2695 water alliance system fitted with 2996 photo diode array (PDA) detector with Empower 2 software. Analytical column used for this method was Inertsil ODS 3V with the dimensions 150 mm × 4.6 mm, 5 µm particle size.

Preparation of mobile phase A (buffer)

Mobile phase A was prepared by dissolving 6.8 g of potassium dihydrogen orthophosphate (0.05 mol) and 1 g of 1-octane sulfonic acid into a 1000 ml of water, pH was adjusted to 4.0±0.05 with diluted orthophosphoric acid. Mobile phase B was prepared by mixing the above phosphate buffer (pH 4.0) and acetonitrile in 20:80 (% v/v). Gradient mode was used with the flow rate of 1.0 ml/minutes, and the peaks were monitored at 260 nm.

Preparation of mobile phase B

Mobile phase B was prepared by mixing of above buffer and acetonitrile in 20:80 (% v/v).
Preparation of diluent
Diluent was prepared by mixing of above buffer and methanol in 80:20 (%/v).

Standard solution preparation
About 10 mg of Fampridine was taken into a 100 ml volumetric flask, and 75 ml of diluent was added and sonicated for 5 minutes to dissolve and made up to volume with diluent. Standard solution was prepared by taking 2 ml of the above solution into a 200 ml volumetric flask and made up to volume with diluent. The final concentration of Fampridine is 1 µg/ml.

Sample preparation
The amount equivalent to 10 mg of Fampridine, finely tablet powder was weighed and transferred into a 25 ml volumetric flask, added 15 ml of diluent and sonicated for 20 minutes to dissolve, made up to volume with diluent and filtered through 0.45 um nylon filter and the final concentration of Fampridine is 0.4 mg/ml.

Optimized chromatographic conditions
The analysis was performed on Inertsil ODS 3V (150 mm x 4.6 mm, 5 µm) column maintained at 35°C using mobile phase A and mobile phase B in gradient mode (Table 2) with a flow rate of 1.0 ml/minutes. Before delivering the mobile phase into the system, it was degassed and filtered through 0.45 µm nylon filter using a vacuum. The injection volume was 10 µl, and the detection was performed at 260 nm using a PDA detector.

RESULTS AND DISCUSSION
Optimum separation between Fampridine and related substances was achieved with the above-optimized conditions. The aim of method validation is to confirm that the present method is suitable for its intended purpose as described in International Council of Harmonization (ICH) guidelines Q2 (R1). The described method has been validated in terms of specificity, forced degradation, limit of detection (LOD) and limit of quantification (LOQ), linearity, accuracy, precision, robustness, and solution stability.

System suitability
To ensure that the system is working correctly during the analysis, tailing factor, theoretical plates and % RSD of six standard injections were checked. The parameters such as tailing factor should not be more than 2.0; theoretical plate should be not <4000 and % RSD for six replicate injections of standard solution should not be more than 5.0. The results of system suitability are summarized in Table 3, and the corresponding chromatogram is shown in Fig 1.

Specificity
Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the method is established by injecting blank, placebo and the impurity spiked sample and their corresponding chromatograms are shown in Figs. 2-4. The results of specificity are presented in Table 4. The chromatograms in figures show that there was no interference of the blank, placebo and main drug substances with impurities and the developed method was successfully separated all the impurities with each other and with the main drug. Hence, the present RP-HPLC method used for the estimation of related substances in Fampridine tablets is very selective and specific.

Forced degradation studies
Forced degradation studies were performed to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to the samples under conditions such as thermal at 60°C for 7 days, humidity (90% relative humidity) for 7 day, photolytic condition (1.2 million lux h), acid hydrolysis (using 1.0 N HCl at room temperature for 2 hrs), base hydrolysis (using 1.0 N NaOH at room temperature for 2 hrs), and oxidative degradation (using 10.0% H2O2 at room temperature for 2 hrs) to evaluate the ability of the proposed method to separate degradation products from each other and active ingredients as well. To check and ensure the homogeneity (peak purity) of all peaks in the stressed sample solutions, a wide range of wavelength (200-400 nm) was applied using PDA detector, and the corresponding results are tabulated in Table 5. In forced degradation, it is observed that Fampridine is susceptible for degradation in oxidation stress condition, and found to be stable in all other stress conditions.

LOD and LOQ
LOD and LOQ for Fampridine and two impurities are determined by injecting a series of solutions of known concentration till the signal-to-noise ratio became as 3:1 and 10:1, respectively, and the corresponding values are given in Table 6. The found LOQ values are sufficient to quantify these impurities below 0.2% of the drug concentration as per the limits defined by pharma regulating agencies. The corresponding results are tabulated in Table 6.
Linearity and relative response factor

The series of solutions were prepared by diluting the Fampridine and impurity stock solution at different concentrations from LOQ to 150%, i.e., with respect to sample concentration (0.4 mg/ml). The correlation coefficients ($r^2$), y-intercepts and relative retention factor values are given in Table 7, which shows that there is an excellent correlation...
(r^2 < 0.995) exist between peak areas and concentration of all analytes.

**Precision**

The precision of the method was verified by injecting six individual preparations (n=6), spiked with two impurities at 0.5% level with respect to the sample concentration (0.4 mg/ml). Results of method precision are given in Table 8. Results in Table 8 indicate that the % RSD for two impurities was found below 10.0%. Results of intermediate precision at different days with different lots of analytical columns are included in Table 9, which shows that the % RSD for all impurities was below 10%.

**Accuracy**

The accuracy of the method was determined by spiking the respective impurities in the sample at LOQ, 50%, 100%, and 150% of specification level (0.5% with respect to the sample concentration 0.4 mg/ml) in triplicate and corresponding recovery values were calculated. The

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**Table 4: Specificity results of spiked sample**

| Name                  | RT  | RT ratio | Resolution | Purity angle | Purity threshold |
|-----------------------|-----|----------|------------|--------------|-----------------|
| Isonicotinamide       | 4.457 | 0.32    | 6.2        | 0.15        | 0.325           |
| Fampridine N-oxide impurity | 11.977 | 0.85    | 3.5        | 0.125       | 0.645           |
| Fampridine            | 14.025 | -       | 0.031      | 0.978       |                 |

**Table 5: Forced degradation results**

| Sample details | Degradation (%) | Assay (%) | Mass balance | Peak purity |
|----------------|----------------|-----------|--------------|-------------|
| As such sample | 0.05           | 100.5     | -            | Pass        |
| Thermal       | 0.12           | 99.2      | 98.8         | Pass        |
| Photolytic    | 0.21           | 99.9      | 99.6         | Pass        |
| Humidity      | 0.32           | 98.2      | 98.0         | Pass        |
| Acid          | 0.21           | 100.2     | 99.9         | Pass        |
| Base          | 0.18           | 98.9      | 98.5         | Pass        |
| Oxidative     | 5.25           | 94.2      | 98.9         | Pass        |

**Table 6: LOD and LOQ values of analytes along with S/N ratios**

| Name                  | LOD (%) | S/N ratio | LOQ (%) | S/N ratio |
|-----------------------|---------|-----------|---------|-----------|
| Isonicotinamide       | 0.03    | 3         | 0.10    | 12        |
| Fampridine N-oxide impurity | 0.03    | 3         | 0.10    | 11        |
| Fampridine            | 0.02    | 3         | 0.07    | 10        |

**Table 7: Linearity results for Fampridine and its impurities**

| Name                  | Correlation coefficient (r^2) | Y-intercept at 100% level | RR % RRF |
|-----------------------|-------------------------------|---------------------------|---------|
| Isonicotinamide       | 0.995                         | -1.1                      | 1.12    |
| Fampridine N-oxide impurity | 0.997                         | 0.5                       | 1.35    |
| Fampridine            | 0.999                         | 1.2                       | -       |

**Table 8: Method precision results (% of impurities)**

| Sample id | Isonicotinamide | Fampridine N-oxide impurity | Total impurities |
|------------|-----------------|-----------------------------|------------------|
| Spl-1      | 0.49            | 0.51                        | 0.95             |
| Spl-2      | 0.46            | 0.49                        | 1.01             |
| Spl-3      | 0.49            | 0.52                        | 0.95             |
| Spl-4      | 0.48            | 0.47                        | 1.03             |
| Spl-5      | 0.52            | 0.51                        | 1.02             |
| Spl-6      | 0.51            | 0.51                        | 0.95             |
| Mean       | 0.49            | 0.50                        | 0.99             |
| SD         | 0.02            | 0.02                        | 0.04             |
| % RSD      | 4.3             | 3.7                         | 3.5              |

**Table 9: Intermediate precision results (% of impurities)**

| Sample id | Isonicotinamide | Fampridine N-oxide impurity | Total impurities |
|------------|-----------------|-----------------------------|------------------|
| Spl-1      | 0.52            | 0.49                        | 1.01             |
| Spl-2      | 0.51            | 0.52                        | 1.03             |
| Spl-3      | 0.48            | 0.47                        | 0.95             |
| Spl-4      | 0.50            | 0.49                        | 0.99             |
| Spl-5      | 0.49            | 0.50                        | 0.99             |
| Spl-6      | 0.51            | 0.52                        | 1.03             |
| Mean       | 0.50            | 0.50                        | 1.00             |
| SD         | 0.01            | 0.02                        | 0.03             |
| % RSD      | 2.9             | 3.9                         | 3.0              |

Spl: Sample, SD: Standard deviation, n=6 sample preparations, RSD: Relative standard deviation
recovery values are found within the range of 98.5-104.5% with the % RSD of <1.7, which indicate that the method is more reliable and accurate. The results of accuracy study are summarized in Table 10.

**Robustness**

The robustness of the method was checked by intentional changes in flow rate, column temperature and pH of the mobile phase. The flow rate of the mobile phase was changed to 0.9 ml/minutes and 1.1 ml/minutes. The effect of pH was studied at pH 3.9 and 4.1. The effect of column temperature was studied at 25°C and 35°C. The resolution between adjacent peaks of all samples was evaluated, and it was found >2.0.

**Solution stability**

To check the stability, both standard and impurity spiked samples were kept at refrigerator condition (5°C) and room temperature (25°C). Much change was not observed in the area of the respective impurities. The results of solution stability studies confirmed that both standard and test solutions were stable up to 24 hrs.

**CONCLUSION**

A specific, linear, precise and accurate stability indicating HPLC method has been developed for the quantification of two impurities in the tablet dosage form. The method has been validated for specificity, linearity, accuracy, precision, robustness, and stability. The method is linear in the range of LOQ to 150% of the specification concentration for two impurities with a correlation coefficient not <0.995. The accuracy of the method is in the range of 98.5-104.5% for two impurities. As the method is validated according to ICH guidelines, it can be used for the analysis of all these two impurities in the tablet dosage forms.

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| Sample name             | LOQ level | 50% level | 100% level | 150% level | % mean | SD | % RSD |
|-------------------------|-----------|-----------|------------|------------|--------|----|-------|
| Isonicotinamide         | 103.2     | 102.5     | 99.5       | 101.5      | 101.7  | 1.6| 1.6   |
| Fampridine N-oxide impurity | 100.2     | 101.9     | 98.5       | 103.2      | 100.2  | 1.7| 1.7   |
| Total impurities        | 103.5     | 102.2     | 100.4      | 104.5      | 102.7  | 1.8| 1.7   |

SD: Standard deviation, RSD: Relative standard deviation, LOQ: Limit of quantification