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"DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR ESTIMATION OF DALFAMPRIDINE, IN BULK AND PHARMACEUTICAL DOSAGE FORM."

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ABSTRACT

A rapid and precise reverse phase high performance liquid chromatographic method has been developed for the validated of Dalfampridine in its pure form as well as in tablet dosage form. Chromatography was carried out on ODS C18 (4.6 x 250mm, 5µm) column using Acetonitrile and water in the ratio of 80:20 v/v,as the mobile phase at a flow rate of 1.0mL/min, The retention time obtained for DFP was 2.98 min. The method produce linear responses in the concentration range of 20 µg/ml of Dalfampridine . The method precision for the determination of assay was below 2.0%RSD. The method is useful in the quality control of bulk and pharmaceutical formulations.

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INTRODUCTION

Pharmaceutical Analysis Plays a very vital role in the quality assurance and quality control of bulk drugs and their formulations. Pharmaceutical analysis is a specialized branch of analytical chemistry which involves separating, identifying and determining the relative amounts of components in a sample of matter. It is concerned with the chemical characterization of matter both quantitative and qualitative.

SPECTROPHOTOMETRIC METHODS

Spectrophotometry is generally preferred especially by small-scale industries as the cost of the equipment is less and the maintenance problems are minimal. The method of analysis is based on measuring the absorption of a monochromatic light by colorless compounds in the near ultraviolet path of spectrum (200-380nm). The photometric methods of analysis are based on the Bouger-Lambert-Beer's law, which establishes the absorbance of a solution is directly proportional to the concentration of the analyte. The fundamental principle of operation of spectrophotometer covering UV region consists in that light of definite interval of wavelength passes through a cell with solvent and falls on to the photoelectric cell that transforms the radiant energy into electrical energy measured by a galvanometer.

The important applications are

- Identification of many types of organic, inorganic molecules and ions.
- Quantitative determination of many biological, organic and inorganic species.
- Monitoring and identification of chromatographic of effluents.

HPLC METHOD DEVELOPMENT

The term 'Chromatography' covers those processes aimed at the separation of the various species of a mixture on the basis of their distribution characteristics between a stationary and a mobile phase.

MODES OF CHROMATOGRAPHY

Modes of chromatography are defined essentially according to the nature of the interactions between the solute and the stationary phase, which may arise from hydrogen bonding, Vander walls forces, electrostatic forces or hydrophobic forces or basing on the size of the particles (e.g. Size exclusion chromatography).

Different modes of chromatography are as follows:

♦ Normal Phase Chromatography
♦ Reversed Phase Chromatography
♦ Reversed Phase – ion pair Chromatography
♦ Ion-Exchange Chromatography
♦ Size Exclusion Chromatography

METHOD VALIDATION

Method validation can be defined as (ICH) "estabilishing documented evidence which provides a high degree of assurance that specific activity will consistenty produce a desired result or product meeting its predetermined specifications and quality characteristic, Method validation is an integral part of the method development; it is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, an and drug products. Simply, method validation is the process of proving that and potency of the drug substances analytical method is acceptable for its intended purpose.

For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters

(a) Recovery (b) Response function (c) Sensitivity (d) Presicion (e) Accuracy (f) limits of detection (g) Limit of quantitation (h) Ruggedness (i) Robustness (j) stability (k) system suitability

Recovery:

The absolute recovery of analytical method is measured as the response of a processed spiked matrix standard expressed as a percentage of the response of pure standard which has not been subjected to sample pre treatment and indicates whether the method provides a response for the entire amount of analyte that is present in the sample.

$$\text{Absolute recovery} = \frac{\text{Response of an analyte spike into matrix (processed)}}{\text{Response of analyte of pure standard (unprocessed)}} \times 100$$

Sensitivity:

The method is said to be sensitive if small changes in concentration cause large changes in response function. The sensitivity of an analytical method is determined from the slope of the calibration line. The limits of quantification (LOQ) or working dynamic range of bio analytical method are defined as the highest and lowest concentrations, which can determined with acceptable accuracy. It is suggested that, this be set at ± 15% for both the upper and lower limit of quantitation respectively.

Precision:

The purpose of carrying out a determination is to obtain a valid estimate of a 'true' value. When one considers the criteria according to which an analytical procedure is selected, precision and accuracy are usually the first time to come to mind. Precision and accuracy together determine the error of an individual determination.

Precision refers to the reproducibility of measurement within a set, that is, to the scatter of dispersion of a set about its central value. The standard deviation S, is given by

$$S = \sqrt{\frac{1}{n-1}\sum_{i-1}^{n}(x_i - \bar{x})^2}$$

The square of standard deviation is called variance (S^2). Relative standard deviation is the standard deviation expressed as a fraction of the mean, i.e., S/x. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision.

% Relative standard deviation = S x 100 / x

Accuracy:

Accuracy normally refers to the difference between the mean x****, of the set of results and the true or correct value for the quantity measured. According to IUPAC accuracy relates to the difference between results (or mean) and the true value. For analytical methods, there are two possible ways of determining the accuracy, absolute method and comparative method.

$$\%Bias = \frac{(\text{measured value} - \text{true value})}{\text{true value}} X\,100$$

Limit of detection (LOD):

The limit of detection (LOD) of an analytical method may be defined as the concentration, which gives rise to an instrument signal that is significantly different from the blank. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (Sa), which may be related to LOD and the slope of the calibration curve, b, by:

LOD = 3 Sa/ b

Limit of quantitation (LOQ)

The LOQ is the concentration that can be quantitate reliably with a specified level of accuracy and precision. The LOQ represent the concentration of analyte that would yield a signal-to-noise ratio of 10.

LOQ = 10 Sa/ b

Where, Sa- the estimate is the standard deviation of the peak area ratio of analyte to IS (5 injections) of the drugs. b -is slope of the corresponding calibration curve.

Ruggedness

Method Ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments, source of reagents, chemicals, solvents etc..

Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small but deliberate variations in method parameters". The robustness of a method is the ability to remain unaffected by small changes in parameters such as pH of the mobile phase, temperature, %organic solvent strength and buffer concentration etc

System suitability

MATERIAL AND METHODS

Raw material characterization

Characterization of Dalfampridine (DFP)

Determination of Melting point

The melting point of Dalfampridine was determined by digital melting point apparatus The reference melting point for DFP is 157 to 161°C.

Determination of λ max by UV

Stock solution of DFP was prepared by accurately weighing 10mg of DFP dissolved in 100ml Acetonitrile: water (50:50) mixture to yield a concentration of 100µg/ml. From the stock solution of DFP 1.0ml was pipette out and diluted up to 10ml by using Acetonitrile: water (50:50) to yield resultant concentration stock solution of DFP as 10µg/ml. The solution was filtered through 0.45µ membrane filter. Further, the solution was scanned using UV Visible spectrophotometer (Schimadzu) between 200-400nm. The spectrum was recorded and analyzed the peak to determine wavelengths and their corresponding absorbances. An appropriate conclusions were drawn from the results obtained.

Characterization of reagents and chemicals

Table 1 Characterization of reagents and chemicals.

Ingredients	Grade	Suppliers	Characterization
Methanol	HPLC	Merck	As HPLC grade no further characterization performed
Acetonitrile	HPLC	Merck	As HPLC grade no further characterization performed
Water	HPLC	Merck	As HPLC grade no further characterization performed
Ortho phosphoric acid	HPLC	Avra	As HPLC grade no further characterization performed

All reagents and chemicals used were HPLC grade. Hence no further characterization was performed.

Experimental work

HPLC method development for Dalfampridine (DFP)

Preparation of standard stock solutions DFP

Weighed accurately 10mg of DFP and transferred to 100ml volumetric flask containing a mobile phase as Acetonitrile: Buffer in the ratio of 70:30v/v. The volume was made up to the mark using same composition of solvent to get resulting solution of 100µg/ml. The resulting solution was filtered through 0.45µ membrane filter paper and ultrasonicated for 30 min in three cycles each of 10min.

Preparation of working solution of DFP

0.5ml of standard solution prepared as above, (100µg/ml) was pipette out and diluted to 10ml (in 10 ml volumetric flask) to obtain working solution of 5µg/ml. This solution was filtered through 0.45µ membrane filter and ultra-sonicated for three cycles each of 10min.The resultant solution was used for further method development using RP-HPLC.

Optimization of chromatographic conditions

The optimization of chromatographic conditions was achieved by trial and error on C_{18} stationary phase (250mm x 4.6mm, 5µ id). Trial runs were performed with chromatographic conditions as given in **Table 3** below.

Table 2 Changes in chromatographic conditions for trail studies.

Trial	Mobile phase (% v/v)	Flow rate (ml/min)	Wavelength (nm)	pH
1	Methanol Water (50:50)	0.9	262	--
2	Acetonitrile: Water (50:50)	0.9	262	--
3	Acetonitrile: Phosphate buffer (55:45)	0.9	262	3.0
4	Acetonitrile: Phosphate buffer (80:20)	0.9	262	3.0

System suitability testing (SST)

5µg/ml standard working solution was selected for SST. The working solution of DFP was prepared as per procedure laid down in section 2.2.1.2 of this chapter. The six repeated measurements of working solution (5µg/ml) were recorded using RP-HPLC in following optimized chromatographic conditions as shown in Table 4. The detection was carried out at 262nm with UV detector. The chromatograms obtained in each case were integrated to determine peak area, retention time, number of theoretical plates (NOP) and USP tailing factor. The results recorded were subjected to statistical analysis by determining parameters like mean, standard deviation (SD) and percent relative standard deviation (%RSD). The results obtained after statistical analysis were compared for their compliance as per ICH guideline Q2R1.

Table 3 Optimized chromatographic conditions.

Chromatographic conditions	
Buffer preparation	1.36 g Potassium dihydrogen phosphate into 1 liter pH adjusted to 3.00 using Orthophosporic acid
MOBILE PHASE	Buffer : Acetonitrile (20:80)
Column	250 x 4.6 mm C18
Flow rate	0.90 ml/min
Wavelength	262 nm
Run time	7.0 min
Injection Volume	10 µl
Temperature	25°C
HPLC MAKE	Thermo Fischer Scientifics
Soft ware	Chromequest 4.1

HPLC method validation

Preparation of stock solution

This standard stock solution was prepared in the comparable method as given section 2.2.1.1 and standard working solution was prepared from the standard stock solution as per necessities of particular experiment.

Linearity and Range

From standard stock solution of DFP, aliquots of 0.5ml, 1.0ml, 1.5ml, 2.0ml, 2.5ml and 3.0ml were pipetted out in six different 10ml volumetric flask and volume was made up to the mark of the flask with mobile phase, Acetonitrile: Water (80:20) to attain ultimate concentrations of 5, 10, 15, 20, 25 and 30µg/ml. These standard working solutions of DFP were injected to chromatographic conditions as set above in Table 4 and chromatograms were recorded at 262nm. The calibration curve was plotted between concentrations of DFP verses comparative peak area of each standard concentration. Results were recorded for equation of line, correlation coefficient and intercept from the calibration curve of DFP. The general equation of line is given by:

$$Y = mX + c$$

Where,

Y- Area under the chromatogram

X- Unknown concentration

m- Slope of calibration curve and 'c' is intercept.

Precision

Precision of the method was established across range of calibration curve (5-30µg/ml). As range was noted to be 5-30µg/ml for DFP, the tree quality control (QC) standards were defined across this range viz. 7, 17 and 27ppm for precision experiment. The working solutions of these QC standards were prepared from standard stock solution of DFP by withdrawing 0.7, 1.7 and 2.7ml in three 10 ml volumetric flasks respectively. The volume of each flask was made up to the mark using mobile phase to get secondary solutions of 7, 17 and 27µg/ml respectively. The precision of the method was studied by repeatability and intermediate precision. The repeatability was assessed by intra-day measurements of three QC standards selected as above in triplicate at an interval of three hrs. However, intermediate precision was studied on three different days with identical solutions of three QC standards. The chromatograms recorded were integrated to determined peak area, retention time, NOP and tailing factor. The peak area was then subjected to statistical analysis by calculating mean, standard deviation (SD) and percent relative standard deviation (%RSD). The %RSD was then compared with standards as per ICH Q2R1 guideline.

Accuracy

% Accuracy can be estimated by more than one method as per ICH Q2R1 guidelines. In this project, % accuracy was studied two different methods firstly, by minimum nine determinations three levels across the given range. Secondly, by percent recovery method as discussed in later section of this project work. Data obtained for inter-day precision experiment was used here for the determination of percent accuracy by first method. The % accuracy of DFP was determined by using subsequent formula as given below. The results obtained for % accuracy were compared with pharmacopoeial standards for their compliance.

$$\% \text{ Accuracy} = \frac{\text{Mean measured concentration}}{\text{Nominal Concentration}} \times 100$$

Robustness

Robustness of the presented method of DFP was studied by decisive disproportion in method limits. In this case, the method limits like mobile phase flow rate (in ml/min) and wavelength (in nm) were varied as per **Table 5**. The 5µg/ml concentration of standard solution of DFP was selected for this study and it was kept indistinguishable all the way through all system parameters varied. The selected concentration (5µg/ml) of DFP was injected to given chromatographic conditions in triplicate at each level of variation and chromatograms recorded. From the chromatograms obtained mean peak area was determined in all measurements made.

Table 4 Design of robustness experiment.

Method parameters	Standard	Variation 1	Variation 2
Temperature in ^{O}C (± 1)	25	26	24
Flow Rate in ml/min (± 0.1ml)	0.9	1.0	0.8

Mean peak area obtained in each case was then used for calculation of mean measured concentration at each level of variation using equation of line. The mean measured concentrations obtained in each case for DFP were then finally used for determination of percent assay of DFP. The percent assay results of DFP were evaluated for compliance with the pharmacopoeial standards of DFP in tablet dosage form.

%Recovery

Preparation of stock from API

10 mg of DFP (API) was precisely weighed and transferred to 10ml volumetric flask containing few ml of mobile phase (80:20 Acetonitrile: buffer at pH 3.0) and volume was made up to the mark (10ml) using mobile phase to obtain final concentration standard solution of DFP 100µg/ml. The resulting solution was filtered through 0.45µ membrane filter and ultra-sonicated30 min in three cycles each of 10 min. From the main stock solution DFP (100µg/ml) 1.0ml was withdrawn using micro-pipette three times and kept in three 10ml volumetric flasks. The content of each flask was diluted to 10ml to obtain solution of 10µg/ml. These standard working solutions of DFP were injected for given chromatographic system in triplicate and mean peak area was determined.

Preparation of standard stock solution from dosage form

Ten tablets (Label claim 50mg of Dalfampridine, Instgra, Emcure pharmaceuticals Ltd.) were weighed and average weight was determined. Powder equivalent to 10mg of DFP was transferred to 100ml volumetric flask containing few ml of mobile phase. The content of the flask was then diluted with mobile phase to 100ml to obtain resultant solution of 100µg/ml as sample stock solution of DFP. The substantial solution was filtered through 0.45µ membrane filter and ultra-sonicated for 30min in three cycles each of 10 min. From the sample stock sample solution of DFP aliquots of 0.8, 1.0 and 1.2ml were withdrawn in three different 10ml volumetric flasks and the aliquot solution of each of these flasks was diluted up to 10ml using mobile phase to obtain resultant working sample solutions of 8, 10, 12µg/ml respectively.

Preparation of test solution of DFP for % recovery by spike method

10µg/ml standard solution (prepared formerly) of DFP (API) was spiked into each of above sample solutions of DFP viz. 8, 10 and 12µg/ml to get test solutions at 80%, 100% and 120% levels respectively. Each of these three levels test solutions of DFP was injected in triplicate to the given chromatographic conditions and mean peak area for each level was determined. The mean peak area obtained for standard solution of DFP injected (previously determined) was deducted from the mean peak area of each of these three levels to acquire peak area equivalent to each sample solutions of DFP. % recovery was calculated from the sample area and standard area using following formula as given below.

$$\% \text{ Recovery} = \frac{Sample\ Area}{Standard\ Area} \times \frac{Standard\ Concentration}{Sample\ Concentration} \times 100$$

LOD and LOQ

Limit of detection (LOD) and Limit of quantitation (LOQ) were calculated from the next formulae. The LOD and LOQ should be articulated in µg/ml for DFP.

$$LOD = \frac{3.3 * STEYX}{Slope}$$

$$LOQ = \frac{10 * STEYX}{Slope}$$

Where, STEYX = Standard error of Y and X axis and calculated from calibration curve of DFP.

RESULTS AND DISCUSSION

Melting point determination of Dalfampridine (DFP)

The melting point of a substance is the temperature at which it alters state from solid to liquid. At the melting point the solid and liquid phase, exist in equilibrium. The melting point of a substance depends on pressure and is frequently specified at a standard pressure such as 1 atmosphere or 100 kPa.

The melting point of Dalfampridine was determined by digital melting point apparatus and was found to be 159 °C. The reference melting point for DFP is 157 to 161°C. The observed melting point was in agreement was in consistency with reference value. Therefore, it was concluded from this study that the sample obtained for Dalfampridine was possessed sufficient purity.

Determination of absorption maxima (λ_{max}) using UV Visible spectrophotometer

The absorption maximum (λ_{max}) of DFP was determined in the mobile phase of Acetonitrile and Water in the ratio of 50:50. The 10µg/ml standard working solution of DFP was selected for this study and the same was prepared as per procedure laid down in chapter 6. The UV spectrum recorded for DFP was as shown in Figure 14.

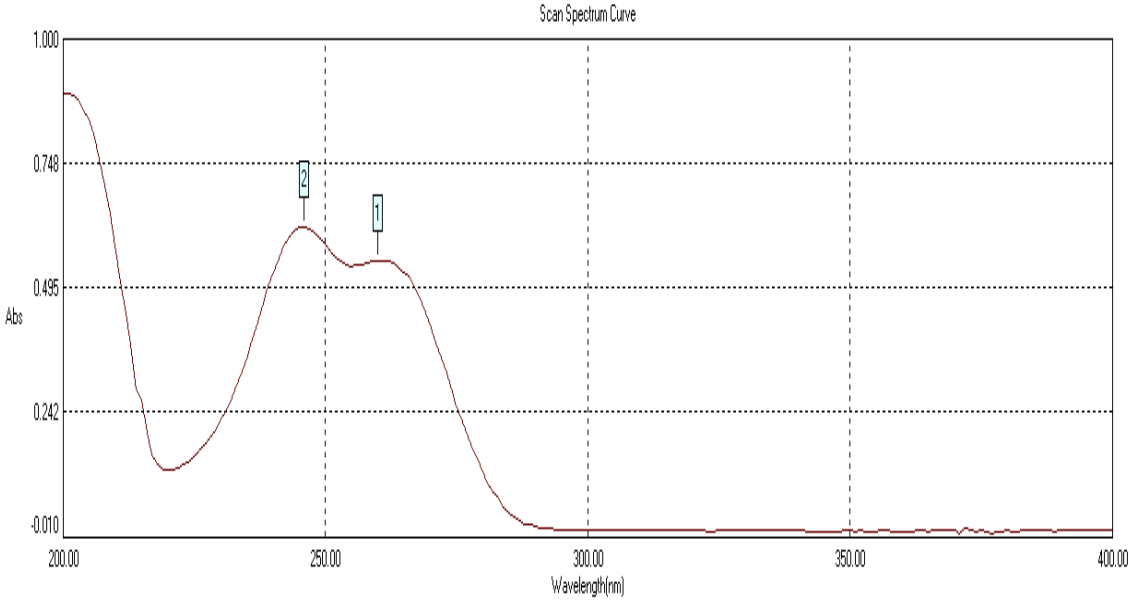

Figure 1 UV spectrum of Dalfampridine obtained in Mobile phase.

The UV spectrum obtained showed two absorbance for DFP. Considerable absorbance was measured at wavelength 246 and 262nm and same was as demonstrated in Table 6. Consequently, the highest wavelength of 262nm was selected for the analysis of DFP with HPLC. Hence, HPLC detector was tuned to 262nm to measure the concentrations of DFP injected while RP-HPLC method development.

Table 5 Observation for UV spectrum analysis of DFP.

Sr. No.	Wavelength (nm)	Absorbance
1.	262	0.552
2.	246	0.620

Characterization of reagents and chemicals

As all reagents and chemicals used as per **Table 2** for the RP-HPLC method development of DFP were HPLC grade, hence no further characterization was performed.

Experimental work

RP-HPLC method development

Methods of analysis are routinely developed, improved, validated, collaboratively studied and applied. Compilations of these developed methods then appear in large compendia such as USP, BP and IP, etc. In most cases as desired separation can be achieved easily with only a few experiments. In other cases a considerable amount of experimentation may be needed. However, a good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result(s). The development of a method of analysis is usually based on prior art or existing literature using almost the same or similar experimentation. The development of any new or improved method usually tailors existing approaches and instrumentation to the current analyte, as well as to the final need or requirement of the method.

Method development usually requires selecting the method requirements and deciding on what type of instrumentation to utilize and why. In the HPLC method development stage, decisions regarding choice of column, mobile phase, detectors, and method quantitation must be considered. So development involves a consideration of all the parameters pertaining to any method.

Therefore, development of a new HPLC method involves selection of best mobile phase, best detector, best column, column length, stationary phase and best internal diameter for the column[i]. The analytical strategy for HPLC method development contains a number of steps[ii].

Preliminary HPLC method development

Preliminary HPLC method development was achieved by trial and error approach. Various combinations of mobile phase and flow rate were tested in order to obtain better results in terms of retention time, peak area, number of theoretical plates and tailing factor.

Selection of mobile phase

The mobile phase selection was based on the use of different mobile phases or different composition of mobile phase. It was also consisted of use of different flow rate in ml/min as per the requirement of the experiment. The trial runs were executed as per conditions laid down in Table 3. The chromatogram resulted after trial 1 was as shown in Figure 15.

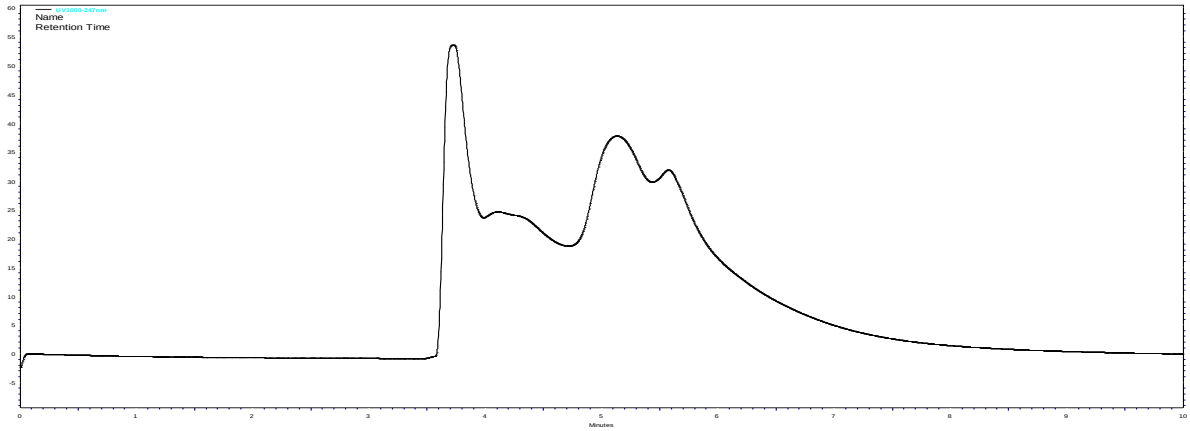

Figure 2 Chromatogram for Trial 1 using Methanol Water in the ratio of 50:50 at 262 nm.

As shown in Figure 15, no peak for DFP was observed hence it was rejected. Another trial was executed by changing mobile phase from Methanol: Water (50:50) to Acetonitrile: Water in the ratio of 50:50 and the chromatogram was recorded as shown in Figure 16. As shown in Figure 16, peak shape was not in acceptable limits and dimensions. Therefore, this was also rejected. Trial 3 was run using mobile phase composition of Phosphate buffer: Acetonitrile in the ratio of 45:55. Furthermore, the pH of the aqueous phase of the mobile phase was also adjusted to 3.0 using ortho phosphoric acid solution. The flow rate was 0.9ml/min.

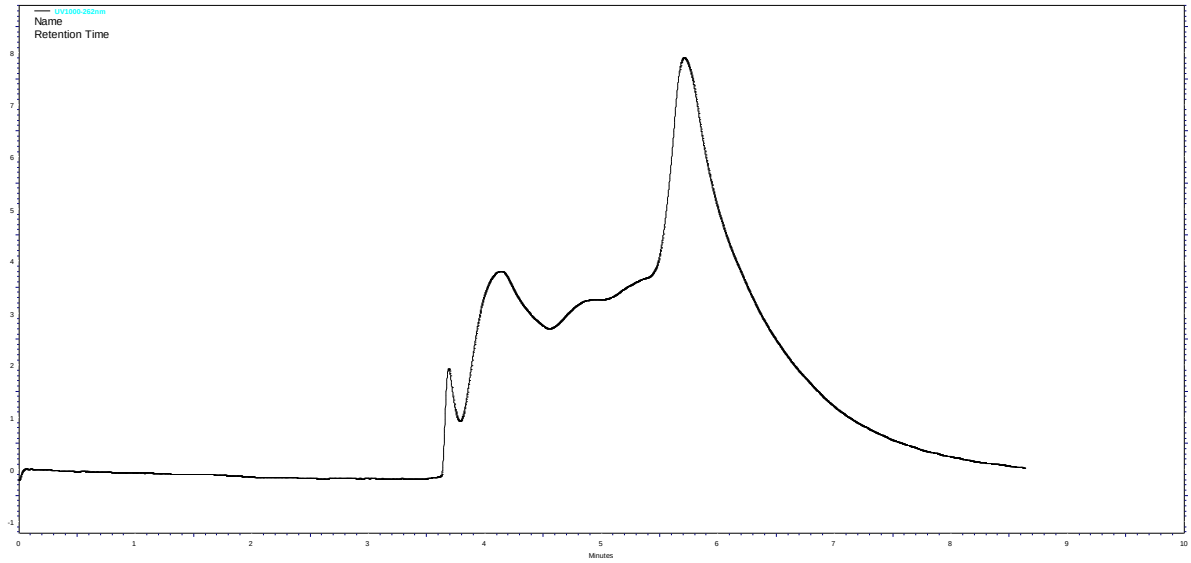

Figure 3 Trial 2 chromatogram obtained with Acetonitrile and Water in the ratio of 50:50 at 262nm.

Trial 3, chromatogram was obtained as shown in **Figure 17**. The chromatogram obtained was then integrated to determine retention time in minutes, peak area, number of theoretical plates. tailing factor. The results obtained were as shown in peak Table 7.

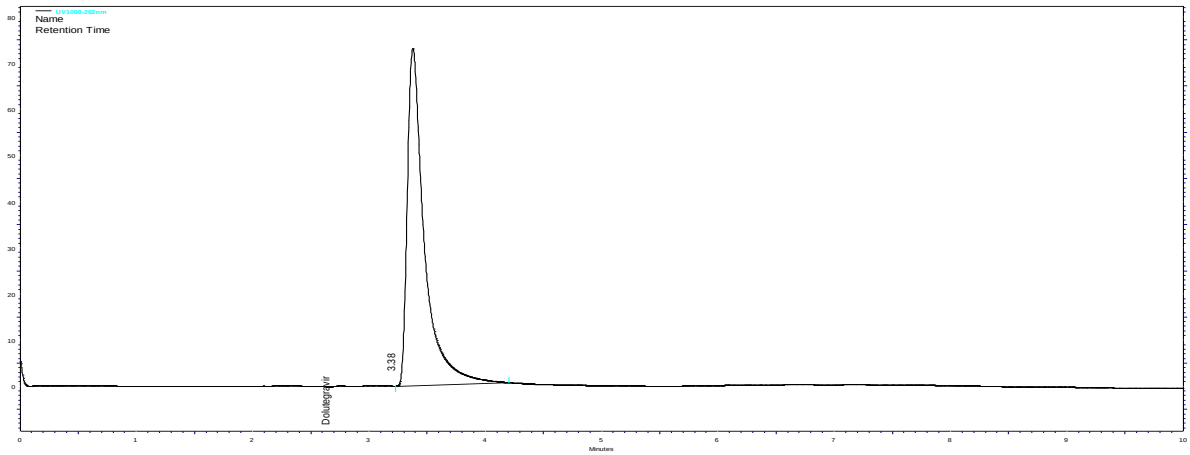

Figure 4 Trial 3 chromatogram using Acetonitrile and Phosphate buffer in the ratio of 45:55 at pH 3.0 and wavelength 262nm.

Table 6 Peak table for Trial 3 of DFP.

Name	Retention Time (min)	Area	No. of Theoretical Plates	Tailing Factor
DFP Trial 3	3.38	226388	3232	2.19

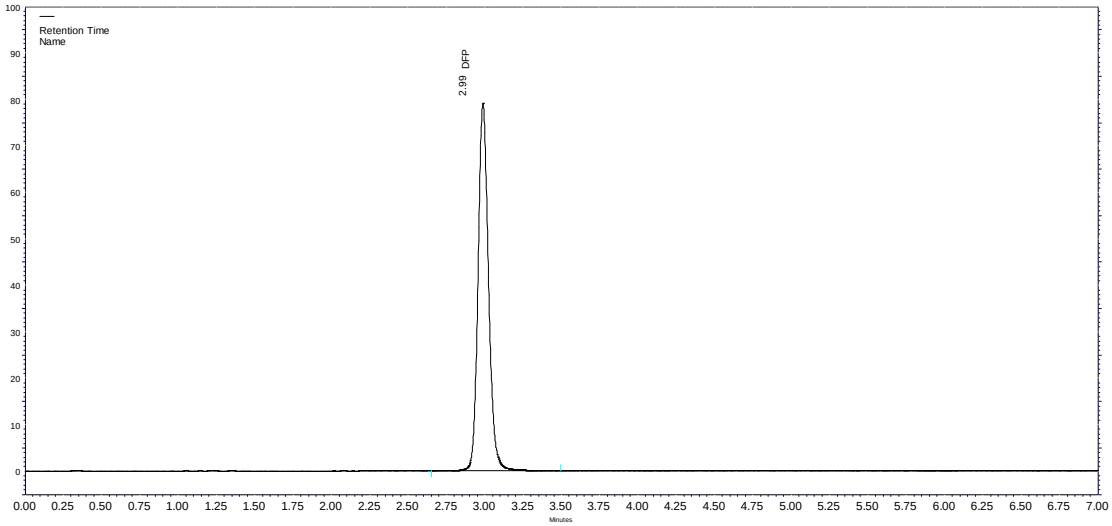

Figure 5 Trial 4 chromatogram using Acetonitrile and Phosphate buffer in the ratio of 80:20 at pH 3.0 and wavelength 262nm.

Table 7 Peak table for Trial 4 of DFP.

Inj. No.	Name	Retention Time(min)	Area	No. of Theoretical Plates	Tailing Factor
1.	DFP Trial 4	2.99	391087	9333	1.14

Trial 4, chromatogram was obtained as shown in Figure 18. The chromatogram obtained was then integrated to determine retention time in minutes, peak area, number of theoretical plates and tailing factors. The results obtained were as shown in peak Table 8.

As shown in Table 8, the retention time was observed at 2.99min with corresponding peak area of 391087. The peak was within limits of USP tailing factor and observed as 1.14. Also, numbers of theoretical plate notes were in the prescribed limits as per ICH Q2R1 guidelines. Therefore, this method conditions were selected for further RP-HPLC method development in the later sections of this research work.

System suitability testing

System suitability testing (SST) is required by USP and FDA to check and ensure on-going performance of analytical systems and methods. Both USP and EP have chapters with recommendations for system suitability tests that are enforced by FDA and other agencies. Related chapters have been updated by USP and EP and they also answer the question as to how

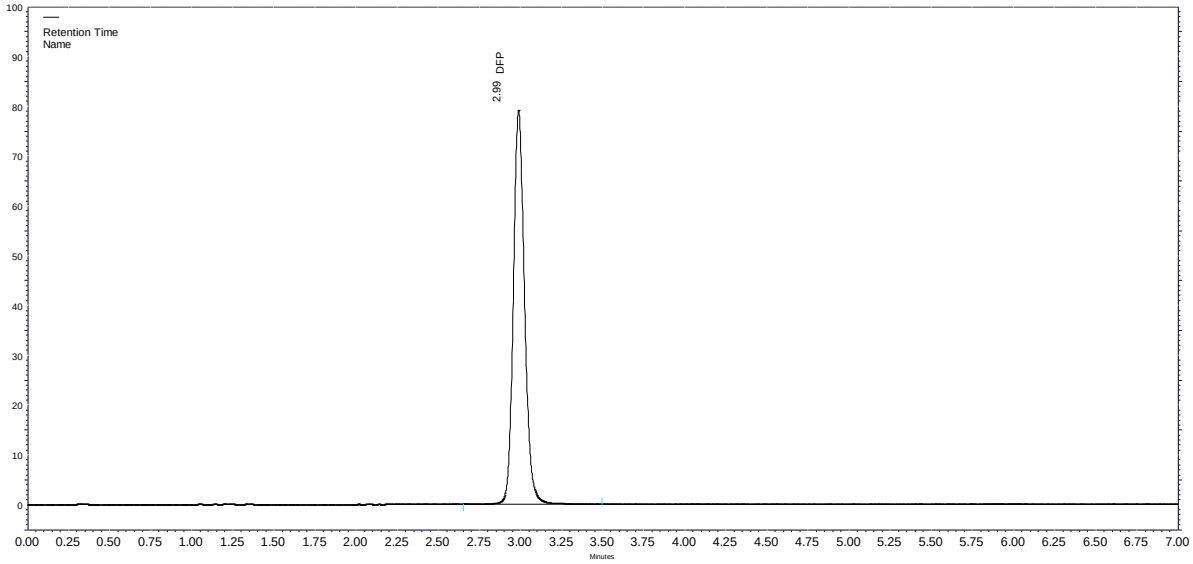

Figure 6 Chromatogram obtained for Dalfampridine in SST.

Table 8 Peak table of DFP SST.

Name	Retention Time (min)	Peak Area	No. of Theoretical Plates	Tailing Factor
DFP SST 1	2.99	396592	9334	1.15

The most excellent separation and peak shape, devoid of unnecessary tailing, were seen by use of chromatographic conditions. The reasonable retention time of 2.99min was obtained with mobile phase containing Acetonitrile: Phosphate buffer, at pH 3.0 maintained using ortho phosphoric acid (80:20). A representative chromatogram was shown in **Figure 20**.

The results obtained for peak area, retention time were assessed by calculating statistical parameters like standard deviation (SD) and percent relative standard deviation (%RSD). The outcome of SD, %RSD, tailing factor and number of theoretical plates (NOP) were as described in Table 10. The results observed for the test of system suitability of DFP were seen in synchronization with the ICH guideline Q2R1.

Table 9 Observations obtained for SST study of DFP.

Sr. No.	Parameter	Mean observations	SD	%RSD	Acceptance criteria	Inference
1	Peak Area	394241.83	5289.56	1.34	< 2	Pass
2	Retention time	2.98	0.0081	0.27	< 0.5	Pass
3	Number of Theoretical plates*	9334	--	--	> 2000	Pass
4	Tailing factor*	1.15	--	--	< 2	Pass

*The values are for the representative chromatogram as shown in Figure 20.

Consequently, from system suitability testing it was concluded that the system with set chromatographic parameters in Table 10 was fitting for quantitative estimation of DFP at 262nm.

RP-HPLC method validation

Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

A linear relationship should be evaluated across the range (see section 3) of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighing of synthetic mixtures of the drug product components, using the proposed procedure. The latter aspect can be studied during investigation of the range.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample.

For the establishment of linearity, a minimum of six concentrations is recommended. Other approaches should be justified.

A linear relationship was observed across the range of 5-30µg/ml by RP-HPLC method of DFP. Linearity was studied by visual examination of a plot of mean peak area as a function of DFP concentration as well as statistical analysis. Results were evaluated by calculation of a regression line by the method of least squares. Data from the regression line i.e. correlation coefficient (r^2 = 0.999) itself was proved to be supportive to offer mathematical knowledgeable supposition of the extent of linearity.

The calibration curve obtained for DFP was as shown in Figure 21. The notes of mean peak area parallel to standard solutions of DFP 5-30µg/ml were as shown in Table 11.

From the results obtained the equation of line was obtained and was witnessed to be Y = 84843x – 42823.

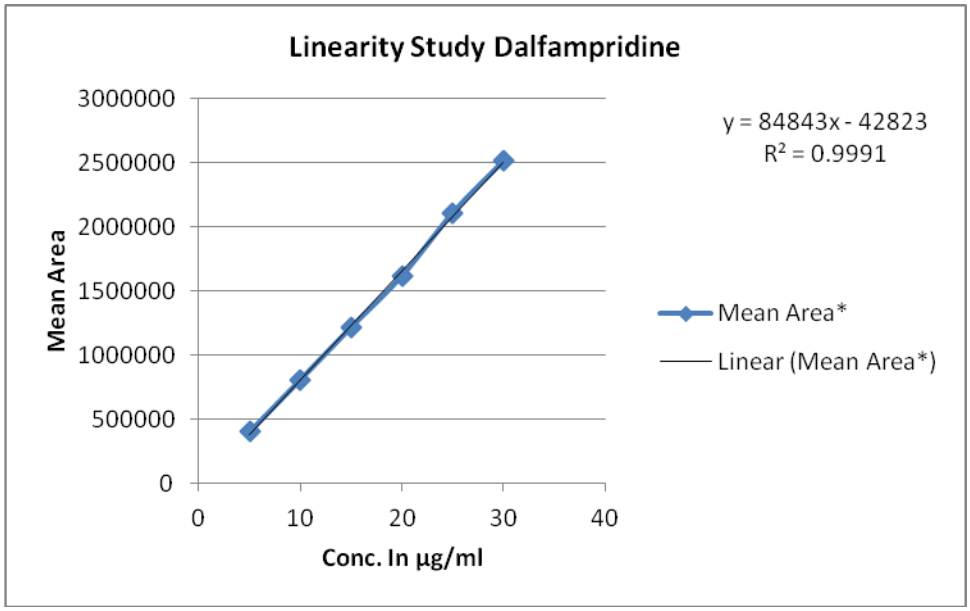

Figure 7 Calibration Curve of Dalfampridine indicated linearity between test results.

Table 10 Observations obtained for Calibration Curve of DFP.

Sr. No.	Conc. of DFP (µg/ml)	Mean Peak Area*
1	5	399592
2	10	806411
3	15	1215231
4	20	1615111
5	25	2105585
6	30	2509600

The correlation coefficient (r^2) was found to be 0.999 suggesting paramount linear association among mean peak area of DFP and corresponding standard concentrations of DFP in the range of 5-30µg/ml.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Repeatability should be assessed using:

a) A minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each);

or

b) A minimum of 6 determinations at 100% of the test concentration.

Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. The use of an experimental design (matrix) is encouraged Repeatability (Intra-day precision) was estimated with nine determinations across the defined range (5-30µg/ml) for the RP-HPLC method of DFP. Three concentrations labeled as QC standards were 7, 17 and 27µg/ml defined for this experiment of DFP. Three repetitive injections were made in a day (at an interval of three hrs) in order to establish repeatability of the RP-HPLC method developed for DFP.

Furthermore, proposed RP-HPLC method was established to be meticulous by assessing it for intermediate precision. In this experiment of RP HPLC method of DFP, an intermediate precision was established by studying a precision experiment on three different days three QC standards as above. The assessments were done on three days with identical standard concentrations of QC standards of DFP and the chromatograms were recorded to determine mean peak area. The mean peak area was then evaluated by calculating SD and %RSD. The results calculated for repeatability and intermediate precision were as demonstrated in Table 12. From the results as shown in Table 12, it was seen that the calculated %RSD was observed for three QC standards as 0.56, 0.55, 0.57 and 0.66, 0.93, 0.31 for intra-day (repeatability) and inter-day precision (intermediate precision) in that order. As %RSD obtained in both studies of precision was obtained in obedience with the standards approved in ICH guideline Q2R1. It was fulfilled that the developed RP-HPLC method ascertained to be precise in the specified range of 5-30 µg/ml for DFP.

Table 11 Observations noted for repeatability and intermediate precision for DFP.

Conc. (µg/ml)	Intra-day precision (Repeatability)			Inter-day precision (Intermediate precision)		
	Mean area ± SD	% RSD	Inference	Mean area ± SD	% RSD	Inference
7	582940.00 ± 3266.64	0.56	Passed	582118.66 ± 3882.46	0.66	Passed
17	1420474.00 ± 7789.86	0.55	Passed	1414545.00 ± 13121.39	0.93	Passed
27	2274106.33 ± 12999.91	0.57	Passed	2282160.33 ± 7136.11	0.31	Passed

% Accuracy

Table 12 Observations for percent accuracy estimated from precision data.

Sr. No.	Conc. (µg/ml)	Mean Peak Area*	Mean Measured Conc. (µg/ml)	% Accuracy (w/w)	Inference (std. for DFP 90-110 % w/w)
1	7	582118.67	7.37	105.23	Passed
2	17	1414545.00	17.18	101.04	Passed
3	27	2282160.33	27.40	101.49	Passed

*Mean peak area of three replicate injections

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.
This is sometimes termed trueness. Accuracy should be established across the specified range of the analytical procedure.

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/3 replicates each of the total analytical procedure).

Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

The accuracy of the proposed method was determined from results of mean peak area for inter-day precision experiment for three QC standards DFP. Firstly, mean measured concentrations for each response (mean peak area) obtained for corresponding QC standards viz. 7, 17 and 27µg/ml were determined from equation of line and found to be 7.37, 17.18 and 27.40µg/ml likewise, **Table 13**. in addition, the mean measured concentrations were applied to compute percent accuracy by using the following formula.

$$\% \text{ Accuracy} = \frac{\text{Mean measured concentration}}{\text{Nominal Concentration}} \times 100$$

For this reason, it was deduced that the projected developed RP-HPLC method passed for test of % accuracy for the results of precision experiment at three QC standards viz. 7, 17and 27µg/ml from corner to corner of range.

Robustness

Table 13 Observations for robustness expt. with temperature variation.

Wavelength (nm)	Standard Conc. (µg/ml)	Mean peak area*	Mean measured conc. (µg/ml)	% Assay (%w/w)	Inference (std. for DFP 90-110 % w/w)
25	5	399592	5.21	104.29	Passed
24	5	390275	5.10	102.09	Passed
26	5	391502	5.12	102.38	Passed

*Mean peak area of three repeated injections

Table 14 Observations for robustness experiment with deliberate variations in flow rate.

Flow Rate (ml/min)	Standard Conc. (µg/ml)	Mean peak area*	Mean measured conc. (µg/ml)	% Assay (%w/w)	Inference (std. for DFP 90-110 % w/w)
0.9	5	399592	5.21	104.29	Passed
0.8	5	388562	5.08	101.69	Passed
1	5	391270	5.12	102.33	Passed

*Mean peak area of three repeated injections

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

The study was executed to check an effect of deliberate variations in the method parameters on method performance. Two parameters viz. temperature ($\pm 1C^0$) and flow rate (±0.1ml/min) of mobile phase were selected for this study. The 5µg/ml concentration

was preferred for the study and it was kept identical during the experiment regardless of changes in method parameters as stated herein above. The design of this study was as per **Table 5** (Section 6.2.3.5).

Results found in the form of peak area were then utilized to calculate mean measured concentrations followed by % assay. The % assay values were found in the range of 102.09 to 104.29 % w/w for temperature variation, **Table 14**. Whereas percent values for variation in flow rate were seen in the range of 101.69 to 104.29 % w/w, **Table 15**. Results for both variations in system parameters were found in consistency with compendial standards for DFP tablets (within 90-110 % w/w).

Therefore, it was concluded that the presented method can tolerate deliberate variation of ±1 C^0 in temperature and ±0.1ml/min in mobile phase flow rate.

% Recovery

Percent recovery experiment was planned to study three different objectives of the present method as given below.

a) Accuracy
b) Specificity
c) Applicability of the method for estimation of Dalfampridine in Marketed formulation (tablet dosage form).

Estimation of accuracy by percent recovery method:

In this study, the percent accuracy was estimated by percent recovery method. The method consists of spiking the standard solution of known concentration to the sample concentration prepared from tablet dosage form. This study was established at three levels of standard concentration viz. 80%, 100% and 120%. The test solutions prepared at these three levels were injected to given set of chromatographic conditions in triplicate and mean total peak area was then determined. The peak area corresponding to standard concentration (previously estimated) was then deducted from total area to get area equivalent to sample concentration. From the resulting sample peak area, parallel mean measured concentration was calculated using regression equation of calibration curve. The mean measured concentration corresponding to three sample concentrations viz. 8, 10 and 12µg/ml were found to be 8.28, 10.44 and 12.36µg/ml respectively.

Table 15 Observations for percent recovery experiment by spike method.

% Recovery Level	Conc. of standard spiked (µg/ml)	Conc. of sample (µg/ml)	Total Mean Peak Area*	Mean peak Area of sample conc.*	Amount recovered# (µg/ml)	% Recovery (%w/w)	Inference (std. for DFP 90-110 % w/w)
80	10	8	1516734	712323	8.28	103.46	Passed
100	10	10	1649026	842615	10.44	104.58	Passed
120	10	12	1812049	1005638	12.36	104.01	Passed

*Mean are of three repeated measurements of sample. #Amount recovered and percent recovery is for sample only and calculated from sample area.

Percent recovery obtained for three levels was in the range of 103.46 to 104.58 % w/w, Table 16. The observed results for percent recovery were seen in agreement with compendial standards (90-110% w/w). The representative chromatogram of percent recovery experiment observed at 80% recovery level was as shown in Figure 22.

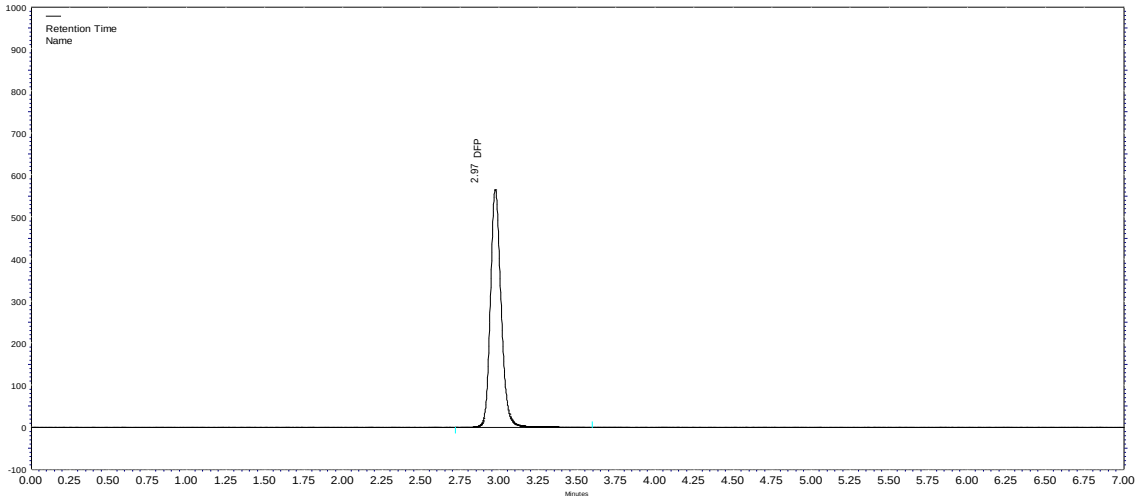

Figure 8 Chromatogram observed at 80% recovery level for DFP.

Table 16 Peak table of DFP % recovery.

Name	Retention Time (min)	Peak Area	No. of Theoretical Plates	Tailing Factor
DFP	2.97	1516734	6749	1.16

Specificity: The chromatogram obtained as in Figure 22, was at 80% recovery level. The test sample for this study was prepared dosage form. As shown in Figure 22, there is no additional peak for any interfering substance of the commonly used excipients was noted. This indicated that the present proved to be specific for estimation of DFP in presence of common excipients used in tablet formulation while manufacturing. Therefore, it was concluded that the presented method passed for test of specificity as per ICH guideline Q2R1.

Applicability of the method for estimation of Dalfampridine in Marketed formulation (tablet dosage form): The presented method obtained test results in compliance with the compendial standards for DFP from marketed tablet dosage form. Therefore, from this observation it was concluded that the presented method can be successfully employed for routine analysis of DFP from marketed tablet dosage during its life cycle.

LOD and LOQ

The detection limit is an characteristic of boundary test. It is an usual amount of analyte in a sample that can be detected, but not essentially quantitated, under the said tentative conditions.

$$\mathbf{LOD} = \frac{3.3 * 26730.08}{84843}$$

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

The detection and quantitation limit was calculated from standard error of responses (peak area) equivalent each standard concentration of DFP in calibration curve. The STEYX was found to be 26730.08. Slope and intercept were found to be 84843 and 42823 respectively. The values of STEYX and slope were substituted in subsequent formulae to estimate LOD and LOQ. The results obtained were as tabulated in Table 18. From the observations as shown in Table 18, the present method can be successfully employed to detect and quantify amount of DFP as 1.04 and 3.15µg/ml respectively.

$$\mathbf{LOQ} = \frac{10 * 26730.08}{84843}$$

Table 17 Observations showing estimated LOD and LOQ.

Standard Drug Solution	LOD (µg/ml)	LOQ (µg/ml)
Dalfampridine	1.04	3.15

From the results obtained for LOD and LOQ, it was concluded that the method was proved to be sensitive for estimation of DFP.

SUMMARY AND CONCLUSION

The anticipated project work was to develop accurate, precise, specific, sensitive and economic RP-HPLC method for estimation of Dalfampridine (DFP) as API. Furthermore, method was projected to test an applicability of the method for analysis of DFP in marketed tablet dosage form. Assortment of Acetonitrile and water in the ratio of 80:20 v/v, at pH 3.0 of aqueous phase on a C18 stationary (250mm x 4.6mm, 5µ id) phase was found to be the most suitable. In view of the chromatographic peaks were better defined and tailing factor within limit. The retention time obtained for DFP was 2.98 min (mean of SST study) with C18 stationary phase.

System suitability study was proposed to assess a competence of the system and to make it fitting for additional experiment with six repeated measurements of standard solution of the DFP. The calculated statistical parameters were within the acceptance criteria as per ICH Q2R1 guidelines for DFP. The equivalent peak areas and retention time of DFP were reproducible as indicated by % RSD within limit (<2 for area and <0.5 for RT).

Linearity of the method was established by observed regression coefficient near to unity (r^2=0.999) between the standard concentration of DFP and the respective peak areas. The regression curve was constructed by linear regression fitting and its regression equation was y = 84843x – 42823 (Where, Y gives peak area and X is the concentration of the DFP).

The proposed RP-HPLC method was validated as per ICH guideline Q2R1 in order to make importunate for program examination of DFP for successive parameters

✓ Accuracy
✓ Precision
✓ Linearity and Range
✓ Robustness
✓ Limit of Detection
✓ Limit of Quantitation
✓ Percent recovery

Precision of this RP-HPLC method was studied by carrying out practical of repeatability and intermediate precision. When DFP standard solutions containing 7, 17 and 27µg/ml of DFP weremeasured by this developed RP-HPLC method for estimating intra-day and inter-day precision, % RSD values obtained by this study were less than 2 for all above standards measured.

Robustness of the method was assessed for the parameters like variation in wavelength in nm (±1nm) and flow rate (±0.1ml/min) variation. The outcome of focused disproportion in method parameter was not affected the quantification of DFP. Therefore, it was estimated that the developed RP-HPLC could attain the reproducible results as per compendial standards even after above variations.

Applicability of the method was established in tablet dosage form of DFP (marketed). Recovery values observed in terms of percent assay were in the range of 103.46 to 104.58 %w/w. The results obtained were in concurrence with the compendial restrictions prescribed for DFP tablets. Moreover, nonattendance of auxiliary peaks pointed out non-interference of normal excipients used in the tablet dosage form. The drug content in tablet dosage form was successfully enumerated by the presented RP-HPLC method of DFP.

Also, method was seen to be specific for estimation of DFP in presence of ordinary excipients. Low LOD and LOQ values obtained with the presented method suggested method was sensitive for particular concentrations of DFP tested during various parts of this project.

Accordingly, it was concluded that this RP-HPLC method was proved to be sensitive, simple, precise, economic and reproducible for the quantification of DFP as API. Also, it was susceptible for estimation of DFP in marketed tablet dosage form even in the presence of excipients.

Eventually, author attained all predetermined objectives set at the beginning of this project work

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