RESEARCH PAPER

Analytical Method Development Optimization And Validation Of Dalfampridine By Rp-Hplc

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ABSTRACT:-
A selective, precise, sensitive, and economical RP-HPLC method was developed for the quantitative analysis of Dalfampridine (DFP) as an active pharmaceutical ingredient and in tablet dosage form. Chromatographic separation was achieved using a C18 column (250 mm × 4.6 mm, 5 µm) with a mobile phase comprising acetonitrile and water (80:20 v/v), adjusted to pH 3.0. The optimized conditions produced well-resolved peaks with acceptable %CV and tailing factor. System suitability testing showed a retention time of 2.98 ± 0.25 min for DFP, indicating efficient and rapid separation. The method was validated in accordance with ICH Q2(R1) guidelines. The %RSD for peak area and retention time was within acceptable limits (<2% and <0.5%, respectively), confirming repeatability and reproducibility. Linearity was established with a satisfactory regression coefficient over the studied range. Precision was evaluated through intra-day and inter-day studies at concentrations of 7, 17, and 27 µg/mL. Robustness testing, involving small variations in wavelength (±1 nm) and flow rate (±0.1 mL/min), showed no significant effect on analytical performance. The recovery ranged from 103.46% to 104.58%, demonstrating good accuracy. The developed RP-HPLC method was successfully applied for the quantification of DFP in tablet dosage form and proved to be reliable for routine analysis.

Keywords: RP-HPLC; DFP; API; RSD; LOD; LOQ.
How To Cite This Article: Mohan Rs, Jagdale Rc, Kudhekar Ar, Dhakne Jr, Jadhav As, Tagad Rr. Analytical Method Development Optimization And Validation Of Dalfampridine By Rp-Hplc. Int J Drug Deliv Technol. 2026;16(25s):926-948. Doi: 10.25258/ijddt.16.25s.109

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1. INTRODUCTION

1.1.1 Analytical Methods

The promotion of analytical methods is aimed at finding the identity of a drug, its purity, and physical and chemical characteristics, and its strength or potency of the drug, including its bioavailability and stability.

Types of Pharmaceutical Analytical Methods

1.1.2.1 Spectral methods

1.1.2.2 The spectral methods are employed to determine the electromagnetic radiation which in most cases is emitted or absorbed by a sample.

1.1.2.3 Electro-analytical methods

1.1.2.4 These methods center on the measurement of the voltage, current and resistance.

1.1.2.5 Chromatographic methods

These are the postmodern chromatographic procedures most often employed for the analysis of multi-component drugs in their matrices. Chromatographic methods are described in terms of three basic methods of techniques. These are GLC, HPLC and HPTLC.

All these methods employed in chromatography are used for analysis of the complex mixtures and to get the quantitative analysis.1

1.2 Chromatography

"Chromatography is a A procedure wherein a sample, or an extract thereof, is dissolved in a 'mobile' phase and propelled through a 'immobile', 'stationary' phase. These phases are chosen to ensure that certain components of the sample exhibit greater solubility in one phase compared to the others. A component that exhibits partial solubility in the stationary phase will necessitate more time than a component that exhibits limited solubility in the stationary phase but substantially dissolvable in the mobile phase. Consequently, mobility disparities, the sample components will be segregated from one another. in the stationary phase.

Examples of chromatographic techniques are the techniques of H.P.L.C. (High Performance Liquid Chromatography) and GC (Gas Chromatography) where a column of relatively small internal diameter containing The stationary phase is basically shifted through by the mobile phase. The sample is eluted through the column alone by the continuous addition of the mobile phase. This procedure is referred to as elution. The starch gel is allowed to cool down a little before the elution begins. The observed average velocity with the passage of an analyte through the column is contingent upon the duration it resides in the mobile phase.

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1.2.1 Principles of separation in chromatography

1.2.1.1 Chromatography is a process that was specifically designed to allow resolution of a mixture with high degree of accuracy. However, it can be used to differentiate two parts that may be extremely close in their function, say, two types of the same protein with one differing in one or the other amino acid. The nature and identifying factors that characterise the separation process occurs are also not very stringent and chromatography can be used on sensitive products. Chromatography, in its fundamental essence, may purify any liquid or volatile compound under the right type of material and conditions.When conducting all forms of chromatography, the sample or part of the examined combination is placed and made to stick in a substance referred to as the stationary phase, or the adsorbent. Another constituent, more commonly referred to The mobile phase, carrier fluid, or eluent is subsequently directed through the adsorbent. Since the individual molecules of the analyte have differing strengths While adhering to the adsorbent, they also penetrate it to varying degrees when the eluent passes through the adsorbent., i.e., the component molecules have a strong adhesion proceed at a reduced pace compared to those with weak adhesion. In actual functioning, this process isolates each of the constituent parts of the analyte into discrete samples suitable for analysis.

1.2.1.2 Adsorption chromatography

E.g.: Column chromatography

The separation principle is that the components adsorb differently on a solid phase (adsorbent). Stationary phase: tube containing adsorbent (= column)

Mobile phase: various solvents (eluents)2.

1.2.1.3 Partition chromatography

Eg:-Paper chromatography

Principle of separation: variations between two liquids in the partition coefficients of the mixture's constituent parts: A thin layer of water adsorbed onto the cellulose fibers of a produced filter paper is known as the stationary phase. (10-20%)

Mobile phase: solvent, partly miscible or immiscible with water3.

1.2.1.4 Ion exchange chromatography

Ion-exchange: RH + K+A- RK + H+A- :

The ions in the electrolyte solution will be exchanged for an equivalent number of ions with the same charge from an ion exchanger.

Principle of separation: The mixture's ions have varying affinities for the ion exchanger.

Stationary phase: zeolithes (sodium aluminum silicate) or cation or anion exchangers (cation exchanger) resins that act as cation and anion exchangers.

Mobile phase: Ionic compound solution in water [4].

1.2.1.5 Size exclusion or gel permeation chromatography

Eg- Size-exclusion chromatography

Principle of separation: Smaller particles of a mixture have an easier time getting into the packing material's pores than larger molecules.

Stationary phase: granules of a three-dimensional swelling polymer with a certain pore size (Sephadex gels, modified polysaccharides).

Mobile phase: solution of components to be separated [5.]

1.2.1.6 Affinity chromatography

The idea behind affinity chromatography is that a ligand and receptor have mutual recognition forces. Shape complementarity, electrostatics, hydrogen bonds, and van der Waals interactions between the groups on the ligand-receptor pair are the main factors that contribute to specificity (Figure 34.1). A ligand (L) and receptor (R) engage mutually to produce a ligand-receptor complex (RL), which has a dissociation constant Kd that may be written as follows-

$$\mathbf{Kd} = \frac{[\mathbf{R}][\mathbf{L}]}{[\mathbf{RL}]}\ \mathrm{R} + \mathrm{L} \leftrightharpoons \mathrm{RL}$$

The number of interactions between the receptor and ligand determines the dissociation constant. The ligand on one molecule and the receptor on the matrix react when a crude combination is run through an affinity column. Mutual collisions between the matrix's receptor and ligands from various molecules determine their affinity for one another; as a result, the best option binds to the receptor while the others do not and instead show up as flow through. A wash process eliminates any last weakly attached molecules from the matrix. A competition between the matrix-bound molecule and the counter ligand is then employed to elute the bound molecule. [6]

Advantages of Affinity chromatography-

1.Specificity: Unlike other purification techniques that use molecule size, charge, hydrophobic patches, or isoelectric point, among other factors, affinity chromatography is unique to the analyte..

2.Purification Yield: Compared to other purification methods, affinity purification provides a high level of purity and yield. Typically, affinity purification can yield over 90% recovery.

3.Reproducible: Affinity purification yields reliable findings when there are no contaminating species present.

4.Easy to perform: Affinity purification is highly effective and relies on the force that governs the development of ligand-receptor complexes. Affinity purification does not require column packing, a particular purification apparatus, or sample preparation, unlike other procedures.

Table 1 Criteria for selection of chromatographic technique based on various factors

Molecular Characteristics Technique for Separating Physical Properties		
Polarity	Volatility	Gas-liquid chromatography
	Solubility	Liquid-liquid chromatography
	Adsorptivity	Liquid-solid chromatography
Ionic	Charge	Ion-exchange chromatography
		Electrophoresis
Size (mass)	Diffusion	Gel permeation Chromatography
		Dialysis
Shape	Sendimentation	ultracentrifugation
	Liquid Binding	Affinity Chromatography

Chromatographic Techniques

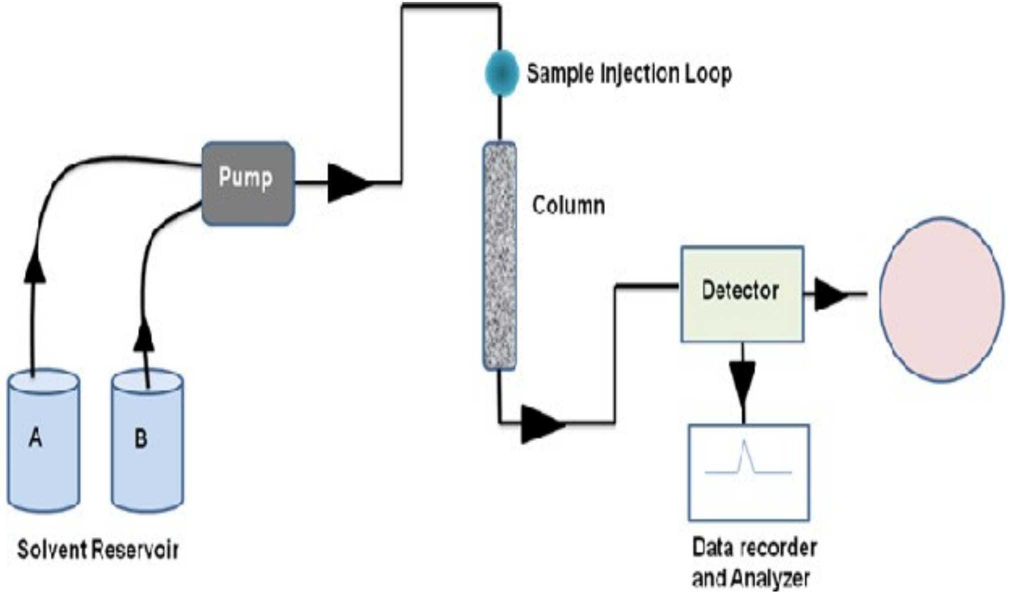
Chromatographic techniques are characterized contingent upon the configuration of the bed, the physical properties of the mobile phase, and the separation methods utilized.

a) Column Chromatography
b) Paper Chromatography
c) Thin Layer Chromatography
d) Gas Chromatography
e) High Performance Liquid Chromatography (HPLC)
f) Fast Protein Liquid Chromatography
g) Supercritical Fluid Chromatography
h) Affinity Chromatography
i) Reversed Phase Chromatography
j) Two Dimensional Chromatography
k) Pyrolysis Gas Chromatography and
l) Counter Current Chromatography.

1.3 High performance Liquid chromatography (HPLC)

HPLC can work at pressures greater than 50–350 bar. A common polysaccharide bead is unable to endure Elevated pressure during High-Performance Liquid Chromatography (HPLC). Consequently, silica-based beads are advised for HPLC applications. The elevated pressure and reduced dimensions of the silica beads yield an increased quantity of theoretical plates. This provides HPLC with exceptional resolution. capacity for separating complicated biological samples.

Figure 1 Components of HPLC instrument



This chromatography method enables rapid structural and functional investigation, as well as purification of various compounds. This method yields superior outcomes in the segregation and characterization of amino acids, carbohydrates, lipids, nucleic acids, proteins, steroids, and othersbiologically active compounds. In HPLC, the mobile phase traverses columns at elevated flow rates (0.1-5 cm/sec) and under pressures ranging from 10 to 400 atmospheres. The utilization of minute particles in this methodology, coupled with the application of elevated pressure on solvent flow rates, enhances HPLC separation efficacy and facilitates expedited analysis.

A HPLC device Must incorporate a solvent reservoir, a high-pressure pump, a commercially produced column, a detector, and a recording device. A automated system controls the duration of the separation, and material accumulates.[7.]

1.3.1 HPLC Instrumentation

Figure 2 various components of HPLC instrument

1.3.1.1 Pump

The pump's function is to drive a liquid (referred to as the mobile phase) is passed through the liquid chromatograph at a specified Flow rate quantified in milliliters per minute (mL/min). Typical HPLC flow rates vary from 1 to 2 mL/min. Pumps typically attain pressures ranging from 6000 to 9000 psi (400 to 600 bar). In the chromatographic experiment, a pump can deliver essentially a single solvent composition to the column (isocratic) or a solvent composition that changes during the course of the experiment (gradient).

1.3.1.2 Injector

It pumps the liquid specimen into the flow stream of the mobile phase Sample volumes generally vary from 5 to 20 microliters (µL). The injector must withstand the high pressures of the liquid system. An autosampler is employed when a user needs to assess a substantial quantity of samples or when manual injection is unfeasible.

1.3.1.3 HPL Column

The column's stationary phase, known as the "heart of the chromatograph," use diverse physical and chemical factors to isolate the components of interest within the sample. The elevated backpressure at standard flow rates is attributable to the minute particles within the column. The pump must provide enough force to drive the mobile phase through the column. The column's stationary phase, known as the "heart of the chromatograph," use diverse physical and chemical factors to isolate the components of interest within the sample. The elevated backpressure at standard flow rates is attributed to the minute particles within the column. The pump must apply considerable force to propel the mobile phase through the column, resulting in high pressure within the chromatograph. This resistance creates high pressure within the chromatograph.

Reverse Phase Columns ODS column

Silica gel treated with functional groups is commonly utilized as the packing material for reversed-phase columns. In the context of post-work engagements (Lesson 1), Individuals may opt for a beverage. because they know the bar, However, if a promoter is positioned in front of the bar distributing fliers, it is likely that more individuals will visit for a drink. The promoter serves as the functional group in the RP column, with octadecyl being the most often employed functional group. The octadecyl functional group comprises a linear chain of 18 carbon atoms. The chemical structure is depicted below..

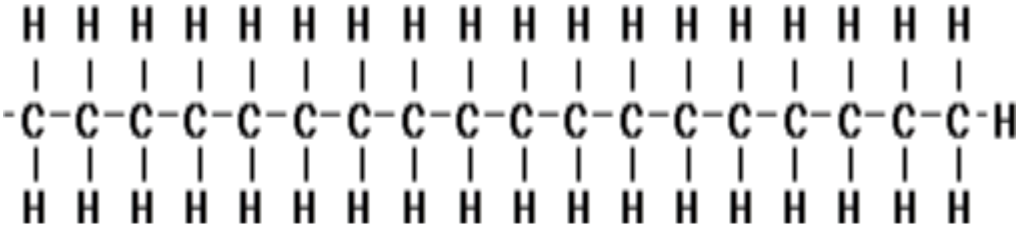


Figure 3 Octadecyl functional group

It is also known as the C18 column. The term octadecyl derives from "octa," signifying the number 8, and "deci," denoting the number 10. A significant quantity of this hydrocarbon chain is adsorbed onto the surface of the silica gel.. Something like the aforementioned structure when looked at might appear to be a lengthy chain; however, in comparison The size of silica gel is rather diminutive.. Hence, one could change An endless quantity of chains on a single silica gel. Moreover, these chains are altered even within the outer confines of the silica-gel pores.

Now as far as what pertains to our "famous" story the following may happen: some of the people may take the flyer while others ignore it completely. Likewise, Certain chemicals may be inhibited by the functional group, while others may not be affected. This provides the definition of the elution timing of each of the components. If, by adopting these terminology therein, it can say "a separation is due to the variation in the interaction between the gel and the specimen constituents. This process is termed partition or adsorption, so this method of separation is also known as partition/adsorption chromatography. Reversed phase mode is, in reality, a subtype of partition/adsorption mode.

The gel synthesized from silica and treated with an octadecyl functional group is termed octadecyl silica (ODS) gel. The column filled with ODS gel is referred to as an ODS column, or alternatively, a C18 column. Currently, generally about 80 per cent of silica-based column sold in the market are ODS column.

For the best of cases, the whole top surface of ODS gel is endowed with C18 functionality; but there will always be pocket space that remains unoccupied. Such parts are referred to as 'residual silanol' and the fact that residual silanols may affect the separation. At times, an end capping is used on the gels in order to anchor the remaining active silanol. Over 95% of ODS columns currently in use are end-capped though depending on kind of analyte, there may be silanol.

Other Silica-Based Columns

ODS is the most commonly utilized RP column; yet, due to its long-chain C18 structure, it excessively stores chemicals., and therefore the analysis takes very long. Therefore, in such instances, it is preferable to employ a functional group with a truncated chain, like C8, C4 and C3. C8: Octyl functional group -CH2CH2CH2CH2 CH2CH2CH2CH3 C4 : Butyl functional group -CH2CH 2CH2CH3C3: Trimethyl functional group -CH2C H2CH3Modified silica gels containing phenyl and cyanopropyl functional groups may be advantageous for separation processes.

1.3.1.4 Detector

A detector can observe (or 'see') the single molecule in a sample as the molecule passes (elutes) through the column. A detector is used to determine the concentration of those molecules to allow the chemist determine quantity of the different elements of the specimen. The detector outputs data to a recorder or computer that generates the liquid chromatogram, which is the graphical representation of the detector's reaction.

1.3.1.5 Recorder

The computer, referred to as the data system, not only controls all components of the HPLC equipment but also receives signals from the detector to ascertain the elution time (retention time) of the sample components (identity analysis) and the sample quantity (concentration analysis).

1.3.2 Types of separation in HPLC

1.3.2.1 Normal phase HPLC

The phospholipids can be well separated using normal phase HPLC. Multiple silica gel columns provide highly effective separations of the principal phospholipid classes, and in numerous instances the minor components are also resolved The separation is facilitated if the silica gel is prepared and packed in a particular way.

The chemical entities within the distinct phospholipid classes can be characterized using normal-phase HPLC with online MS. The complete positive ion current spectra of choline and ethanolamine phosphatides containing the principal molecular species. appear to be amenable to provide single ion chromatograms. With the growing technology make use of this normal-phase system has been freshly identified gyrated diradyl glycerophospho ethanol amine moved with the front of the phosphatidylcholine peak.lipid classes that often offer a clear distinction of the lesser components.

Normal-phase HPLC coupled with online mass spectrometry can be utilized to evaluate the molecular species within several phospholipid classes. Single ion chromatograms can be obtained from the total positive ion current spectra for the predominant Molecular species of choline and ethanolamine phospholipids. In this normal-phase system, the recently discovered gyrated diradyl glycerol sphophoethanol amine co-migrates with the leading edge of the phosphatidylcholine peak. The computer regenerated the single ion chromatograms of total negative ion current to facilitate the measurement of the predominant molecular species of acidic glycerophospholipids The three HPLC fractions, eluted from the normal-phase column, comprise the alkylacyl, alkenylacyl, and diacyl subclasses of ethanolamine glycerophospholipids, represented as tri-nitrophenyl derivatives [10].

1.3.2.2 Reverse phase HPLC

Phase in reverse One of the characteristics of In HPLC, the mobile phase possesses greater polarity than the stationary phase. The phrase "Reversed Phase" originated as it was the second chronological mode of chromatography, succeeding the Normal (or "Straight") phase, It utilizes a polar stationary phase in conjunction with a less polar mobile phase. The surface of a silica support particle is often coated with hydrophobic reversed phase stationary phases that are chemically bound to it (Figure 1). Figure 2 displays the most widely used stationary phases. There are additional support materials and bonded stages available. [1 1]

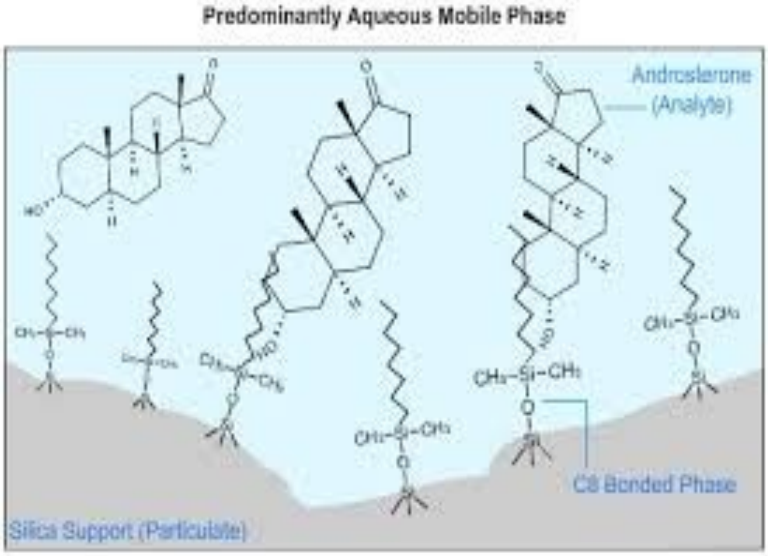


Figure 4 Schematic representation of reversed phase HPLC.

1.3.3 Types of elution in HPLC

1.3.3.1 Isocratic

The disadvantage of isocratic elution is that late-eluting peaks become significantly flattened and broadened., which may prevent them from being identified as peaks due to their shape and width. Peak width grows linearly with retention time in accordance with the equation for N, the number of theoretical platesutilizing the equation for N, representing the number of theoretical plates[13].

1.3.3.2 Gradient

Preparative reversed phase chromatography of biomolecules employs gradient elution as the most common method. Ceramic supports cannot be used in preparative reversed phase chromatigraphy of proteins and peptides because of their low capacity It is common to use linear gradients and binary solvents in preparative reversed phase chromatography. Slight convex-concave gradients are employed now and then for analytical needs especially when separating multicomponent samples when increased resolution is needed at the initial or final stage of a gradient.

Mobile phase A is more rich in the content of the organic solvent as compared to the mobile phase B and has low concentration of the solvent. The gradient is then, regardless of the change in the relationship of the percent organic modifier in terms of differential percentage points, always from the former or of higher relativity of polarity or higher aqueous content, and lower concentration of organic modifier or the latter of lower relativity of polarity or lower aqueous content and greater concentration of organic modifier.

Other factors include the Linear gradient, gradient slope and gradient volume combined with any isocratic condition For the first time when faced with a complicated sample, a broad gradient is run and the gradient shape is initially fixed [14.]

1.3.4 Guide to HPLC method development

As previously mentioned, most of the time, it is impossible to perform HPLC method without the input of knowledge and experience. In addition, the quick method development makes this field and its development move forward rapidly. Using a generic Internet-based method scouting approach, anyone can rather Rapidly and effortlessly create a methodology for general method scouting to thoroughly evaluate separation conditions that can be systematized.

1.3.4.1 Workflow of method development

Prior to method optimization data concerning the compounds to be tested and the conditions of the analysis are obtained. This information, when not available, makes method scouting efficient. For instance, method scouting is helpful for the all-encompassing evaluation of analysis conditions, for instance the mobile phase and or the column. The method scouting to optimize the mobile phase and optimum column conditions facilitate rapid method development with the setting of the basic condition.

Method development for reversed phase separation

Step 1: Method scouting

While scouting for reversed-phase HPLC method, conditions of mobile phase other than organic solvent; the pH of the buffers (solutions) influences the resolution. Therefore, the number of

Unavoidably, the number of issues to solve during method scouting rises. Reduced to 50mm high and with adequate length to complete the analysis in short time, the columns are ideal for instances where many points have to be addressed. The iart series has excellent peak shape, and excellent durability, and there are different sizes available for use in method scouting. The basic components of quick method scouting include Triart columns, the use of and organic solvents, as well as the pH of the mobile **phase.**

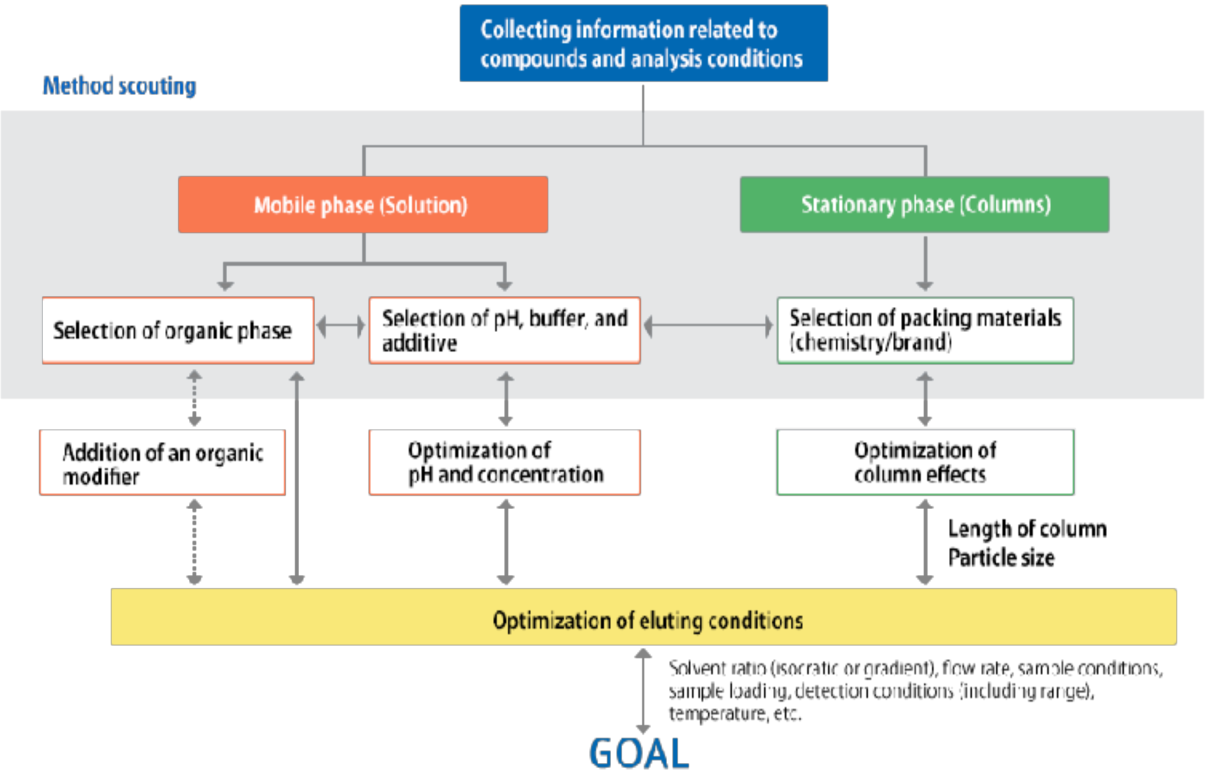


Figure 5 Workflow of method development

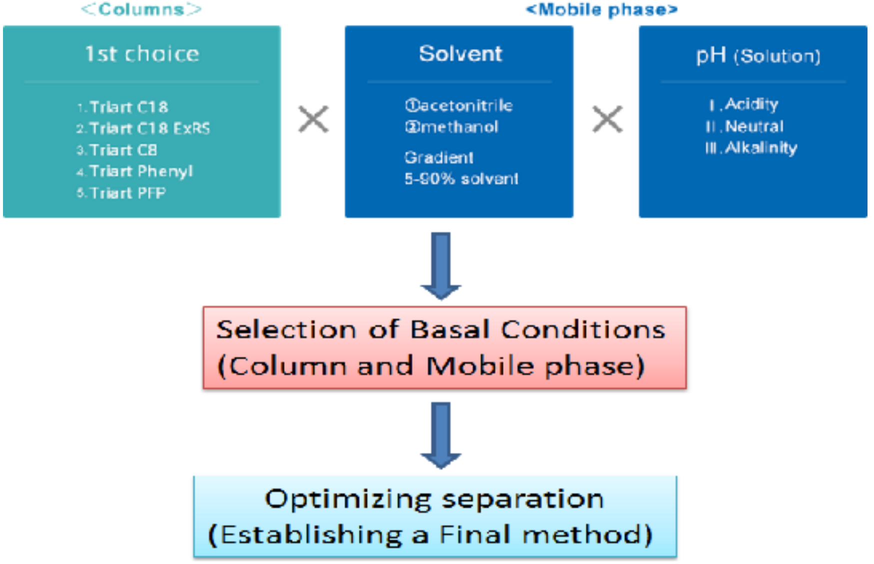


Figure 6 Method scouting

Optimization of conditions

Optimization of conditions is then carried out depending on the results of method scouting that has been conducted earlier. The combination of mobile phase and gradient slope, use of an organic modifier, column temperature, etc., affect the resolution of the peaks and the retaining time.

Optimization of mobile phase ratio and gradient slope

The primary drawback of gradient elution is the time required for column equilibration each time it is employed for the investigation of several components. When deciding on the grade incline The gradient composition is established to elute the components with the greatest retention time. time while letting the components with short retention time be retained by the initial composition of the gradient. To attain preparative enhancement and occupy less analytical time, using A gentle gradient slope to increase resolution and/or a sharp gradient slope to improve elution time is appropriate. The concentration of the organic solvent is 15%–20% lower than that employed to elute the starting component in gradient mode. as established during the method scouting is considered as the starting point in the transition to isocratic mode.

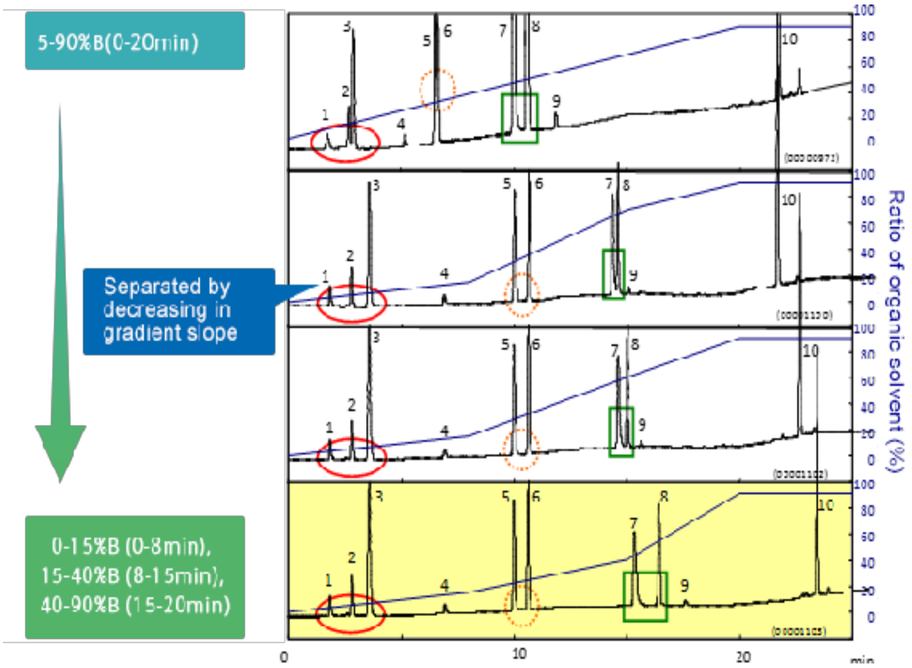


Figure 7 Gradient slope optimization in analysis of commercially available cold medicine extract

Addition of an organic modifier

In order to optimize, The inclusion of an organic modification like tetrahydrofuran may be beneficial. in this formulation. By increasing the proportion of the Utilizing an organic modifier in the mobile phase, while maintaining the total amount of the organic solvent, could enhance preparation by selectively adjusting the selectivity ^{15}.

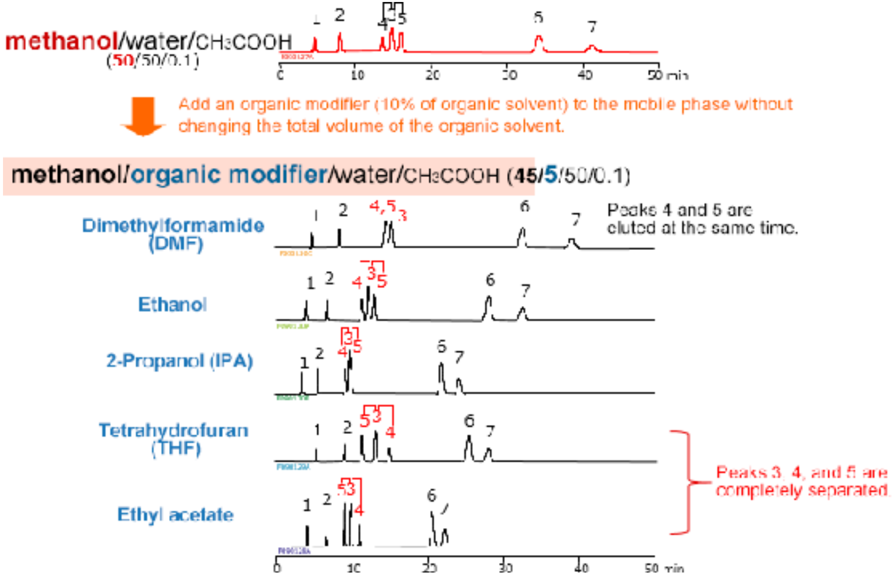


Figure 8 Use of organic modifiers to attain optimum separation

1.3.5 HPLC Method Validation

"Method validation, therefore, is an exercise whereby it is apparent that each The analytical process is appropriate for its intended purpose."The validation of an analytical method might be defined as: method via which it is evidenced through laboratory experiments indicating the performance features of the approaches under consideration are suitable for its proposed analytical utilization.

Retest is necessary To validate the accuracy and precision of any new or changed approach, it must be confirmed by different operators utilizing the same equipment in the same or alternative laboratories. More specifically, the nature of validation program needed is completely determined by the certain method as well as its intended usage.

Typical analytical parameters used in validation include:

- ✓ Specificity
- ✓ Precision
- ✓ Accuracy
- ✓ Linearity
- ✓ Range
- ✓ Robustness
- ✓ Limit of detection
- ✓ Limit of quantification

It is a capability of quantitating without doubt the analyte within matrix where other expected componenets are likely to be found. Normally such may encompass impurities, degradants, matrix, among others.

1.3.5.1 **Precision**

The precision of an analytical technique indicates the level of concordance among measurements obtained from several samplings of the same uniform sample under defined conditions. Precision can be assessed at three levels:

Repeatability: articulates the accuracy maintained during same operational settings over a brief duration. Repeatability is often designated as intra-assay precision.

Intermediate precision: articulates the accuracy of intra-laboratory variability: varying days, distinct analysts, diverse equipment, etc.

Reproducibility: articulates the accuracy among laboratories (collaborative investigations, typically utilized for the standardization of methodologies).

1.3.5.2 Accuracy

The precision of an analytical technique denotes the extent to which the obtained result aligns with an acknowledged true or reference value. This is sometimes termed trueness In the last respect, one might say that black is white and vice versa — this is sometimes termed 'trueness'.

1.3.5.3 Linearity and Range

An analytical method's convenience lies in its ability to generate test findings that are linearly correlated with the analyte concentration in samples within a defined range. It examines the detector performance on the chosen method of this parameter.

1.3.5.4 **Robustness**

Whenever a small portion of method is intentionally altered, then the measure of method's ability to remain unaffected is a measure of its robustness and provides reliability under normal operating conditions.

The validation of methods is a laborious process; yet, it is crucial for newly developed methodologies as it ensures the integrity of the generated data, which is directly correlated with the quality of the technique. Due to short time intervals, frequent method validations are not possible in most of the cases.

1.3.5.5 **Limit of Detection**

The reporting limit of a certain analytical procedure is the lowest concentration of analyte in a sample that can be identified. and identified intuitively, however cannot be properly defined as a precise, limited value.

$DL = 3.3\sigma/S$

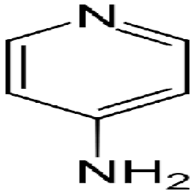
1.3.5.6 **Limit of Quantitation**

The qualities of the analyte that gives the lowest concentration when measured by the proposed method. In general, it is defined based on the concentration of this analyte of a sample [16].

$QL = 10\sigma/S$

2. DRUG PROFILE:

Table 1 Characterization of reagents and chemicals

Name	Dalfampridine (DFP)
Structure	
Description	This compound can be classified into the category of the heterocyclic compounds, aminopyridines and derivatives. These are organic heterocyclic compounds in which the parent nucleus is pyridine ring and an amino group is present. Dalfampridine, a potassium channel blocker, is utilized to aid ambulation in people with multiple sclerosis. This is the first drug which has been produced for the purpose of improving gait in these patients in the first place. Approved by the FDA on January 22, 2010.
IUPAC Name	1,4-dihydropyridin-4-imine
Chemical Formula	C5H6N2
Molecular Mass	94.1146 g/mol
Physical Sate	Solid
Melting Point	157 to 161 °C
Solubility	Freely soluble in water 74 g/ L
pKa	11
t1/2	Immediate release form = 3.5 hours; Extended release form = 5.47 hours;
Therapeutic Use	Dalfampridine is a neurofunctional modifier which assists in movement velocity in multiple sclerosis patients.
Mechanism of Action	In Multiple Sclerosis (MS), myelin sheath is gradually removed thus laying bare potassium channels at the axon. In consequence, there occurs an efflux of potassium ions and the cells are repolarized and neuronal excitability is reduced. Cumulatively, it results in the reduced

	neuromuscular transmission which is demonstrated when it is more difficult to depolarise the membrane sufficiently to produce an action potential. Dalfampridine acts on the CNS by blocking voltage gated potassium channel to keep up the transmembrane potential and to increase action potential. In other words, dalfampridine ensures that the current available is adequate to activate conduction in exposed demyelinated axons in patients affected by multiple sclerosis. Moreover, it enhances neuromuscular and synaptic interaction by overcoming conduction interruptions in demyelinated axons.
Pharmacokinetics Absorption	Dalfampridine which is administered orally absorbs rather rapidly and, in all probability, with almost cent per cent efficiency from the gastro-intestinal tract. Tmax, immediate release form = 1 hour; Tmax, extended release form = 3.5 hours; Cmax, 10 mg extended release =17.3 - 21.6 ng/mL; Relative bioavailability of 10 mg extended-release tablets compared to aqueous oral solution = 96%.
Distribution:	When 10 mg of dalfampridine is administered the calculated apparent volume of distribution is equal to 2.6 L/kg.
Metabolism	by the liver therefore Patient Specific Drugs which influences the cytochrome P 450 enzyme system and those administered along with dalfampridine will not interfere with one another. Derivatives are 3-hydroxy-4-aminopyridine and 3-hydroxy-4- aminopyridine sulfate and both are ineffective. Unlike some other drugs, the increase in M1 formation due to CYP2E1 results in ''3-hydroxylation' 'of dalfampridine.
Elimination	Since it is primarily catabolized in the liver and most of its metabolites are aromatic acids, the total elimination in twenty-four hours is almost of the amount of the dose and is entirely through the kidney. Urine (96% of dose, 90% in the form of unchanged drug) and feces (only 0.5% of the dose).
Pharmacodynamics	Dalfamprdine is used as board-spectrum lipophillic potassium channel blocker that shows a favourable affinity for the open configuration compared to the closed configuration of the potassium channel in the central nervous system. The pharmaceutical target is the potassium channels identified in multiple sclerosis patients. Does not elevate the QTc interval beyond typical limits.

3. MATERIAL AND METHODS

3.1 Raw material characterization

3.1.1 Characterization of Dalfampridine (DFP)

3.1.1.1 Determination of Melting point

The melting point range of Dalfampridine was determined using a digital melting point equipment, The known reference melting point of DFP is 157 to 161°C.

3.1.1.2 Determination of λ max by UV

3.1.2 Stock solution of DFP was prepared by accurately weighing 10mg of DFP dissolved in 100ml Acetonitrile: to prepare a 100µg/ml concentration of the standards, dilutions of fresh human serum pools were prepared by adding distilled water (1:1) to the frozen aliquots. From the stock solution of DFP 1.0ml was pipetted and diluted to a final volume of 10 ml by using Acetonitrile: water (1:1) to give a resultant concentration stock solution of DFP as 10µg/mL. The solution was also filtered via what was 0.45µ membrane filter. In addition, the solution was analyzed using a UV Visible spectrophotometer (Schimadzu) in the wavelength range of 200-400nm. The spectrum was captured and from the peak at the absorbance axis highest wavelengths and the corresponding absorbances were determined. A conclusions were made based on the results that were gotten.

3.1.3 Characterization of reagents and chemicals

Table 2 Characterization of reagents and chemicals

Ingredients	Grade	Suppliers	Characterization
Methanol	HPLC	Merck	As HPLC grade no further characterization performed
Acetonitril	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. Consequently, no more characterisation was conducted.

3.2 Experimental Work

3.2.1 HPLC method development for Dalfampridine (DFP)

3.2.1.1 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 70:30 parts vogative to parts volumetric. The solution was diluted to prepare 1 ml of cosmetics to the specified mark using the same solvent, resulting in a final concentration of 100 µg/ml. The resultant solution was subjected to a 0.45µ membrane filter paper and ultrasonicated for a total duration of 30 minutes, divided into three cycles of 10 minutes each.

3.2.1.2 Preparation of working solution of DFP

To create a working solution of 5µg/ml, 0.5 ml of the standard solution (100µg/ml) was pipetted out and diluted to 10 ml (in a 10 ml volumetric flask). After passing through a 0.45µ membrane filter, this solution was ultrasonically sonicated three times for ten minutes each.RP-HPLC was utilized to further refine the process utilizing the resulting solution.

3.2.1.3 Optimization of chromatographic conditions

The C18 stationary phase (250 x 4.6 mm, 5µ id) was used to optimize the chromatographic conditions by trial and error. The chromatographic conditions used for the trial runs are listed in Table 3 below.

Table 3 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:Phosphatebuffer (80:20)	0.9 3.	262	3.0

3.2.2 System suitability testing (SST)

The SST was prepared using 5µg/ml of the standard working solution. The working solution of DFP was prepared using the methods described in section 6.2.1.2 of this chapter. The contents of six repeated measurements of working solution (5µg/ml) were analyzed using RP HPLC which was run under the following optimal chromatographic conditions mentioned in Table 4. Identification was done at 262nm using UV detector. In each case obtained chromatogram, the peak areas, retention time, number of theoretical plates (NOP), and USP tailing factors were quantified. The results obtained were statistically analysed and calculated by numerical parameters such as mean, standard deviation (SD) and percent relative standard deviation (%RSD). Both the results which were obtained after statistical analysis were subsequently compared for their compliance based on ICH guideline Q2R1.

Table 4 Optimized chromatographic conditions

Chromatographic conditions	
Buffer preparation	1.36g Potassium Dihydrogen phosphate in to 1 litre orthophosporic acid using pH 3.00
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	10µl
Temperature	25°C
HPLC MAKE	Thermo Fischer Scientifics
Soft ware	Chromequest 4.1

Column	Cosmosil C18 (250mm x 4.6ID, Particle size: 5 micron)
Detection of wavelength	262nm
Flow rate	0.90 ml/min
Retention time	2.99
Temperature	Ambient
Sample size	10µl
Mobile phase	Acetonitrile : Phosphate buffer (80:20), at pH 3.0 adjusted with OPA
HPLC System	Thermo Fischar Scientifics
Software	Chromequest 4.1

3.2.3 HPLC Method validation

3.2.3.1 Preparation of stock solution

The standard stock solution was generated using the identical technique outlined in section 6.2.1.1. and Standard working solution was made from standard stock solution according to requirement of that particular experiment.

3.2.3.2 Linearity and Range

Aliquots of 0.5ml, 1.0ml, 1.5ml, 2.0ml, 2.5ml, and 3.0ml were extracted from a standard stock solution of DFP and transferred into six distinct 10ml volumetric flasks, which were then filled to the calibration mark with the mobile phase, Acetonitrile: Deionised water (80:20) to achieve final concentrations of 5, 10, 15, 20, 25, and 30 µg/mL. These standard working solutions of DFP were injected to chromatographic conditions as set above in Table 4 and the chromatograms where recorded at 262nm. The calibration curve was plotted against concentrations of DFP and was compared with reference to the peak area for each concentration level. Observations were made for slope, regression coefficient and intercept on the calibration curve of DFP. The standard equation of a line is expressed as:

Y= mX+c

Where,

Y- Area under the chromatogram

X- Unknown concentration

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

3.2.3.3 Precision

The precision of the procedure was assessed within the calibration curve range. of 5-30µg /ml. Since it was observed range was 5-30µg/ml for DFP, the tree QC standards were set up across this range that is 7;17 and 27ppm for precision experiment. The working solutions of these Quality control standards were created using a standard stock solution. of DFP by withdrawing 0.7, 1.7 and 2.7ml respectively in three different 10 ml volumetric flasks. A specified volume of each flask was filled to the designated mark. using mobile phase to provide working standards solutions of 7, 17 and 27µg/ml respectively. The method's reliability was evaluated through assessments of repeatability and intermediate precision. Intraday precision was determined and the repeatability was evaluated using three QC standards chosen as above analyzed in triplicate at time intervals of 3 hours. Intermediate accuracy was assessed across three distinct days, with three QC standards each simultaneously prepared and examined. The chromatograms obtained were integrarted to obtain peak area, retention time, NOP and tailing factor. The peak area was then evaluated in terms of mean, standard deviation (SD) and percent relative standard deviation (%RSD). The percentage relative standard deviation was then compared with standard as per ICH Q2R1 guideline.

3.2.3.4 Accuracy

From the ICH Q2R1 guideline, it is possible to estimate the accuracy of a method by more than one method. In this project, % accuracy was compared two methods for the first time via minimum nine determinations at the three levels across the given range. Secondly, by the percent recovery method as shall be discussed in the later section of this project work. The percent accuracy by first method was determined using data obtained for inter-day precision experiment. From the subsequent formula the % accuracy of DFP was established; The Pareto charts were plotted and the measurements made on % accuracy were compared with that of the pharmacopoeial standards to test their conformity.

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

3.2.3.4 Robustness

stability of the presented method of DFP was tested by decisive disproportion in method limits. In this case, the method control factors such as Flow rate of the mobile phase in milliliters per minute and wavelength in nanometers were adjusted in accordance to Table 5 below. The standard solution of DFP used in this study was 5µg/ml and was maintained constant and unchanged throughout while the following system parameters were altered. At each level of variation and the given chromatographic conditions, the selected concentration (5µg/ml) of DFP was injected in triplicate and the chromatograms were obtained. In all measurements provided in this study average peak area of the sample was calculated from chromatograms derived.

Table 5 Design of robustness experiment

Method parameters	**Standard**	**Variation 1**	**Variation 2**
Temperature in OC (± 1)	25	26	24
Flow Rate in ml/min (± 0.1ml)	0.9	1.0	0.8

The average peak area acquired in each instance was subsequently utilized to calculate the mean measured concentration at each level of variation employing the equation of the line. The mean measured concentrations were then used in each case for determination of percent assay of DFP. Percent assay values of DFP were also compared to the pharmacopoeial values of DFP in tablet dosage form.

3.2.3.5 % Recovery Preparation of stock from API

10 mg of DFP (API) was accurately measured and put to a 10 mL volumetric flask containing a small volume of mobile phase (80:20 Acetonitrile: buffer at pH 3.0) The volume was adjusted to 10 ml with the mobile phase to achieve a final concentration of the standard solution of DFP at 100 µg/ml. The resultant solution was further filtered using a 0.45µ membrane filter and then subjected to ultrasonication for 30 minutes. with interval of 10minutes in three cycles. From stock solution I 100µg/ml DFP, 1.0 ml was removed three times in triplicate using a micro-pipette and diluted to 10.0 ml in three separate volumetric flasks. Each flask was then diluted to an 10 ml volume in order to obtain solution of 10 ∝g/mL. These standard working solutions of DFP was injected into the specified chromatographic system three times, and the mean peak area was calculated.

Preparation of standard stock solution from dosage form

Label claim: 50 mg of Dalfampridine, Instgra, Emcure Pharmaceuticals Ltd. Ten tablets were measured for weight and average weight was found to be. Ten milligrams of powder was weighed then transferred to a 100 ml volumetric flask containing several milliliters of the mobile phase. The flask's contents were subsequently diluted to 100 ml with the mobile phase to obtain a sample stock solution of DFP at a concentration of 100 micrograms/ml. The considerable solution obtained was filtered through a 0.45µ membrane filter and subjected to ultrasonication for thirty minutes, divided into three intervals of ten minutes each. From each of the 10ml volumetric flasks 0.8, 1.0 and 1.2ml portions of the sample stock sample solution of DFP in duplicate as contained in the sample preparation The note below was transferred into respective 10ml volumetric flasks; the aliquot solution from each flask was thereafter diluted to ten millilitres with the mobile phase to produce the resultant working sample solutions of**Preparation of test solution of DFP for % recovery by spike method**

10µg/ml of standard solution of DFP(API) previously prepared was added to the above prepared DFP sample solutions at 8µg/ml, 10µg /ml and 12µg/ml to achieve test solution 80%, 100% and 120% respectively. Five binary mixtures of DFP were prepared at each of these three levels test solutions of DFP was administered in triplicate under the specified chromatographic conditions, and the mean peak area for each concentration was calculated. The mean peak area secured for standard solution of DFP injected (previously determined) was then deducted from the mean peak area of each of the three levels to get the peak area corresponding to each sample solution of DFP using the following formula:

% recovery = (sample area/ standard area) *100

3.2.3.6**LOD and LOQ :**

LOD and LOQ were determined from the following formulae; LOD = 3.3 SD of blank/sample and LOQ = 10 SD of blank / sample. It is suggested that LOD and LOQ should be described in the µg/ml for DFP.

LOD = 3.3* STEYX
Slope
LOQ = 10 * STEYX
Slope
STEYX = Standard error of Y and X axis and calculated from calibration curve of DFP.

4. RESULTS AND DISCUSSION

4.1.1.1 Melting point determination of Dalfampridine (DFP)

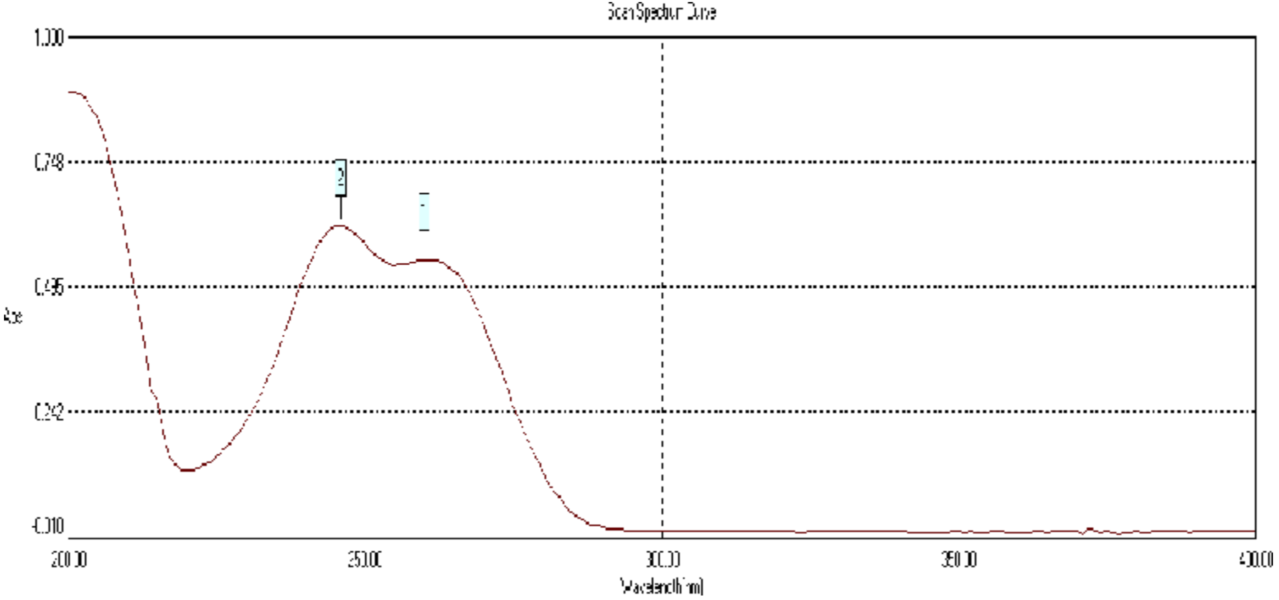
The melting point of a given compound is the temperature to which the compound undergoes change of state from solid to liquid. At the melting point the state of aggregation of a substance are in a state of equilibrium with each other that is, there is a given proportion of the solid and liquid phase. Melting point is influenced by pressure and an elevated pressure of 1 atmosphere or 100 kPa is usually indicated.

This melting point was determined using digital melting point apparatus and was obtained as 159 °C. The reference melting point for DFP is listed as 157 to 161°C. The melting points that was observed were in the same order of the reference values. Thus, and as a result of this study, sample of Dalfampridine used in this study was considered of a sufficient purity.

4.1.1.2 Determination of absorption maxima (λmax) using UV Visible spectrophotometer

Acetonitrile and water in a 50:50 ratio were used to measure the DFP absorption maximum (λ_{max}). For this investigation, the 10µg/ml standard working solution of DFP was used, and it was made in accordance with the steps outlined in chapter 6. Figure 14 displays the UV spectrum that was recorded for DFP.

Figure 9 UV spectrum of Dalfampridine obtained in Mobile phase



Two absorbances for DFP were visible in the acquired UV spectrum. Significant absorbance was recorded at wavelengths of 246 and 262 nm, as Table 6 illustrates. Thus, for the HPLC measurement of DFP, the maximum wavelength of 262 nm was used. In order to evaluate the DFP injection concentrations during the development of the RP-HPLC technology, the HPLC detector was adjusted to 262 nm.

Table 6 Observation for UV spectrum analysis of DFP.

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

4.1.2 Characterization of reagents and chemicals

No additional characterization was carried out because all of the chemicals and reagents employed in the RP-HPLC technique development of DFP were of HPLC quality, as shown in Table 2.

4.2 Experimental work

4.2.1 RP-HPLC Method Development

Methods of analysis are created, optimized, verified, discussed with others and used regularly. These developed method compilations then emerge in large ones like United States Pharmacopeia, British Pharmacopoeia, and Indian Pharmacopoeia, etc. In most instances, the desired spacing can be attained with minimal experimentation. In alternative instances a design model may take considerable amount of experimentation before it becomes effective. A robust method development technique requires only the necessary number of experimental runs to achieve the desired final result(s). Innovativeness of a method of analysis is often found based on the prior art or articles basically similar or equivalent to the experimentation. Population of any new or improved method takes into account the current analyte as well as the final use or purpose of the method from current established approaches and instrumentation.

When developing methods, one usually has to choose the requirements of the certain methods as well as determine the kind of instrumentations to apply and why these instrumentations are suitable. When establishing the HPLC method development, some factors like the selection and kind of column, mobile phase, detector, and quantity method should be taken into account. Thus development is the consideration of all factors belong to any method.

Thus, the creation of a novel HPLC technology requires the selection of the optimal mobile phase, the most appropriate detector, the most suitable column, column length, stationary phase, and the ideal internal diameter for the column. [25]. Chromatographic analysis of the sample for HPLC method development presents a number of stages [26].

4.2.1.1 Preliminary HPLC method development

Initial screening of the HPLC method was done randomly through error-prone method or trial method. To achieve better results in aspects such as retention time, peak area, number of theoretical plates and the appearance of the peak (tail in particular), the author investigated the effects arising from the use of different mobile phase and flow rate conditions.

4.2.1.2 Selection of mobile phase

The choice of the mobile phase was predicated on the utilization of several mobile phases or variations in their composition. As required by the experiment, it also included the use of various flow rates in milliliters per minute. The conditions specified in Table 3 were followed when conducting the experimental runs. Figure 10 displays the chromatogram that was produced following trial 1.

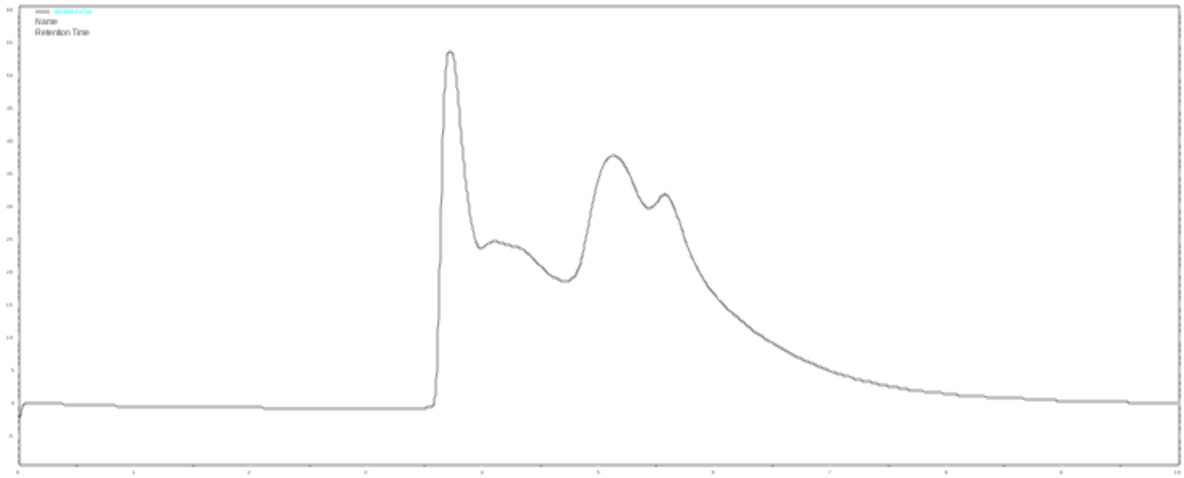


Figure 10 Chromatogram for Trial 1 using Methanol Water in the ratio of 50:50 at 262nm

As shown in Figure 10, 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 obtained was as recorded in the Figure 16 Figure 16: Chromatogram of the compound. From the figures reflected below in figure 16, it is clearly evident that there was inadequacy in the peak shape, as well as its limits and dimensions. Therefore, this also was rejected. Trial 3 was run using mobile phase composition of Phosphate buffer: T/S solvent was found to be acetonitrile with a ratio of 45% : 55%. In addition, the pH of the aqueous phase of the mobile phase was also set to 3.0 by using ortho phosphoric acid solution.

The flow rate was 0.9ml/min

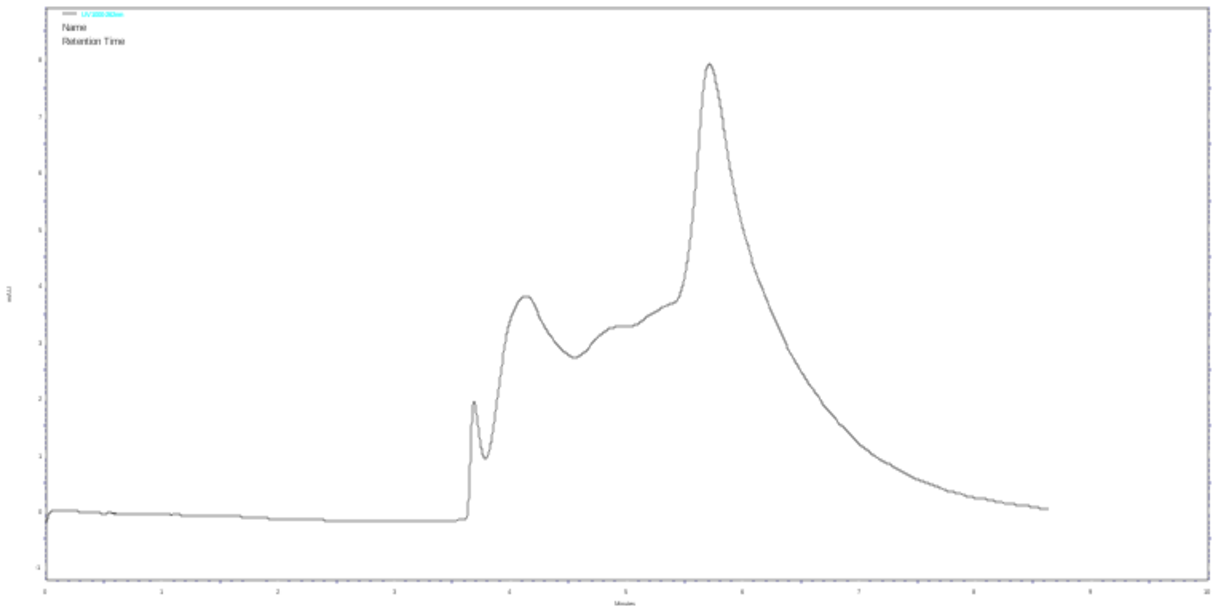


Figure 11 Trial 2 chromatogram obtained with Acetonitrile and Water in the ratio of 50:50 at 262nm

Trial 3, chromatogram results are presented in the Figure 12. The chromatogram obtained was then integrated for purpose of assessing the retention time in minutes, peak area and number of theoretical plates, tailing factor. The results which were obtained were as depicted in peak table 7.

Table 7 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

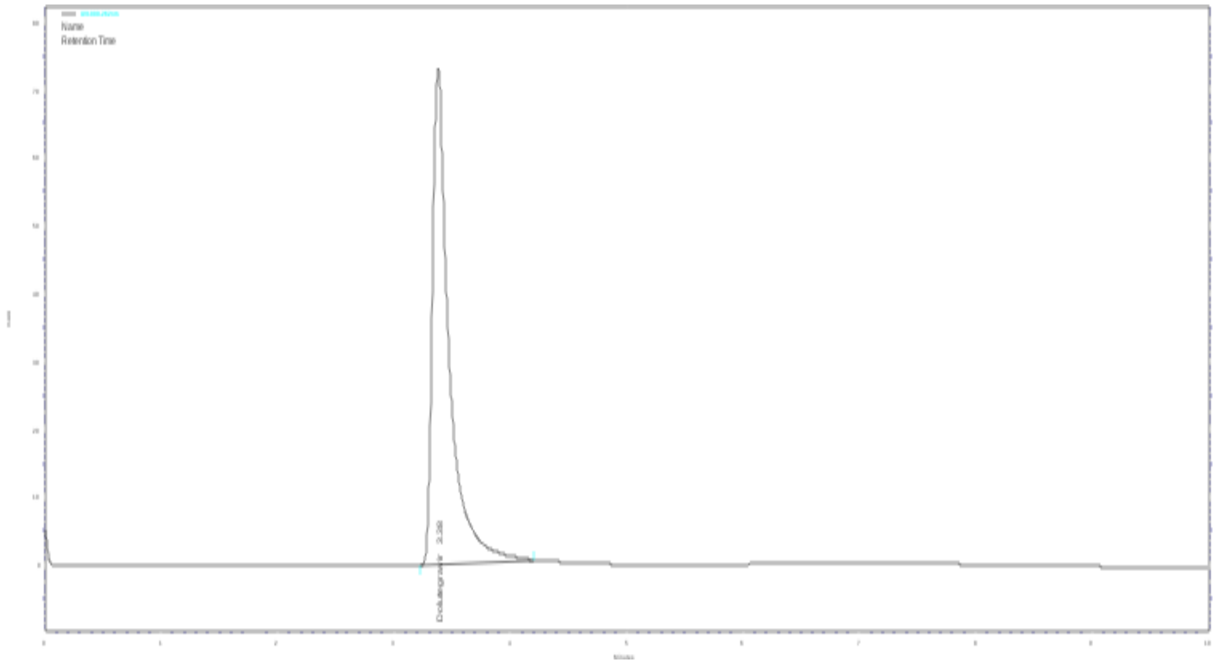


Figure 12 Trial 3 chromatogram using Acetonitrile and Phosphate buffer in the ratio of 45:55 at pH 3.0 and wavelength 262nm

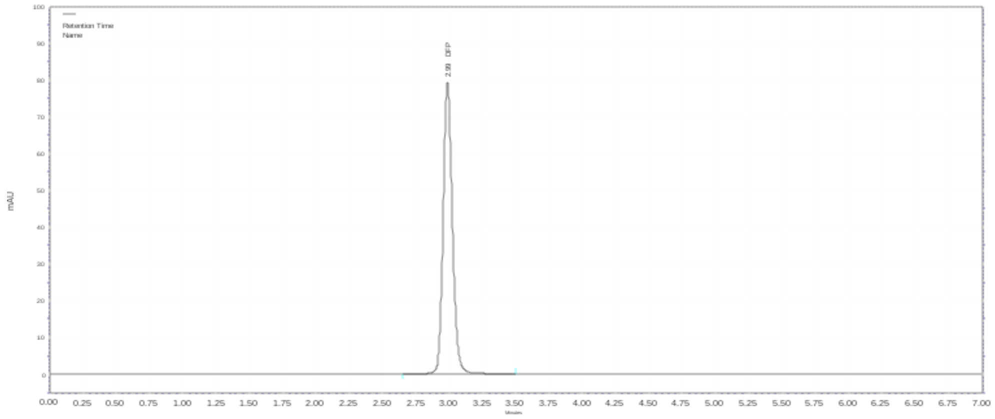


Figure 13 Trial 4 chromatogram using Acetonitrile and Phosphate buffer in the ratio of 80:20 at pH 3.0 and wavelength 262nm

Table 8 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

The chromatogram in trial 4 was as shown in the following figure; Figure 13. The chromatogram obtained was integrated to arrive at retention time in minutes, the area under the curve, number of theoretical plates and measure of tailing. The results obtained were as shown in peak Table 8 Result summaries of structures resulting from two new strategies The first of the two new strategies pertained to node N1 and entailed maximising the number of times it was targeted to increase its importance as a hotspot for identifying epileptic seizures.

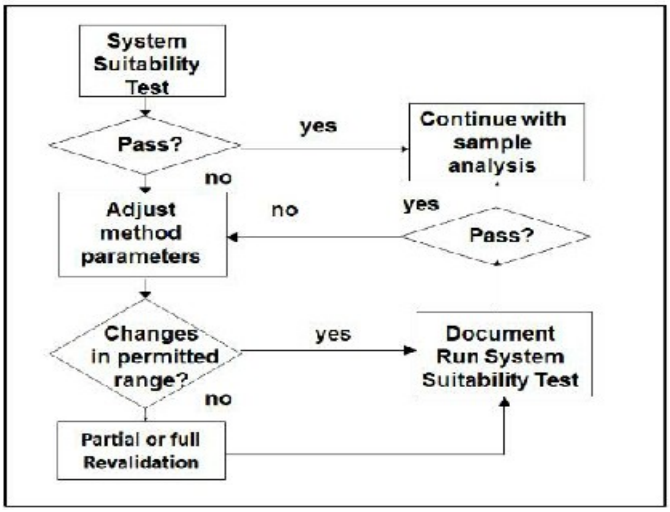
From table 8, the RT was found at 2.99 min along with the peak area of 391087. The maximum was within manageable standard of the USP tailing factor and taken as 1.14. Also, numbers of theoretical plate notes were in prescribed limit by ICH Q2R1 regulation. Accordingly, the method conditions described above were chosen for further RP-HPLC method development in the subsequent parts of this thesis.

4.2.1 System suitability testing

SST is mandatory in USP and FDA as a means of verifying on going performance of the systems and methods used for analysis. USP and EP both contain chapters that offer suggestions of system suitability tests, which agencies such as the FDA uphold. Both USP and EP have revised the related chapters and we also get some indication of how much a method may be altered without having to be revalidated. Moreover, the USP chapter <1058> contains a declaration that SST can replace the performance qualification of an instrument, but there are no additional recommendations. So there are many questions.

It keeps pointing out that resolution, column efficiency, and peak symmetry are achievable, but it doesn't provide standards that are ideal for these attributes. For accuracy, the USP says: Unless the specific monograph specifies otherwise, information from five replicate injections

Figure 14 System suitability testing flow chart



The test was performed by using 5µg/ml standard solution of DFP in the entire experimental procedure. The chromatograms obtained from six repeated injections were stored and used to determine chromatographic performance measurements including the retention time, DFP peak area, theoretical plates and tailing factor. From the chromatographic conditions featured in Table 9, the prevalence symmetrical factor is within the limits of < 2, and the values of peak shape and theoretical plates were obtained. Hence, the chromatographic conditions as indicated in Table 9 were deemed more suitable to be applied in the accompanying work in determining DFP.

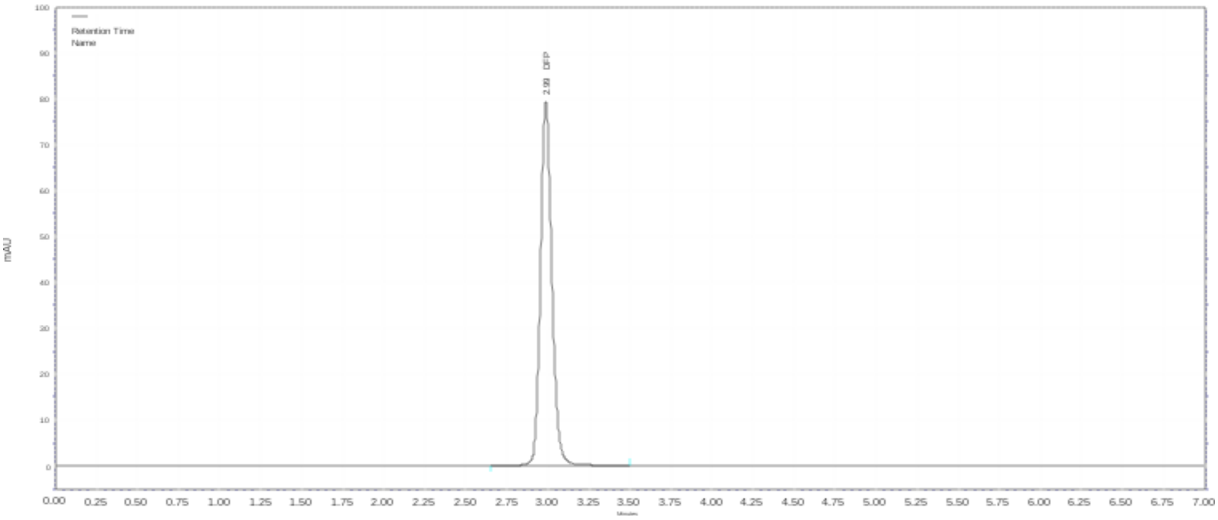


Figure 15 Chromatogram obtained for Dalfampridine in SST Table 9 Peak table of DFP SST

Table 9 Peak Table DEP SST

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

Best separation and peak shape with out excessive tailing were observed by application of chromatographic conditions. The reasonable retention time of 2.99min was obtained with mobile phase containing Acetonitrile: There was phosphate buffer at 3.0 pH obtained with ortho phosphoric acid in the ratio 80:20. A representative chromatogram was presented in figure 15.

The results for peak area and retention time were assessed using statistical measures, including standard deviation and percent relative standard deviation. The results for SD, %RSD, tailing factor and NOP were as tabulated in Table 10. Thus the results observed for the test of system suitability of DFP were obtained in accordance with the ICH guideline Q2R1.

Table 10 Observations obtained for SST study of DFP

Sr. No.	Parameter	Mean observations	SD	% RS D	Acceptance cr iter ia	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 15.

Thus, based on system suitability testing, it was determined that the system for quantitative determination of DFP at 262 nm with the chromatographic parameters listed in Table 10 was appropriate.

4.3.1 RP-HPLC Method Validation

4.3.2 Linearity and range

The measure of linearity of an The analytical procedure pertains to its capability, for the said concentration range, of producing test results which have a direct relation regarding the concentration of analyte in the sample.

A linear relationship must be evaluated throughout the entire range, which is detailed in section 3, of the analytical procedure. Examples of direct demonstration are through the dilution of a standard stock solution of the pharmaceutical compound in the relevant buffer and/or by discrete weighing of synthetic mixes of the different components of the drug product employing the outlined procedure. The The latter element might be examined during the analysis of the range.; identifications and deletions can be explored during examination of a number of possibilities.

The linearity should be assessed by eye balling a graph showing a relationship between signals and concentration of content of the analyte. If the correlation is absolute, then test results should be assessed by suitable statistical methods, for example, a regression line to be calculated by the least squares method. Sometimes, in order for the test data to produce linearity of the assay with sample concentration it might be necessary to perform data transformation before actually doing the regression analysis. Such data may include assistance Utilize the regression line to provide quantitative estimations of linearity statistics.

Residual sum of squares, The y-intercept, slope of the regression line, and correlation coefficient must be generated. It is advisable to have a plot of the data. Thus, graphical techniques may also be useful for assessing linearity if one provides an analysis of the scatter of the multiple data points from the regression line.

There is a sentiment that certain analytical procedures especially immunoassays are not linear even after some transformation. Here the analytical response should be expressed through a suitable function of concentration (amount) of analyte in sample.

To establish linearity, a minimum of six concentrations is the most preferable. Others should be justified.

A straight line relationship existed in DFP concentration level of 5-30µg/ml by RP-HPLC method. Linearity was tested by both visual analysis of a graph of mean peak area versus DFP concentration, and through statistics. Findings were analyzed by determination of a regression line by the least squares technique. Using data of the regression line that is the correlation coefficient (r2 = 0.999), it could be proven it is helpful to providing mathematical knowledgeable assumption of the degree of linearity.

The calibration curve that was generated for DFP was as indicated in figure 16. Therefore, the notes of mean peak area parallel to the standard solutions of DFP 5-30µg/ml were in the following table 11.

From these results the equation of line was worked out and as observed it equalled Y = 84843x – 42823

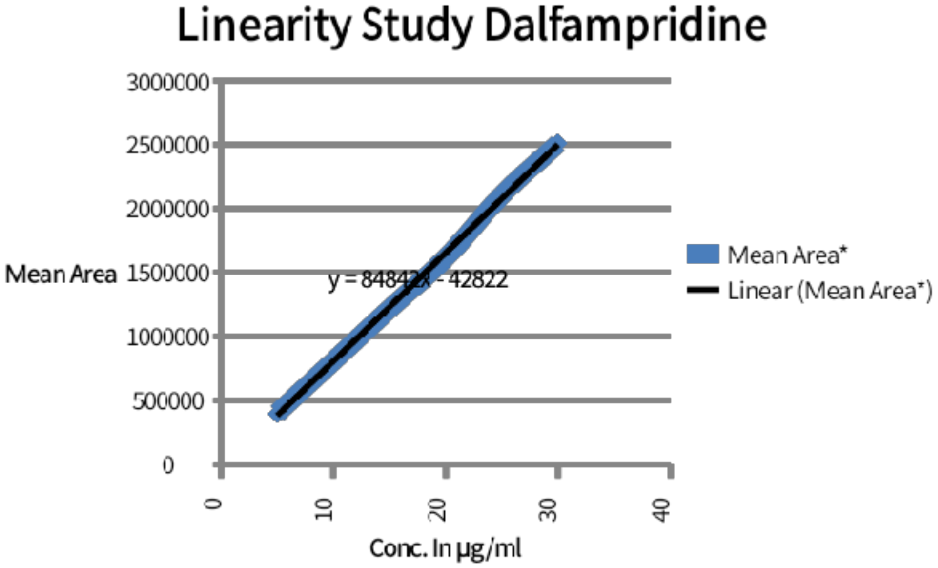


Figure 16 Calibration Curve of Dalfampridine indicated linearity between test results

Table 11 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 value (r^2) was determined to be 0.999, indicating a significant linear relationship between the mean peak area of DFP and the corresponding standard concentrations of DFP within the range of 5-30 µg/ml.

4.3.2.1 Precision

The accuracy of an analytical technique assesses the proximity of results; specifically, the extent of variability across replicate measurements conducted on the same homogenous sample according to established protocols. Precision can be categorized into three levels: Accuracy, which is further broken into repeatability, intermediate precision, and reproducibility.

It is advised that precision be investigated with respect to differential and essential low groupings. However, if obtaining a homogeneous sample is impractical, it may be analyzed using an artificially generated sample or sample solution.The accuracy of an analytical technique can be defined by the variance, standard deviation, or coefficient of variation of a series of measurements.

4.3.2.2 Repeatability

Repeatability refers to the capacity to achieve precision within a short timeframe under identical operational conditions. Repeatability is often referred to as intra-assay precision.

Repeatability should be assessed using:

1. At least nine determinations that span the procedure's designated range (for example, three conc/3 replicates each); or
2. At least six determinations that are at 100% of the test concentration.

Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analyst, different machinery etc.

Whether, and to what degree, it is necessary to specify intermediate precision depends on situations in which the procedure is supposed to be applied. The applicant should explain how randomness influences accuracy of the analytical process. The usual synchronic variations which it is required to investigate are days, analysts, equipment, etc. It is not thought advisable to investigate these effects separately. Preferably, an experimental design (matrix) must be employed.

Intra day precision was determined by replicate measurements nine times across the range of 5-30 µg /ml for the RP-HPLC method of DFP. Three concentrations labelled as QC standards were 7, 17 and 27µg/ml set for this experiment of DFP. To achieve the data reproducibility, three repetitive injections were done in day constantly at an interval of three hrs to make the RP-HPLC method developed for DFP repetitive.

In addition, proposed RP-HPLC method was checked whether it is accurate or not by determining intermediate precision. Thus, in this experiment of RP HPLC method of DFP, worldwide intermediate precision was defined based on a precision experiment on three of the above three days QC standards. The above-mentioned assessments were carried out on three different days to standard concentrations of the QC standards of DFP; the chromatograms are shown below for determination of the mean peak area. The mean peak area was then computed for SD and %RSD. The values determined for repeatability and intermediate precision were in the following manner as shown in Table 12. As depicted from the above finding from table 12 it was observed that the calculated %RSD was observed for three QC standards as 0.56, 0.55, 0.57 & 0.66, 0.93, 0.31 for inter day(precision between run also)repeatability & inter day precision respectively. the %RSD obtained in both studies of precision was achieved in compliance with the standards set in ICH guideline Q2R1. The developed RP-HPLC method shown to be accurate for the calibration range of 5-30 µg/ml for DFP was fulfilled.

Table 12 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

4.3.2.3 % Accuracy

Table 13 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 precision of an analytical procedure indicates the degree of consensus between the value which is accepted either as the conventional true value or the accepted reference value and the value obtained.

It may be called trueness at times. Anaanas should be of a fixed accuracy over the range defined for the analytical procedure.

’Repeatability should be measured from at least 9 different measurements at the low, medium and high spikes/ concentrations minimum (e.g. three spikes of the total analytical method at a total of three concentrations). It should be expressed as the percentage recovery based on the assay of the known additional quantity of the analyte in the sample, or as the mean alongside the real value and confidence intervals.

Percent relative standard deviation and percent coefficient of variation were calculated from the mean peak area of the inter- day precision experiment of three QC standards, DFP. Firstly, the mean measured concentrations for each response (mean peak area) obtained for corresponding QC standards viz 7, 17 and 27µg/ml was obtained from the equation of line and was found to be 7.37, 17.18 and 27.40µg/ml Likewise table 13enor, using the same percent accuracy was calculated by applying the mean measured concentrations by the following formula.

% Accuracy = Mean measured concentration x 100
Nominal Concentration

The proposed produced RP-HPLC technique was therefore found to pass the accuracy test for precision experiment findings at three QC standards, namely 7, 17, and 27 µg/ml from corner to corner of the range.

4.3.2.4 Robustness

Table 14 Observations for robustness expt. with temperature variation

Wavelength (nm)	Standar d 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 15 Observations for robustness experiment with deliberate variations in flow rate

Flow Rate (ml/min)	Standar d 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

Multiple metrics assess the quality of an analytical technique, with precision being the most critical, since it indicates the extent to which the average result of a sample analysis diverges from the mean value of a substantial number of analogous samples.

In the case of robustness assessment, there should be specified ideas concerning this point at the time of development and, according to the kind of procedure which will be analyzed. It should demonstrate the accuracy of an analysis concerning deliberate manipulations of method factors.

If measurements are influenced by variability in analytical circumstances, such variables should be appropriately standardized, or a disclaimer should be added to the technique. One evaluation outcome of robustness should be the establishment of a set of system appropriateness parameters. (e.g., resolution test) that guarantees the analytically valid application of the procedure whenever applicable.

The study was performed to observe whether a contemplated influence of changes in the method parameters on the method behaviour can be demonstrated. Two factors namely temperature and flow rate of the mobile phase were chosen for this work with their tolerance limits being ±1C0 and ±0.1ml/min respectively. The concentration used in this study was 5µg/ml and it did not change during the experiment irrespective of changes in method parameters as described herein above. This study was designed as per the plan outlined in Table 5 described under section six point two three five.

The values obtained in terms of peak area were used to make estimates of the mean measured concentrations as well as the % assay. The % assay values were found in the range of 102.09 to 104.29 % w/w for temperature variation, Table 14. While, percent values for variation in flow rate as 101.69 % to 104.29 % w/w have been recorded as given in Table 15. Both the variations in system parameters yielded results within compendial requirement limit values of 90-110 % w/w for DFP tablets.

Hence, it was concluded that the presented method can accommodate deliberate change of ±1 C0 variation in temperature and ± 0.1 ml/min change in the flow rate of the mobile phase.

4.3.2.5 % 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).

a) Estimation of accuracy by percent recovery method:

In this study the percent accuracy was estimated by percent recovery method. The method includes addition of a definite amount of the standard solution of a solution of known concentration to the sample concentration obtained from tablet dosage form. This study was conducted at three concentration levels of standard that include 80%, 100% and 120%. These test solutions prepared at these levels of injection were run in triplicate at the selected set of chromatographic conditions and mean total peak area was computed. The peak area corresponding to standard concentration (as estimated earlier) was then subtracted from total peak area to arrive at the area which would represent sample concentration only. The mean measured concentration in parallel was calculated from the resulting sample peak area of using the regression equation of the calibration curve. The mean measured concentration obtained from three sample concentration namely: 8, 10 and 12µg/ml, were8.28, 10.44 and 12.36µg/ml.

Table 16 Observations for percent recovery experiment by spike method

% Reco very 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 17.

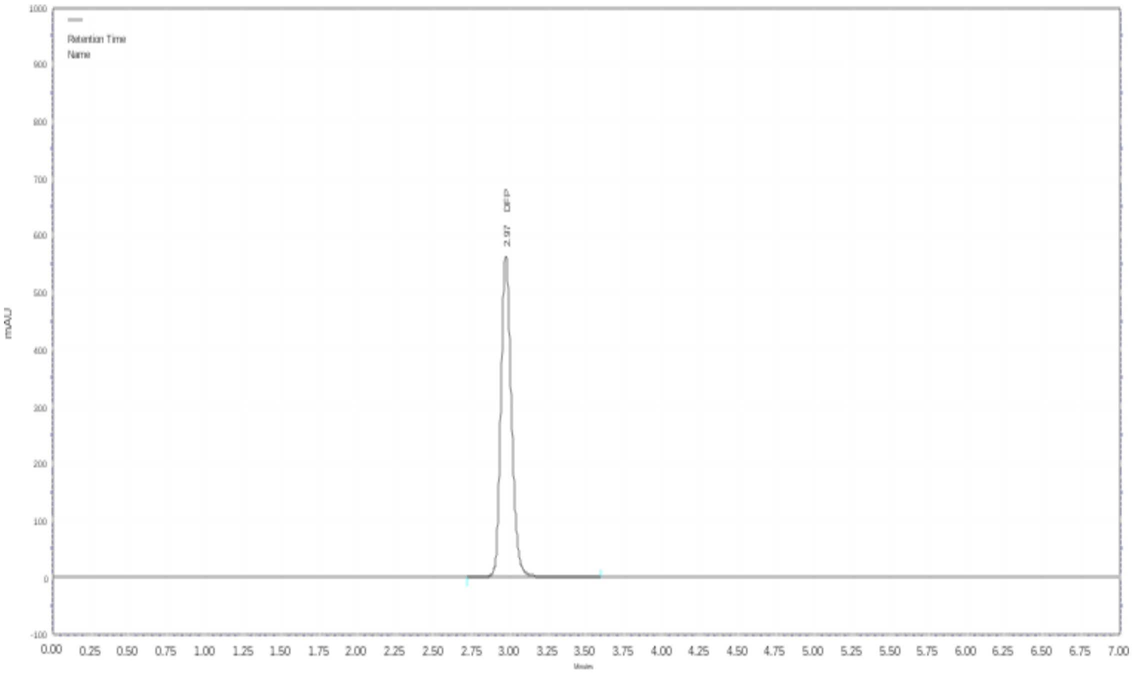


Figure 17 Chromatogram observed at 80% recovery level for DFP

Table 17 Peak table of DFP % recovry

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

The chromatogram that was obtained as shown in figure 17 was at 80% recovery level. The test sample for this study was a prepared dosage form. As may be seen from Figure 17, there is no further peak for any interfering substance of the commonly used excipients observed. This pointed to the possibility of using the present to accurately estimate DFP when it is presented along with generic excipients usually used in tablet compression during manufacture. Hence it was concluded that the presented method was specific as per the test of specificity of ICH guideline Q2R1.

Applicability of the method for estimation of Dalfampridine in Marketed formulation (tablet dosage form): For DFP, test results using the presented method were within the compendial standards from the marketed tablet dosage form. Hence from this observation it can be concluded that the presented method can be effectively used for method development for analysis of DFP from marketed tablet dosage during its life cycle.

4.3.2.6LOD and LOQ

One feature of a boundary test is the detection limit. It is a typical amount of analyte in a sample that, under the aforementioned provisional conditions, can be detected but not precisely quantified.

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

The reporting limit of a specific analytical process is the minimum concentration of an analyte in a sample that can be quantified with an acceptable level of precision and accuracy. The quantitation limit denotes a property of quantitative assays for substances in sample matrices at trace levels, commonly utilized in the assessment of contaminants and/or degradation products.

The limit of detection and quantitation for DFP was estimated from the standard error of response (peak area) equivalent to each standard concentration of DFP in calibration curve. It was possible to established that the STEYX is equal to 26730.08. For the slope this value was gotten to be 84843 while for the intercept the values was gotten to be 42823. The value of STEYX and slope were then plugged into other formulae in order to calculate LOD and LOQ. The result obtained were as tabulated in Table 18. From observations as highlighted in table 18, the present method can be successfully used for determination of amount of DFP as 1.04 and 3.15μg/ml respectively.

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

Table 18 Observations showing estimated LOD and LOQ

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

Based on the LOD and LOQ values, it was determined that the approach was sensitive enough to estimate DFP.

5. SUMMARY AND CONCLUSION

The expected project work was to establish reliable, efficient, selective and economical RP-HPLC method for quantitative estimation of Dalfampridine (DFP) as an API. In addition, method was planned to check an applicability of the method for determination of DFP in marketed tablet dosage form. Assortment of Acetonitrile and water in the ratio of 80:The optimized method was determined to have 20v/v of the aqueous phase at pH 3.0 on a C18 stationary (250mm x 4.6mm, 5μ id). In light of the above findings, the chromatographic peaks were well resolved, and more importantly the tailing factor was within acceptable limit. The retention time of the analog DFP was established at 2.98 min (mean of SST study) with C18 stationary phase.

Since system suitability study was designed to indicate a competence of the system and to prepare the system for further experiment, six repeat measurements of the standard solution of the DFP were made.

The calculated statistical parameters were being accepted as per the acceptance criteria for DFP as per ICH Q2R1 guidelines. The peak areas and the retention time of DFP were produced to be equally Angelo comparing the data obtained. ≥ % RSD within limit (<2 for area and <0.5 for RT).

The linearity of the method was confirmed by observed regression coefficient close to 1 (r2=0.999) between the standard concentration of DFP and the respective peak areas. The regression curve was plotted by using linear regression fitting method, its regression equation was: y = 84843x – 42823 (Where: Y - peak area X-concentration of DFP).

The proposed RP-HPLC method was validated based on ICH guideline Q2R1 for importunate for program examination of DFP for successive parameters.Accuracy

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

Repeatability and intermediate precision of this RP-HPLC method were determined by working through the practical. This developed RP-HPLC method for estimating intra-day and inter-day precision, when measured DFP standard solutions containing 7, 17 and 27μg/ml of DFP.

All the above measured standards had RSD values of the present study less than 2%.

The reliability of the method was checked for the parameters as variation in the wavelength, ±1 nm and variation in flow rate, ± 0.1 ml/min. The result of focused disproportion in method parameter was not influence the quantification of DFP. Thus, it was anticipated that the developed RP-HPLC would continue to deliver the requisite reproducibility consonant with compendial trends notwithstanding these above changes.

The suitability of this method was demonstrated using tablet dosage form of DFP (marketed). Recovery values in terms of percent assay for the present study varied between 103.46 to 104.58 %w/w. The obtained results were quite reasonable to the compendial limits recommended for DFP tablets. Furthermore, absence of overload side peaks indicated non-contributing behaviour of normal excipients employed within the tablet dosage form. The efficiency of DFP's presented RP-HPLC method for enumeration of drug content in tablet dosage form was confirmed.

Moreover, it was observed that method could be applied specifically for estimation of DFP in presence of ordinary excipients. These LOD and LOQ values representing the sensitivity of presented method were monitored during various part of this project for particular concentrations of DFP.

In view of the foregoing findings, it is suggested that this RP-HPLC method is sensitive, simple, precise, economic and reproducible for the determination of DFP as an API. Also, it was fairly suitable for estimating quantities of DFP in marketed tablet dosage form even over a background of excipients.

At long last, for author every proposed aim that was set before at the start of this project work was achieved.

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