DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR RAPID SIMULTANEOUS ESTIMATION OF CALCIUM PANTOTHENATE AND BIOTIN IN PURE AND TABLET DOSAGE FORM

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MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted by

R. KIRUTHIKA

(REG.NO: 261530352)

Under the guidance of

Dr. R. RAJAPANDI, M.Pharm., Ph.D.,

DEPARTMENT OF PHARMACEUTICAL ANALYSIS,

ARULMIGU KALASALINGAM COLLEGE OF PHARMACY,

ANAND NAGAR, KRISHNANKOIL-626126

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Phone : 91-44-4740 4310 E-mail : fourrts@plant.fourrts.com website : www.fourrts.com

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Industrial Guide

J. Rajesh, M.Sc., PGDQM,
Head – Analytical Research & Development,
Fourrts (India) Laboratories Pvt. Limited,
Kelambakkam,
Chennai- 603 103
Tamilnadu.

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Place: Krishnankoil.
Date:

Dr. R. RAJAPANDI M. Pharm., Ph.D.,
Department of pharmaceutical Analysis,
Arulmigu Kalasalingam College of Pharmacy,
Krishnankoil – 626126.

CERTIFICATE

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Place: Krishnankoil.
Date:

Prof. Dr. N. VENKATESHAN M.Pharm.,Ph.D.,
Professor & Principal,
Arulmigu Kalasalingam College of Pharmacy,
Krishnankoil - 626126.

EVALUATION CERTIFICATE

This is to certify that dissertation work entitled ***"DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR RAPID SIMULTANEOUS ESTIMATION OF CALCIUM PANTOTHENATE AND BIOTIN PRESENT IN PURE AND TABLET DOSAGE FORM"*** submitted by ***Reg.no: 261530352*** was carried out in the Department of Pharmaceutical Analysis, Arulmigu Kalasalingam College of Pharmacy, Anand Nagar, Krishnankoil -626126, which is affiliated to The Tamil Nadu Dr.M.G.R. Medical University, Chennai, under the supervision and guidance of **Dr. R. RAJAPANDI M.Pharm., Ph.D.,** for the partial fulfillment of degree of ***MASTER OF PHARMACY*** in the department of ***PHARMACEUTICAL ANALYSIS*** were evaluated by,

Centre : Arulmigu Kalasalingam College of Pharmacy, Krishnankoil.

Date :

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1.

2.

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R.KIRUTHIKA.

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CHAPTER 1:

INTRODUCTION

INTRODUCTION

This thesis deals with the studies carried for the simultaneous estimation of calcium pantothenate and biotin in tablet dosage form by RP-HPLC Method.

Modern medicines for human consumption are required to meet exact standards which relate to their quality, safety and efficacy. The evaluation of safety and their maintenance in practice is dependent upon the existence of adequate methods for quality control of products. The purity of standard is a must therefore, it can strictly define in such a way as to ensure that successive batches are consistent in composition, irrespective of whether they came from the same or different manufactures. Here lies the work of the analytical chemist.

Definition:

Pharmaceutical analysis is branch of pharmaceutical chemistry, which is define as a process or a sequence of processes to identify and quantify a substance or drugs the components of a pharmaceutical solution or mixture or the determination of the structural of chemical compounds used in the formulation of pharmaceutical products[1].

Pharmaceutical Analysis:

Modern methods of pharmaceutical analysis are extremely sensitive, providing precise and detailed information from small samples of materials. In general they are readily amenable to automation and also this method is very rapid, due to this reasons they are now in widespread use in the product development in the control of manufacturing formulations, as a check on stability during storage and in monitoring the use of drugs and medicine[2, 3].

Role of analytical chemistry in pharmaceutical analysis [4, 5]:

Analytical chemistry may be defined as the science and art of determining the composition of materials in terms of the elements of composition contained. Its prime importance is to gain about the qualitative and the quantitative information of the drug substance or chemical species

i.e., to find out what a substance is composed of and how much. In relation of this context pharmaceutical analysis plays an important role in the quality assurance and quality control of drug samples as well as pharmaceutical formulations. In this connection it should be emphasized that the pharmacists should be aware and capable of developing new methods for the purpose of keeping the pharmaceutical industries and quality control laboratories in viable position. The best way to characterize the quality of the drug is to determine its purity. There are two possible approaches to reach this goal.

1. Determination of Active Pharmaceutical Ingredient (API).
2. Determination of its impurities with a highly accurate and precise specific method.

Drugs and pharmaceuticals are chemicals or like substances, which are of organic, inorganic or other origin. Whatever may be the origin, we use some property of the medicinal agent to measure them qualitatively or qualitatively. Pharmaceutical analytical techniques, which are being used, can be categorized as follows.

Analytical Techniques:

There are three types analytical techniques are used as follows [6]:

1. Spectroscopic analysis.

2. Electrochemical analysis.

3. Chromatographic technique.

SPECTROSCOPY:

Measurement based on light and other forms of electromagnetic radiation are widely used to thought analytical chemistry. The interactions of radiation and matter are the subject of the science called spectrometry. Spectroscopic analytical methods are based on the measurement the amount radiation produce or absorbed by molecular or atomic species of interest [7].

Spectrophotometric Methods:

The light absorption (or) emission characteristic of drugs is measured such as UV-Visible, IR, NMR, ESR, fluorescence and mass spectroscopy.

UV-Visible	-	Changes in electronic energy levels within the molecule.
Infrared	-	Changes in the vibrational and rotational movements of the Molecule.
Microwave	-	Electron spin resonance or electron paramagnetic resonance induces changes in the magnetic properties of unpaired electron.
Radiofrequency	-	Nuclear magnetic resonance; induces changes in the magnetic properties of certain atomic nuclei, notably that of hydrogen and the C^{13} isotope of carbon [8].

Quantitative spectrophotometric assay of medical substance by using UV-Visible spectroscopy:

The assay of an absorbing substance may be quickly carried out by preparing a solution in a transparent solvent and measuring its absorption at a suitable wavelength of maximum absorption where small errors in setting the wavelength scale have little effect on the measurement absorbance of approximately 0.9, around which the accuracy and precision of measurement are optimal. The concentration of the absorbing substance is then calculated from the measured absorbance using one of these principal procedures [9].

1. Use of standard absorptivity value.
2. Use of calibration graph.
3. Single or double point standardization.
4. Assay of substances multicomponent samples.
5. Assay as a single components sample.

6. Assay using absorbance corrected for interference.
7. Assay of after solvent extraction of the sample.
8. Simultaneous equations method.
9. Absorbance ratio method.
10. Geometric correction method [9].

CHROMATOGRAPHY:

Chromatography may be defined as a method of separating mixture of components into individual components though equilibrium distribution between two phases. Especially, the technique of chromatography is based on the difference in the rate at which the components of the mixture move thorough a porous medium under the influence of some solvent or gas. The chromatographic method of separation, in general, it involves the following steps (i.e) adsorption or retention of substance or substance on the stationary phase. Separated substance by a continuous flow of mobile phase, the method is being called elution**.**

High Performance Liquid Chromatography (HPLC):

The technique is based on the same mode of separation as that classical column chromatography that is adsorption, partition, ion exchange and gel permeation, but it differs from column chromatography in that the mobile phase is pumped through the packed column under high pressure. High performance liquid chromatography is a very sensitive analytical technique most widely used for quantitative and qualitative analysis of pharmaceuticals. The principle advantage of HPLC compared to classical column chromatography is improved resolution of the separated substance, faster separation times and the increased accuracy, precision, and sensitivity. High-performance liquid chromatography sometimes referred to as high-pressure liquid chromatography, which is a chromatographic technique used to separate a mixture of compounds in analytical chemistry with the purpose of identifying, quantifying and purifying the individual components of the mixture [10, 11].

Principle involved in HPLC:

The principle of separation in normal phase mode and reverse phase mode is adsorption. The component which has more affinity towards the adsorbent travels slower. The component which has less affinity towards the stationary phase travels faster. Since no two components have the same affinity towards the stationary phase, the components are separated.

Instrumentation:

Solvents must be degassed to eliminate formation of bubbles. The pumps provide a steady high pressure with no pulsating, and can be programmed to vary the composition of the solvent during the course of the separation. Detectors rely on a change in refractive index, UV-VIS absorption, or fluorescence after excitation with a suitable wavelength. The different types of HPLC columns are described in a separate document.

A column (or other support for TLC, see below) holds the stationary phase and the mobile phase carries the sample through it. Sample components that partition strongly into the stationary phase spend a greater amount of time in the column and are separated from components that stay predominantly in the mobile phase and pass through the column faster.

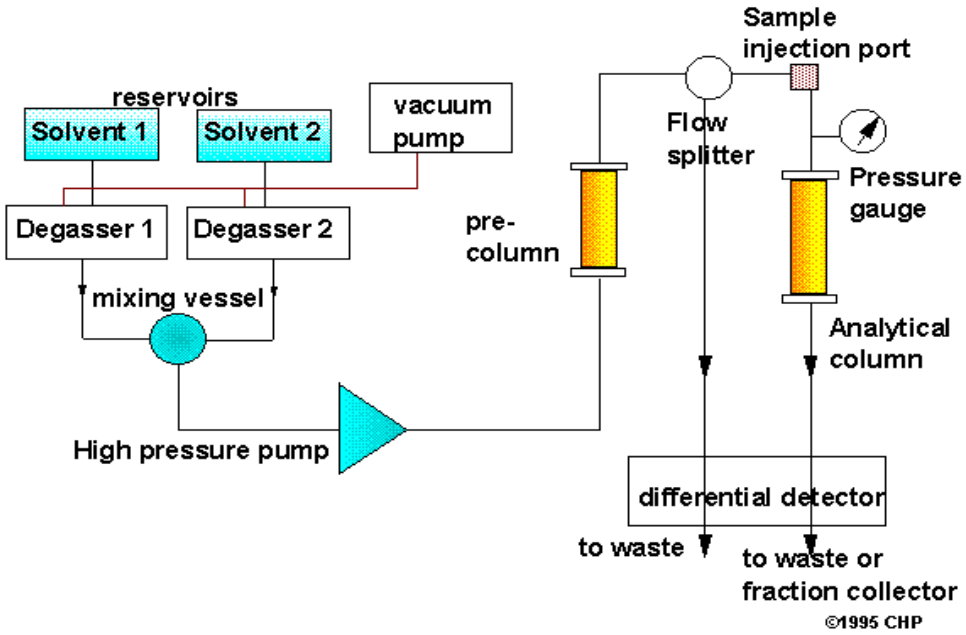


Figure1: Schematic diagram of an HPLC instrument.

Detector:

Optical.

Equipped with a flow cell.

Focus light beam at the center for maximum energy transmission.

Cell ensures that the separated bands do not wide[11, 12].

ELECTROCHEMICAL ANALYSIS:

In electrochemical analysis one or more electrically related parameters e.g.:-voltage, current, or charge are measured and related to the state of the system generating or carrying the charge. In addition these methods can be divided into those relating as in gram to system equilibrium. The combination of electro-analytical methods with chromatography is a powerful tool for both qualitative and quantitative work. It is measured based on the electrochemical property of drugs and classified as potentiometry, conductometry, polarography and amperometry.

Physical methods:

The physical characteristics of drugs are measured such as DSC, DTA, TGA and TMA.

Radio-active methods:

In this method, radiation intensity from a radioactive substance or an induced radioactive substance arising from exposure of the sample to a neutron source is measured using scintillation counters. e.g., radioimmunoassay

Titrimetric methods:

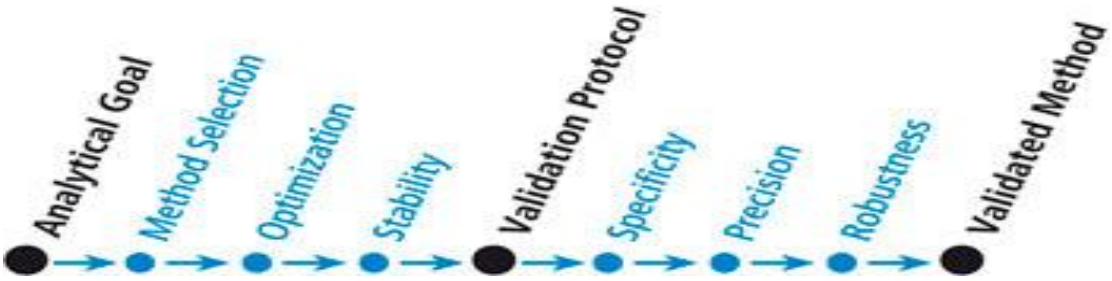
It is based on the reaction between titrant and titrate and the end point detected by the addition of a suitable indicator reagent. It is classified in to several types based on the reaction which is non aqueous, redox, diazotization and complexometric titrations.

X-ray methods:

In this, x-ray is used to identify certain emission peaks, which are characteristics of elements contained in the target. The wavelength of the peak can be related to the atomic numbers of the elements producing them. Other techniques such as combination of two or more methods where separation and identification is possible by using coupled instruments such as GC-MS and LC-MS.

DEFINITION OF VALIDATION:

As per USP, validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for the intended analytical applications [13].



Validation process:

The real goal of the validation process is to challenge the method and determine the limits or allowed deriability for the condition needed to the method.

1. Specificity
2. Linearity
3. Range
4. Accuracy
5. Precision
6. Detection limit

7. Quantification limit

8. Robustness

9. System suitability testing

Methodology:

The real goal of the validation process is to challenge the method and determine the limits or allowed deriability for the condition needed to the method.

Types of analytical procedure to be validated:

Identification test.

Quantitative test for impurities contents.

Limit test for the control of impurities.

Quantitative test of the active molecule in samples of drug substance or drug product.

Analytical procedure:

It refers to the way of performing the analysis.

1. Specificity:

An investigation of specificity should be conducted during the validation of identification test, determination of impurities and assay.

a. Identification:

To ensure the identity of an analyte.

b. Purity test:

To ensure that all the procedure performed allow an accurate statement of the control of impurity of an analyte.

c. *Assay (content of potency):*

To provide an exact result which allow an accurate statement on the content of the analyte in a sample.

2. Linearity:

The linearity of an analytical procedure is of ability (within a given range) to obtain test result while are directly proportional to the concentration of analyte in the dam pole for establishment of linearity. A minimum of 5 concentrations is recommended other approaches would justified.

3. Range:

The range of analytical procedure is the introduce between the upper and lower concentration of an analyte in the sample. The specific range is normally derived from linearity studies and depending on the intended application procedure. The following minimum specified ranges should be considered.

For assay of drug substance: Normally from so to use of the test concentration.

For content uniformity: - 70-80% of the concentration.

For dissolution release product 20% other one hour upto 40% after 24 hour for the validating range would be 0-100% of the label claim.

4. Accuracy:

Accuracy on the method was determined by relative and absolute recovery experiments.

a. Drug substances:

Application of an analytical procedure to analyte of known purity of reference material.

Comparison of request with those of second well characterized procedure, the accuracy of which is stated.

b. Drug product:

Application of an analytical procedure to analyte of known purity of reference the accuracy of which is state.

c. Impurities:

Accuracy should be assessed on sample splinked with known amount of impurities. Compare there to independent procedure.

d. Recommended data:

Accuracy should be assessed using a minimum of a determination over a minimum of 3 concentration levels covering a specified range.

5. Precision:

Validation of test for assay and quantitative determination of impurities includes an included an investigation of precision.

a. Repeatability:

A minimum of a determination covering the specified range for procedure (or) A minimum of 6 determination at 100% of the test.

b. Intermediate precision:

The applicant should establish the affects of random events on the precision of analytical procedure.

c. Reproducibility:

Reproducibility is assessed by means of an inter laboratory test this should be considered in case of standardization of analytical procedure for instance for inclusion, and procedure in I.P.

Standard deviation, relative standard deviation and confidence interval should be reported for each type of precision investigation.

6. Detection limit:

The detection limit of an individual analytical procedure is the lowest amount of analyte is a sample which can be detected but not necessary quantified as an exact value approaches other than those listed below may be acceptable.

Based on visual evaluation.

Based on signal to raise.

Based on the standard deviation on the response and slope.

Based on the standard deviation of the blank.

Based on the calibration curve.

Recommended data.

Detection levels and determination method detection limit should be presented.

The detection limit (LOD) may be expressed as:

$$\mathbf{LOD = 3.3\sigma/S}$$

Where, σ = The standard deviation of the response.

S = The slope of the calibration curve (of an analyte).

7. Quantification limit:

The quantitative limit of an individual analytical procedure is the lowest amount of an analyte in the sample which can be quantitative determined with suitable precision and accuracy. The quantification limit is a parameter of quantitative assay of law levels of compound in sample material and it is used particularly for the determination of impurity.

Quantification Limit (LOQ) may be expressed as:

$$\mathbf{LOQ = 10\sigma/S}$$

Where, σ = The standard deviation of the response.

S = The slope of the calibration curve (of an analyte).

8. Robustness:

The robustness of the method was determined by analyzing the standard solution six times followed by a sample in duplicate with varying HPLC conditions as described below,

Examples of typical variation are:

Stability of analytical solution.

Extraction time.

In case of liquid chromatography examples of typical variation are.

Influence of variation of pH in mobile phase.

Influence of variation of pH in mobile phase components.

Different column.

Temperature.

Flow rate.

9. System suitability testing:

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

The parameters that are affected by the changes in chromatographic conditions are,

Retention (K_A)

Resolution (R_s)

Capacity factor (k')

Selectivity (α)

Column efficiency (N) and

Peak asymmetry factor (A_s)

CHAPTER 2:

LITERATURE REVIEW

LITERATURE REVIEW

Havlikova L et al[14]., **2006** carried out research about HPLC determination of calcium pantothenate and two preservatives in topical cream and reported as simultaneous determination of calcium pantothenate and two preservatives methylparaben and propylparaben present in topical cream. Different analytical columns with various stationary phases were tested. During method development, Supelco Discovery C18 column (125 mm × 4.0 mm, 5 µm) and Zorbax SB-CN column (150 mm × 4.6 mm, 5 µm) were tested. Both were not convenient for analytical separation because of the co-elution of calcium pantothenate with dead volume, and problems with the peak-shape of all components. Good separation was achieved using Zorbax TSM (250 mm × 4.6 mm, 5 µm) and Hypersil ODS column (250 mm × 4.6 mm, 5 µm), the latter was finally used for the analysis. The analysis time was 12 min, at flow rate 0.7 ml min^{-1}. Chromatography was performed using binary mobile phase composed of methanol and phosphoric acid, pH 2.5, 65:35 (v/v). UV detection was accomplished at 214 nm. The method was validated according to ICH guideline recommendations. The method is suitable for practical routine analysis of commercially produced topical pharmaceutical preparations.

Thomas J Franks et al[15]., **2006** developed “A Reverse Phase HPLC Assay for the Determination of Calcium Pantothenate Utilizing Column Switching” and summarized as a reverse phase HPLC assay utilizing column switching has been developed and validated for the determination of calcium pantothenate (CP) in several multivitamin tablet formulations. The reverse phase system utilizes a DuPont Zorbax C-8 analytical column, an automatically switched and back flushed Brownlee RP-18 guard column for the elimination of a highly retained excipient peak, 88:12 0.25M phosphate buffer: CH_3OH mobile phase, and 214 nm detection. Sample preparation and the switched column chromatography cycle each require approximately 15 minutes. A spiked recovery study showed linearity over the 50–150% of theory concentration range. Average recovery was 99.7%. Assay precision studies yielded sample RSD's ranging from 0.8 to 2.3%. Results obtained by this method are comparable to those obtained by the USP method.

Huangmin et al[16]., **2009** carried out research in **"**Measurement of Water-Soluble B Vitamins in Infant Formula by Liquid Chromatography/Tandem Mass Spectrometry." A method has been developed for the simultaneous measurement of multiple B vitamins (i.e., B_1, B_2, B_3, B_5, and B_6) in infant formulas by LC-MS/MS. The vitamins were extracted with acidic solvent, followed by protein precipitation at a pH range of 4.5 to 5.5, and filtered. This simplified procedure eliminates many of the potential sources of laboratory error and facilitates rapid and efficient analysis. As is common in most cases, isotope internal standards were added to account for variations in sample preparation, as well as changes in MS measurement. In this method, isotope-labeled internal standards of B_1, B_3, B_5, and B_6 were used. The factors affecting analytical performance were investigated and optimized. In addition, the stability of these vitamins in the extraction solution was investigated. An acidic condition (5M HCl) was applied to successfully stabilize B_1, which had shown a decrease in signal when other solvents were used. The quantitative extraction and good stability allowed isotope standards to be added to the filtered sample solution, instead of to the extraction solvent. The addition of the isotope to the small portion of the filtered sample solution significantly reduces cost. A comprehensive evaluation of the analysis of the standard reference material and good spike recovery of the vitamins demonstrates the accuracy of the method. The results for commercially available infant formula samples were also compared with those obtained using the current microbiological method.

Karuppiah SP et al[17]., **2015** worked in "Phytochemical evaluation of hydroxy citric acid, catechins and calcium pantothenate present in herbal formulation" and concluded as the analytical method for the estimation of phytochemical active ingredients present in *Garginia combogia* extract with calcium pantothenate for herbal formulations are evaluate for its assay content and dissolution release. This herbal formulation with slim formula consists of several active ingredients such as hydroxy citric acid, catechins and calcium pantothenate. The HPLC method for the estimation of active content an *in-vitro* dissolution release is developed for the study.

Ulrich Holler et al[18]., **2005,** carried out research in ”Quantification of biotin in feed, food, tablets, and premixes using HPLC–MS/MS and summarized as two sensitive and specific methods for quantification of biotin in feed, food, tablets, and premixes based on HPLC–MS/MS have been developed and validated. Depending on sample matrix and biotin content different extraction procedures and HPLC conditions were applied. Key steps in sample preparation were an alkaline extraction or a hydrolysis with sulphuric acid followed by enzymatic digest with papain. For many samples with low biotin content the latter combination of extraction steps was shown to be necessary for an optimal release of biotin from the matrix. The first time synthesis of deuterated biotin for use as internal standard allowed the compensation of losses during sample work-up and ion suppression during HPLC–MS/MS analysis. The new methods are faster than the commonly used microbiological assay using *Lactobacillus plantarum*. Additionally, they have a higher specificity as results for biotin are based on determination of a chemically defined compound, and not of a biological activity. Quantification is applicable to samples with a biotin content >100 µg/kg. Results obtained with the new methods have been compared with those of the microbiological assay, and were in good agreement.

AnthonyE.Ekpe et al[19].,**1997,** developed method for “Liquid chromatographic determination of biotin in multivitamin-multimineral tablets” A reproducible reverse phase high pressure liquid chromatography (RP-HPLC) method for the determination of biotin in multivitamin-multimineral tablets has been developed and validated. This method involves reverse phase separation of the component monitored by absorbance at 200 nm wavelength. The method has excellent precision and accuracy with S.D. 0.83 and 2.9%, respectively. The established linearity range was 0.5–2 µg ml^{-1} (r^2>0.9999). The recovery of biotin from spiked placebo was >97% over the linear range. The extraction procedure is simple and the HPLC conditions separate biotin from its degradation products and excipients. The method has been successfully used in determining biotin content in 4 brands of commercially available multivitamin- multimineral tablets.

Chikakoyomota et al[20]., **2016,** carried out research in**"**Determination of biotin following derivatization with 2-nitrophenylhydrazine by high-performance liquid chromatography with on-line UV detection and electrospray-ionization mass spectrometry" Currently, biotin is typically determined in Japan using a microbiological method. Such microbiological assays are sensitive, but they are not always highly specific and are also rather tedious and time-consuming. In the present study, RP-HPLC and LC–MS methods for the determination of biotin have been developed by derivatizing the carboxyl group with 2-nitrophenylhydrazine hydrochloride. 2-Nitrophenylhydrazine is used for the derivatization of carboxylic acids, and these derivatives are known to be applicable to LC–MS detection. Biotins in tablets were extracted by the addition of water and ultrasonic agitation. In order to clean up the sample solution, the filtrate was applied to an ODS cartridge and eluted with methanol. The conditions for preparing the 2-nitrophenylhydrazide derivatives were modified from a previous report for fatty acids. Good recovery rates of over 70% were obtained for the addition of 5–125 μg of biotin per formulation. The detection limit in HPLC at 400 nm was 0.6 ng per injection, with good linearity being obtained over the concentration range 0.001–0.2 μg per injection. Further, derivatives were determined by LC–MS with electrospray ionization, where the spectra indicated the molecular ions $[M + H]^+$. The detection limit was 0.025 ng per injection in the selected ion monitoring analysis, and linearity was observed in the range of 0.6–6 ng per injection. The proposed method could be used to specifically determine the presence of biotin in relatively clean samples.

Hua-BinLi et al[21]., **2001,** performed research in "Simultaneous determination of nine water-soluble vitamins in pharmaceutical preparations by high-performance liquid chromatography with diode array detection" and concluded as a simple and rapid method for the simultaneous determination of nine water-soluble vitamins was developed by high performance liquid chromatography with diode array detection. The water-soluble vitamins were analyzed by HPLC on a μ-Bondapak C_{18} column (300×3.9 mm, 10 μm) with methanol-0.1 mol/L KH_2PO_4 buffer (pH 7.0) as mobile phase (1.5 mL/min) in gradient mode as follows: in the range 0–7.0 min, 10:90; and in the range

8.0–15.0 min, 40:60 (*v*/*v*). All nine water-soluble vitamins were separated in a single HPLC run within less than 15 min. The linearity of the calibration graphs was compound-dependent with correlation coefficients of 0.9986 to 0.9999 (*n* = 6). The detection limits ranged from 0.02 µg/mL to 0.5 µg/mL. The method was successfully applied to the determination of vitamins in pharmaceutical preparations. The recoveries were from 95% to 102% and the relative standard deviations were in the range of 0.8% to 4.2%.

Yoon young huh et al[22]., **2011,** carried out research in ”Development of Analytical Method of Biotin in Complex Drugs and Dietary Supplements Using HPLC-UV” and concluded as to improve the quality control, and protect consumers by developing prevalently used and efficient analytical tools to determine and quantify target compounds. Because the Korean Pharmacopeia (KP) presents microbiological assays for biotin, which is laborious and time-consuming, this study is focused on applying HPLC-UV to detect and quantify biotin in complex drugs and dietary supplements like multi-vitamin. Biotin in complex drugs was extracted from methanol and analyzed using mobile phase with 10 M potassium phosphate (monobasic, pH=3.0) in distilled water and acetonitrile. Gradient condition was used to successfully detect and quantify biotin within 20 minutes. Validation result for linearity was significant that average r^2 was 0.999 (n=3) and its relative standard deviation (RSD) was 0.0578% which was less than 2%. Using this method, quantification of biotin in complex drugs was completed successfully and recovery tests were finished that recovery percentage greater than 95% with relative standard deviation less than 2%.

Timmons et al[23]., **1987** performed research in “Reverse phase liquid chromatographic assay for calcium pantothenate in multivitamin preparations and raw materials.” A reverse phase liquid chromatographic (LC) method has been developed for the assay of calcium pantothenate in commercial multivitamin tablet formulations and raw materials. The assay was validated according to the Pharmaceutical Manufacturers Association Quality Control HPLC Committee guidelines. The chromatographic system includes a C-18 column and a mobile phase consisting of 0.25M sodium phosphate buffer, pH 2.5, and acetonitrile (97 + 3 v/v). The column effluent is monitored by UV detection at 205 nm. The sample preparation involves only extraction in water

followed by filtration. The method is stability-indicating with a detection limit of approximately 50 ng/mL of the calcium pantothenate in the samples. The system is linear from at least 0.02 to 0.10 mg/mL. The mean recovery of spiked placebos ranged from 98.7 to 99.8%. The within-day precision of the assay on finished products (N = 6) ranged from 0.3 to 2.0% CV. A system suitability criterion for resolution is based on the separation between calcium pantothenate and 2 closely eluting compounds, saccharin and a saccharin degradation product, 2-sulfamoylbenzoic acid.

Ramniwas Dudhwal et al[24]., **2011** carried out research in "Simultaneous Estimation of Ascorbic Acid and Calcium Pantothenate in Multivitamin and Multimineral Tablets by Reverse-Phase HPLC" and concluded as simultaneous estimation of Ascorbic acid (AA) & Calcium pantothenate (CP) in a pharmaceutical Multivitamin & Multimineral unit dosage form was achieved on a reverse phase C_{18} column (5µm; 250x4.6 mm) with an isocratic mobile phase elution order at a flow rate of 1.0 mL/min. The mobile phase was buffer [0.1 %v/v solution of triethylamine in milli-Q water with pH 3.00 ± 0.05 (adjusted with ortho-phosphoric acid)] and methanol in the ratio of 80:20. Detection was performed with UV detector at 210nm. The method was validated with respect to linearity, precision, accuracy, and specificity according to ICH guidelines. The responses were linear in concentration range of 20-60 µg/mL for AA and 2.5-7.5 µg/mL for CP. The values of slope and correlation coefficient were found to be 15202 & 1.000 for AA; and 7583 & 1.000 for CP respectively. The %RSD value for repeatability and intermediate precision studies were 2.9 & 0.8 for AA; and 3.3 & 2.8 for CP respectively. The %recovery of the vitamins ranged between 98.0 to 99.7 for AA and 98.1 to 98.7 for CP.

Thomas J Hudson et al[25]., **1984,** worked in the area of "Determination of pantothenic acid in multivitamin pharmaceutical preparations by reverse-phase high-performance liquid chromatography" concluded as a high-performance liquid chromatographic procedure was developed for the analysis of calcium pantothenate in nutritional supplements. The method involves a simple extraction using phosphate buffer and sonication. Chromatographic separation is obtained using an aminopropyl-loaded silica

gel column in the reverse-phase mode. A UV detector set at 210 nm was used to monitor the effluent. Quantitative recoveries were obtained, and precision of the method is discussed. The method is applicable to multivitamin tablets, calcium pantothenate raw material, and yeast grown in the presence of high levels of calcium pantothenate. The results of the method are compared with results obtained from the USP microbiological method of analysis. It was concluded that the procedure is rapid, accurate, easily automated, and practical for routine quality control use.

Kai-On Chu et al[26]., **1998,** carried out research in the area of ”Analysis of Commercial Multi-vitamin Preparation by HPLC with Diode Array Detector” and reported as a simple and efficient HPLC analysis method has been developed to simultaneously analyze complicated multi-vitamin preparations, including vitamin B complexes, vitamin C, orotic acid, methionine, calcium pantothenate, nicotinamide, biotin, folic acid and rutin, without using expensive ion pairing reagent. Diode array detector and adjustment of the mobile phase to pH 5.6 are required in the study. This method has been validated with good linearity, reproducibility, recovery and accuracy for analysis of commercial multi-vitamin products.

Zerzanova A et al[27]., **2007,** carried out research in ”Using of HPLC coupled with coulometric detector for the determination of biotin in pharmaceuticals” and concluded as the method for the determination of biotin by high performance liquid chromatography (HPLC) coupled with coulometric detector. Chromatographic and detection conditions were tested. A LiChrospher 60RP-select B column (250 mm × 4 mm; 5 μm) and the mobile phase containing 0.24 mol/L aqueous solution of acetic acid and acetonitrile in the ratio 85:15 (v/v) were found as the most suitable. The flow rate was 1 mL/min and the injected volume of the sample was 20 μL. The hydrodynamic voltammogram of biotin was measured and according to obtained data the detection parameters were set – channel I 600 mV, channel II 900 mV, sensitivity 1 μA. The developed method has been validated. The calibration curve is linear in the range 15–3600 ng/mL, correlation coefficient is 0.9998, limits of detection and quantification are 5 and 15 ng/mL, respectively. Recovery of the spiked samples was 98.67% with RSD 0.255%

on average. The developed method has been successfully applied for determination of biotin in pharmaceutical preparations.

Staggs C G et al[28]., **2004,** carried out research in ”Determination of the biotin content of select foods using accurate and sensitive HPLC/avidin binding” and reported as accuracy in estimating dietary biotin is limited both by data gaps in food composition tables and by inaccuracies in published data. The present study applied sensitive and specific analytical techniques to determine values for biotin content in a select group of foods. Total biotin content of 87 foods was determined using acid hydrolysis and the HPLC/avidin-binding assay. These values are consistent with published values in that meat, fish, poultry, egg, dairy, and some vegetables are relatively rich sources of biotin. However, these biotin values disagreed substantially with published values for many foods. Assay values varied between 247 times greater than published values for a given food to as much as 36% less than the published biotin value. Among 51 foods assayed for which published values were available, only seven agreed within analytical variability (±20%). We conclude that published values for biotin content of foods are likely to be inaccurate.

Pei chen et al[29]., **2010** carried out research in “A LC/UV/Vis method for determination of cyanocobalamin (VB_{12}) in multivitamin dietary supplements with on-line sample clean-up” and concluded that the HPLC- UV/Vis method using a two-column strategy with a switching valve for on-line sample clean-up was developed by using two columns (i.e) Agilent zorbax C_8 (150mm× 4.6mm, 5µm particle size) reverse phase column and a waters symmetry C_{18} (150mm× 4.6mm, 5µm particle size) reverse phase column. Chromatographic separation was achieved using a programmed gradient mobile phase consisting of (A) 0.1% formic acid in acetronitrile. Because of the low levels of vitamin-B_{12} in the sample, large injection volumes, and thus much interfering material, must be used to exceed the limit of quantification (LOQ) by UV detection. A switching valve was used to divert most of these early eluting interfering materials to waste, effecting online sample cleanup without excessive sample preparation steps. The recovery of CN-Cbl in the method was 99.5% and the LOQ was 10ng per injection. The method was

successfully applied to the analysis of the NIST SRM 3280 multivitamin/ multimineral dietary supplement tablet. The method is specific, precise and accurate for the intended use. Compared to off-line sample clean-up procedures, it offers the advantage of being easier, more economical and less time consuming.

Gabriela Klaczkow et al[30]., **2001**, developed method for "Elaboration of HPLC method for Biotin determination in multiple vitamin drugs and comparison with microbiological method" and reported the optimal analytical conditions for the biotin determination by HPLC method with detection at the wavelength 200 nm in multiple vitamin drugs. Statistical parameters of the HPLC method and the microbiological method of the biotin determination were compared whose RSD values were 0.31%-1.88%and 2.02%-6.63% respectively.

Tsui-Min Wang et al[31]., **2003**, performed research in "Liquid chromatographic method for determination of calcium pantothenate preparations and related stability studies" and reported that the samples were analyzed in C_{18} column with the mobile phase of acetonitrile and potassium dihydrogen phosphate solution pH adjusted to 2.5 with phosphoric acid at the flow rate of 1.0 mL/min and UV absorbance detection at 204 nm. Ampicilin was used as a internal standard. The retention time of calcium pantothenate and ampicilin were 5.3 and 6.5 min, respectively. An equation was presented for linear relationship between peak height ratios of calcium pantothenate to ampicilin and the calcium pantothenate concentration over a range of 10-50 µg/ml (r = 0.9999). Standard addition recoveries were greater than 98.96% with twelve commercial products. The RSD were lies between 0.1 and 0.9% in inter-day assays, 0.1 and 0.7% in intra-day assays. The result obtained from the HPLC assay method which we developed and the microbiological assay of USP was paired at 95% confidence level. There was no significant difference between these two methods. The proposed HPLC method was a suitable substitute for microbiological method for quantitative assays of calcium pantothenate in commercial products.

Marina Franco et al[32]., **2011**, carried out research in "Application of CZE method in routine analysis for determination of B-complex vitamins in pharmaceutical and veterinary preparations" and concluded that vitamins of interest are thiamine hydrochloride (B_1), thiamine monophosphate chloride (B_{1a}), ribofavine (B_2), riboflavin-5' monophosphate (B_{2a}), nicotinamide (B_3), d-pantothenic acid calcium salt (B_5), pyridoxine hydrochloride (B_6), folic acid (B_9) and 4-amino benzoic acid (B_{10}). These analytes were separated optimizing the experimental condition in 20mM tetra borate buffer p^H = 9.2 as a BGE, on Beckman P/ACE system MDQ instrument, using uncoated fused silica capillary. The effective capillary length was of 49.5 cm, I.D. = 50µm, the applied voltage 20kV and the temperature 25ºc. Detection was performed by diode array detector at 214 nm for all vitamins except B_5 (190nm) and B_{2a} (260 nm). Separation time was about 9 min. After experimental conditions optimization, the proposed method was validated. Precision of migration time and corrected peak area, linearity range, LOD and LOQ, accuracy (recovery), robustness and ruggedness were evaluated for each analyte demonstrating the good reliability of the method. Analysis of the pharmaceutical real samples was performed and confirmed the versatility of this method.

CHAPTER 3: RESEARCH OBJECTIVE & PLAN OF RESEARCH WORK

RESEARCH OBJECTIVE

The drug analysis plays an important role in the development of drugs, their manufacture and the therapeutic use. Pharmaceutical industries rely upon quantitative chemical analysis to ensure that the raw materials used and final product obtained meets the required specification. The number of drugs and drug formulations introduced in to the markets has been increased at an alarming rate. These drugs or formulation may be either in the new entities in the market or partial structure modification of the existing drugs or novels dosage forms or multi component dosage forms.

The drug selected for the present study is biotin and calcium pantothenate in tablet dosage form containing multi-vitamins and multi-minerals. A review of literature reveals that only few chromatographic and spectrophotometric methods have been reported for the estimation of biotin and calcium pantothenate separately but not in simultaneous method. So my prime aim is to develop a simple, rapid, efficient, accurate, reproducible, less time consuming and reliable new validated chromatographic method of analysis for biotin and calcium pantothenate in pharmaceutical dosage form.

The objective of the research is as follows:

- Development of rapid, sensitive and accurate reverse phase HPLC method for the simultaneous estimation of biotin and calcium pantothenate.
- To estimate biotin and calcium pantothenate simultaneously from tablet dosage form by developed method and validation of proposed method.

PLAN OF RESEARCH WORK

1. To collect through knowledge in practical RP-HPLC method development by using various search engines like ACS, Elsevier, Bentham, etc…
2. To proceed a step by step procedure for method development to be implemented and initial chromatographic condition for assay of biotin and calcium pantothenate in tablet dosage form is to be estimated.
3. To optimize the chromatographic conditions and trials the method.
4. To validated a method for analytical quantization of assay in biotin and calcium pantothenate in tablets dosage form.
5. To give a general ICH guidelines for the validation of methods aim for the quantization of biotin and calcium pantothenate in tablets dosage form.
6. To make document from the obtained results with improved accuracy and precision.

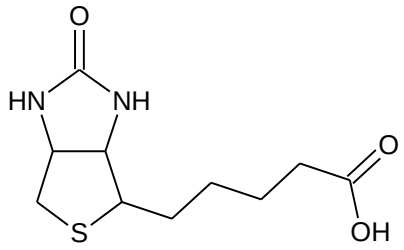
CHAPTER 4:

DRUG PROFILE

DRUG PROFILE

Biotin:

Biotin is a water-soluble B-vitamin, also called vitamin B_7 and formerly known as vitamin H or coenzyme R. Biotin is necessary for cell growth, the production of fatty acids, and the metabolism of fats and amino acids. Biotin assists in various metabolic reactions involving the transfer of carbon dioxide. It may also be helpful in maintaining a steady blood sugar level. Biotin is often recommended as a dietary supplement for strengthening hair and nails, though scientific data supporting this outcome are weak. Nevertheless, biotin is found in many cosmetics and health products for the hair and skin.

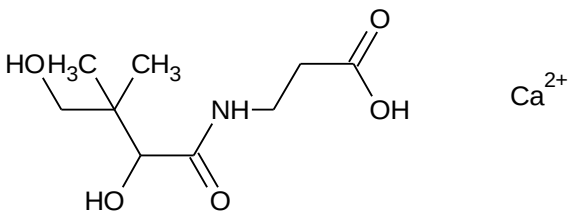
Synonym	:	Vitamin B_7; Vitamin H; Coenzyme R; Biopeiderm
Empirical Formula	:	$C_{10}H_{16}N_2O_3S$
Molecular weight	:	244.31 g·mol^{-1}
Appearance	:	White crystalline needles
Melting Point	:	232 to 233 °C (450 to 451 °F; 505 to 506 K)
Solubility in water	:	22 mg/100ml
Structure	:	

5-(2-Oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl) pentanoic acid

Calcium Pantothenate:

Pantothenic acid, also called vitamin B_5 (B vitamin), is a water-soluble vitamin. Pantothenic acid is an essential nutrient. Animals require pantothenic acid to synthesize coenzyme-A (CoA), as well as to synthesize and metabolize proteins, carbohydrates and fats. The anion is called pantothenate. Pantothenic acid in the form of CoA is also required for acylation and acetylation, which, for example, are involved in signal transduction and enzyme activation and deactivation, respectively. Since pantothenic acid participates in a wide array of key biological roles, it is essential to all forms of life. As such, deficiencies in pantothenic acid may have numerous wide-ranging effects. Calcium pantothenate is often used in dietary supplements because, as a salt, it is more stable than pantothenic acid. Supplementation may improve oxygen utilization efficiency and reduce lactic acid accumulation in athletes.

Molecular formula	:	$C_9H_{17}NO_5$
Molecular weight	:	476.536 g/mol
Appearance	:	Yellow oil ; Colorless crystals (Ca^{2+} salt)
Melting point	:	183.83 °C (362.89 °F; 456.98 K)
Solubility	:	Very soluble in C_6H_6, ether; Ca^{2+} salt: Slightly soluble in alcohol, $CHCl_3$.
Solubility in Water	:	Very soluble 2.11 g/mL (Ca^{2+} salt)
Related Compound	:	Panthenol
Structure	:	



Calcium salt of 3-[(2,4-dihydroxy-3,3-dimethylbutanoyl)amino]propanoic acid

CHAPTER 5:
MATERIALS & EQUIPMENTS

MATERIALS AND EQUIPMENTS

Background:

The RP-HPLC method for the estimation of biotin and calcium pantothenate in solid dosage form has been validated in Fourrts (India) Laboratories Pvt. Ltd. Analytical method validation for the estimation of biotin and calcium pantothenate in solid dosage form by RP-HPLC has been conducted as per the protocol, to ensure that the performance characteristics of the method meet the requirements for its intended application.

Required instrument and reagent:

Materials:

The following table list materials were used in this study.

S.No	Name	Grade	Supplier	Lot No/ B.No	Purity	Expiry
1	KH_2PO_4	AR	Rankem, RFCL Ltd, Mumbai.	J037C16	100.0%	Feb'20
2	KH_2PO_4	AR	Finar Chemical Ltd, Sanand.	21070305J12	99.5%	Oct'17
3	Methanol	HPLC	Fischer Chemicals.	1333170916	99.9%	Oct'18
4	Methanol	HPLC	Finar Chemical Ltd, Sanand.	168071027AP	99.8%	Jun'18
5	H_3PO_4	AR	Rankem, RFCL Ltd, Mumbai.	G025J15	88.0%	Sep'18
6	Ammonia	AR	Fischer Chemicals.	4929/17306-5	25.0%	Jun'17

Working standard:

The following table list standards were used in this study.

S.No	Name	Grade	Lot No/ B.No	Purity	Expiry
1	Calcium pantothenate	BP	WS038/09	97.75	23/10/2017
2	Biotin	BP	WS103/07	99.35	09/02/2018

Instrumentation:

The following table list instruments were used in this study.

S.No	Name of the Instrument	Ref. Number	Make	Model
1	Electronic balance	I/RD/OEB/EB/01	Adventurer OHAUS	AR2140
2	HPLC	I/RD/HPC/02	Shimadzu	Prominence
3	HPLC	I/RD/HPC/04	Shimadzu	LC-2010C HT

Column details:

The following table list column that was used in this study.

Column	Ref. Number	I.D. Number	Make	Specification
1	RD/COL/76	K63402	Nacalai Tesque,Inc.	*Cosmosil* ,5C_{18}-MS-II (250 X 4.6 mm, 5µ)
2	RD/COL/77	K65386	Nacalai Tesque,Inc.	*Cosmosil* ,5C_{18}-MS-II (250 X 4.6 mm, 5µ)

CHAPTER 6: EXPERIMENTAL WORKS

EXPERIMENTAL WORKS – (RP- HPLC)

Method development

1. Selection of wavelength:

Biotin and calcium pantothenate working standards were dissolved in suitable diluent. And the solution were scanned between 200 to 400 nm using uv-visible spectrophotometer then these were subjected to chromatograph containing PDA detector to determine the accurate and optimized wavelength.

After reviewing the uv-visible Spectrum, a wavelength of **210 nm** was selected as the optimum wavelength for this method.

2. Selection of Mobile phase:

A mobile phase of water: methanol in the ratio of 1:1 was run through the HPLC column. But the elution of drug in this mobile phase was poor. So, mobile phase was changed to mono basic potassium dihydrogen orthophosphate buffer and methanol in the ratio of 1:1 with pH of 4, again the elution was poor. So, the concentration of same mobile phase was changed to 60: 40 (buffer: methanol) and adjusts the pH 2.20 ± 0.05 with 10% orthophosphoric acid and it was used.

3. Buffer preparation:

Dissolve 4.1 g of mono basic potassium dihydrogen ortho phosphate in 1000 ml of water. Adjust the pH to 2.20 ± 0.05 with 10 % orthophosphoric acid. Filter through 0.45 µ membrane filter.

4. Mobile phase preparation:

Measure 600ml of buffer and 400ml of methanol to form the concentration ratio of 60:40, mix well and degas it.

5. Standard solution (Calcium pantothenate):

Weigh accurately about 100.0 mg of calcium pantothenate working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 1.0 mg/ml of calcium pantothenate).

6. Standard solution (Biotin):

Weigh accurately about 10.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 0.10 mg/ml of biotin).

7. Standard Solution (Calcium pantothenate+ biotin):

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 1.0 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

Chromatographic conditions:

Column	: *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent
Flow rate	: 1.0 ml / min
Detection wavelength	: 210 nm
Injection volume	: 20 µL
Oven temperature	: 30°C
Mobile phase	: Buffer: methanol (60: 40)

Report:

It shows elution but the peak was not splitted in combined form.

For improving the elution of biotin and calcium pantothenate in combination:

The peaks of biotin and calcium pantothenate were not separated in the combined form. In order to separate peaks either change in ratio of mobile phase or addition of peak modifier or ion pairing agent has to be used. Here changing the buffer concentration should be used to its ability to shift peak in the ratio of 70:30 (buffer: methanol) and thus giving good resolution.

Buffer preparation:

Dissolve 4.1 g of mono basic potassium dihydrogen ortho phosphate in 1000 ml of water. Adjust the pH to 2.20 ± 0.05 with 10 % orthophosphoric acid. Filter through 0.45 µ membrane filter.

Mobile phase preparation:

Measure 700ml of buffer and 300ml of methanol to form the concentration ratio of 70:30, mix well and degas it.

Standard solution (Calcium pantothenate):

Weigh accurately about 100.0 mg of calcium pantothenate working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 1.0 mg/ml of calcium pantothenate).

Standard solution (Biotin):

Weigh accurately about 10.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 0.10 mg/ml of biotin).

Standard solution (Calcium pantothenate+ biotin):

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of Biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 1.0 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

Chromatographic conditions:

Column	: *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent
Flow rate	: 1.0 ml / min
Detection wavelength	: 210 nm
Injection volume	: 20 µL
Oven temperature	: 30°C
Mobile phase	: Buffer: methanol (70: 30)

Report:

Elution of both biotin and calcium pantothenate was found to be extremely good when change in concentration of mobile phase was used.

Selection of phase:

On the basis of RP-HPLC mode and number of carbon atom present in molecule C_{18} bonded stationary phase was tried. *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent, for this stationary phase was selected as it gives good result in case of system suitability parameter i.e. resolution, USP tangent, USP tailing.

Selection of other parameter:

Other parameter like mobile phase, flow rate, column, temperature and wavelength of detector can be selected on the basis of chemical properties of components present in the sample, sensitivity and system suitability requirement of the analytical method.

OPTIMIZED METHOD

Buffer preparation:

Dissolve 4.1 g of mono basic potassium dihydrogen ortho phosphate in 1000 ml of water. Adjust the pH to 2.20 ± 0.05 with 10 % orthophosphoric acid. Filter through 0.45µ membrane filter.

Mobile phase preparation:

Measure 700ml of buffer and 300ml of methanol to form the concentration ratio of 70:30, mix well and degas it.

Standard solution (Calcium pantothenate):

Weigh accurately about 100.0 mg of calcium pantothenate working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 1.0 mg/ml of calcium pantothenate).

Standard solution (Biotin):

Weigh accurately about 10.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 0.10 mg/ml of biotin).

Standard solution (Calcium pantothenate+ biotin):

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 1.0 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

Chromatographic conditions:

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 1.0 ml / min

Detection wavelength : 210 nm

Injection volume : 20 µL

Oven temperature : 30°C

Mobile phase : Buffer: methanol (70: 30)

Calculation:

% Assay of calcium pantothenate:

$$= \frac{\text{Area of sample X Std wt (mg) X 100 X Purity of std. X Avg wt. of tablet (mg) X 100}}{\text{Area of std X 100 X Sample wt (mg) X 100 X 100}}$$

= ---------- % of calcium pantothenate

% Assay of biotin:

$$= \frac{\text{Area of sample X Std wt (mg) X 100 X Purity of std. X Avg wt. of tablet (mg) X 100}}{\text{Area of std X 100 X Sample wt (mg) X 100 X 10}}$$

= ---------- % of biotin

Parameters used for assay validation:

The validation of the assay procedure was carried out using the following parameters.

1. Specificity:

Analyze the *placebo*, calcium pantothenate and biotin separately. A solution of *placebo* was spiked with the calcium pantothenate and biotin at its working concentration. The solution was analyzed as per the RP-HPLC method described.

(i) Placebo solution:

Weigh accurately about 430 mg of powdered *placebo* into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter (Concentration: 4.30 mg/ml of *placebo*).

(ii) Standard solution (Calcium pantothenate):

Weigh accurately about 100.0 mg of calcium pantothenate working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 1.0 mg/ml of calcium pantothenate).

(iii) Standard solution (Biotin):

Weigh accurately about 10.0 mg of biotin working standard into a clean 100ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 0.10 mg/ml of biotin).

(iv) Standard solution (Calcium pantothenate + biotin):

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 1.0 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(v) Standard + placebo solution:

Weigh accurately about 430.0 mg of *placebo*, 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 4.30 mg/ml of Placebo, 1.0 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

Acceptance criteria:

The *placebo* chromatogram should not show any peak at the retention time of calcium pantothenate and biotin.

2. System precision:

A standard solution of 1.00 mg/ml of calcium pantothenate, and 0.10 mg/ml of biotin was prepared and analyzed as per the method.

(i) Standard solution:

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 1.0 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

Procedure:

Inject the standard solution (6 injections). Ensure the following system suitability criteria. **Table 6.1** expresses the criteria for system precision.

Table 6.1: Acceptance criteria

System suitability parameter	Acceptance criteria
Tailing factor	NMT 2.0
Column efficiency	NLT 2000 theoretical plates
Relative standard deviation	NMT 2.0%

3. Linearity and Range:

(a) Linearity of Calcium pantothenate:

The linearity of the RP-HPLC method was demonstrated for calcium pantothenate ranging from 0.4960 mg/ml to 1.4940 mg/ml, which is equivalent to 50% to 150% of the calcium pantothenate working strength. Five standard solutions at the concentrations within the mentioned range were prepared and analyzed as per the method.

(b) Linearity of biotin:

The linearity of the RP-HPLC method was demonstrated for biotin solutions ranging from 0.0520 mg/ml to 0.1500 mg/ml, which is equivalent to 50% to 150% of the biotin working strength. Five standard solutions at the concentrations within the mentioned range were prepared and analyzed as per the method.

Solutions:

(i) Level - I (50%):

Weigh accurately about 50.0 mg of calcium pantothenate working standard and 5.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 0.50 mg/ml of calcium pantothenate and 0.05 mg/ml of biotin).

(ii) Level - II (80%):

Weigh accurately about 80.0 mg of calcium pantothenate working standard and 8.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 0.80 mg/ml of calcium pantothenate and 0.08 mg/ml of biotin).

(iii) Level - III (100%):

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(iv) Level – IV (120%):

Weigh accurately about 120.0 mg of calcium pantothenate working standard and 12.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 1.20 mg/ml of calcium pantothenate and 0.12 mg/ml of biotin).

(v) Level – V (150%):

Weigh accurately about 150.0 mg of calcium pantothenate working standard and 15.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 1.50 mg/ml of calcium pantothenate and 0.15 mg/ml of biotin).

Procedure:

Prepare the above solutions ranging from 50% to 150% and inject each level in duplicate.

Perform the Correlation co-efficient by covering at least five points and report the linearity as the range for determining the assay.

Acceptance criteria:

The plot of concentration versus peak area should be linear with a correlation co- efficient not less than 0.995.

4. Accuracy:

The accuracy of the method was determined by using three solutions containing *placebo* spiked with calcium pantothenate and biotin at approximately 50%, 100% and 150% of its working strength. Each level was analyzed.

Solutions:

(i) Level - I (50%) – 1:

Weigh accurately about 430 mg of *placebo*, 50.0 mg of calcium pantothenate working standard and 5.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 4.30 mg/ml of *placebo*, 0.50 mg/ml of calcium pantothenate and 0.05 mg/ml of biotin).

(ii) Level - I (50%) – 2:

Weigh accurately about 430 mg of *placebo*, 50.0 mg of calcium pantothenate working standard and 5.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 4.30 mg/ml of *placebo*, 0.50 mg/ml of calcium pantothenate and 0.05 mg/ml of biotin).

(iii) Level - I (50%) – 3:

Weigh accurately about 430 mg of *placebo*, 50.0 mg of calcium pantothenate working standard and 5.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 4.30 mg/ml of *placebo*, 0.50 mg/ml of calcium pantothenate and 0.05 mg/ml of biotin).

(iv) Level - II (100%) – 1:

Weigh accurately about 430 mg of *placebo*, 100.0 mg of calcium pantothenate working standard and 10.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 4.30 mg/ml of *placebo*, 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(v) Level – II (100%) – 2:

Weigh accurately about 430 mg of *placebo*, 100.0 mg of calcium pantothenate working standard and 10.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 4.30 mg/ml of *placebo*, 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(vi) Level – II (100%) – 3:

Weigh accurately about 430 mg of *placebo*, 100.0 mg of calcium pantothenate working standard and 10.0 mg of Biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 4.30 mg/ml of *placebo*, 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(vii) Level – III (150%) – 1:

Weigh accurately about 430 mg of *placebo*, 150.0 mg of calcium pantothenate working standard and 15.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 4.30 mg/ml of *placebo*, 1.50 mg/ml of calcium pantothenate and 0.15 mg/ml of biotin).

(viii) Level – III (150%) – 2:

Weigh accurately about 430 mg of *placebo*, 150.0 mg of calcium pantothenate working standard and 15.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 4.30 mg/ml of *placebo*, 1.50 mg/ml of calcium pantothenate and 0.15 mg/ml of biotin).

(ix) Level – III (150%) – 3:

Weigh accurately about 430 mg of *placebo*, 150.0 mg of calcium pantothenate working standard and 15.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 4.30 mg/ml of *placebo*, 1.50 mg/ml of calcium pantothenate and 0.15 mg/ml of biotin).

Procedure

Prepare the above solutions in the range of from 50%, 100% and 150% and inject each solution in duplicate as per the test method.

Calculate the recovery in each level by calculating the measured concentration against theoretical concentration.

Acceptance criteria:

The recovery should be in the range of 98.0-102.0%

5. Method precision:

The method precision was performed by analyzing a sample solution of Hairgro tablets (B.No: RD16047) as per the test method (six replicate sample preparation).

Solutions:

(i) Standard solution:

Weigh accurately about 430 mg of *placebo*, 100.0 mg of calcium pantothenate working standard and 10.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 4.30 mg/ml of *placebo*, 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(ii) Test solution - 1:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter (Concentration: Equivalent to 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(iii) Test solution - 2:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter (Concentration: Equivalent to 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(iv) Test solution - 3:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter (Concentration: Equivalent to 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(v) Test solution – 4:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter (Concentration: Equivalent to 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(vi) Test solution - 5:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter (Concentration: Equivalent to 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(vii) Test solution - 6:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter. (Concentration: Equivalent to 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin)

Procedure:

Prepare the test solution of Hairgro tablets as per the test method and inject each solution. Calculate the precision of the method by calculating % assay of each solution against standard solution. Report the % RSD of all individual assay values.

Acceptance criteria:

The percentage relative standard deviation for the assay values should be less than 2.0.

6. Ruggedness (Intermediate precision)

The ruggedness of the method was performed by analyzing a sample solution of Hairgro tablets (B.No:RD16047) as per the test method (six replicate sample preparation) and injected each solution in duplicate using different instrument, column, reagent, and analyst on different days. The results of set I were compared with the results of set II.

Table 6.2

Parameter	Set I	Set II
Instrument to instrument	Instrument – 1	Instrument – 2
Column to column	Column – 1	Column – 2
Reagent to reagent	Reagent – 1	Reagent – 2
Analyst to analyst	Analyst – 1	Analyst – 2
Day to day	Day – 1	Day – 2

Solutions:

(i) Standard solution:

Weigh accurately about 430 mg of *placebo*, 100.0 mg of calcium pantothenate working standard and 10.0 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of

ammonia solution. Sonicate for 5 minute, and make up the volume with the water (Concentration: 4.30 mg/ml of *placebo*, 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(ii) Test solution - 1:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter (Concentration: Equivalent to 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(iii) Test solution - 2:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter (Concentration: Equivalent to 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(iv) Test solution - 3:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter (Concentration: Equivalent to 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(v) Test solution – 4:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter (Concentration: Equivalent to 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(vi) Test solution - 5:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter (Concentration: Equivalent to 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

(vii) Test solution - 6:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter (Concentration: Equivalent to 1.00 mg/ml of calcium pantothenate and 0.10 mg/ml of biotin).

Procedure:

Prepare the test solution of Hairgro tablets by different analyst with different reagent on different day as per the test method. Inject each solution with different instrument using different column, different reagent, different analyst and different days.

Calculate the ruggedness of the method by calculating % assay of each solution against standard solution.

Report the overall % RSD of all individual assay values in set-I and set–II.

Acceptance criteria:

The overall % RSD should not be more than 2.0%.

7. Robustness

The following table **(Table 6.3)** shows the parameters of the method that were altered to test the robustness of the method. The robustness of the method is to be determined by analyzing the standard solution six times with varying RP-HPLC conditions as described below. **Table 6.4** expresses the criteria for robustness

Table 6.3: Parameters altered for robustness test

Parameter	Actual	Low	High
Flow rate	1.00 ml/min	0.90 ml/min	1.10 ml/min
Mobile Phase ratio	70 : 30	72 : 28	68 : 32
Buffer pH	2.20	2.10	2.30
Column oven temperature	30°C	28°C	32°C

Table 6.4: Acceptance criteria

System suitability parameter	Acceptance criteria
Tailing factor	NMT 2.0
Column efficiency	NLT 2000 theoretical plates
Relative standard deviation	NMT 2.0%

7.1 Chromatographic conditions (Actual):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 1.0 ml / min

Detection wavelength : 210 nm

Injection volume : 20 µL

Oven temperature : 30°C

Mobile phase : Buffer: methanol (70: 30)

Preparation of buffer:

Dissolve 4.1 g of mono basic potassium dihydrogen ortho phosphate in 1000 ml of water. Adjust the pH to 2.20 ± 0.05 with 10 % orthophosphoric acid. Filter through 0.45 µ membrane filter.

Standard solution:

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of Biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water.

Test Solution:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter.

7.2 Chromatographic conditions (Flow-Low):

Column	: *Cosmosil, C18,* 250 X 4.6 mm, 5 µm or Equivalent
Flow rate	: 0.9 ml / min
Detection wavelength	: 210 nm
Injection volume	: 20 µL
Oven temperature	: 30°C
Mobile phase	: Buffer: methanol (70: 30)

Preparation of buffer:

Dissolve 4.1 g of mono basic potassium dihydrogen ortho phosphate in 1000 ml of water. Adjust the pH to 2.20 ± 0.05 with 10 % orthophosphoric acid. Filter through 0.45 µ membrane filter.

Standard solution:

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water.

Test solution:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter.

7.3 Chromatographic conditions (Flow-High):

Column	: *Cosmosil, C18,* 250 X 4.6 mm, 5 µm or equivalent
Flow rate	: 1.1 ml / min
Detection wavelength	: 210 nm
Injection volume	: 20 µL
Oven temperature	: 30°C
Mobile phase	: Buffer: methanol (70: 30)

Preparation of buffer:

Dissolve 4.1 g of mono basic potassium dihydrogen ortho phosphate in 1000 ml of water. Adjust the pH to 2.20 ± 0.05 with 10 % orthophosphoric acid. Filter through 0.45 µ membrane filter.

Standard solution:

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water.

Test solution:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter.

7.4 Chromatographic conditions (Mobile Phase – Ratio - Low):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 1.0 ml / min

Detection wavelength : 210 nm

Injection volume : 20 µL

Oven temperature : 30°C

Mobile phase : Buffer: methanol (72: 28)

Preparation of buffer:

Dissolve 4.1 g of mono basic potassium dihydrogen ortho phosphate in 1000 ml of water. Adjust the pH to 2.20 ± 0.05 with 10 % orthophosphoric acid. Filter through 0.45 µ membrane filter.

Standard solution:

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water.

Test solution:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter.

7.5 Chromatographic conditions (Mobile Phase – Ratio - High):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 1.0 ml / min

Detection wavelength : 210 nm

Injection volume : 20 μL

Oven temperature : 30°C

Mobile phase : Buffer: methanol (68: 32)

Preparation of buffer:

Dissolve 4.1 g of mono basic potassium dihydrogen ortho phosphate in 1000 ml of water. Adjust the pH to 2.20 ± 0.05 with 10 % orthophosphoric acid. Filter through 0.45 μ membrane filter.

Standard solution:

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water.

Test solution:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter.

7.6 Chromatographic conditions (Buffer pH - Low):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 μm or equivalent

Flow rate : 1.0 ml / min

Detection wavelength : 210 nm

Injection volume : 20 μL

Oven temperature : 30°C

Mobile phase : Buffer: methanol (70: 30)

Preparation of buffer:

Dissolve 4.1 g of mono basic potassium dihydrogen ortho phosphate in 1000 ml of water. Adjust the pH to 2.10 ± 0.05 with 10 % orthophosphoric acid. Filter through 0.45 µ membrane filter.

Standard solution:

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water.

Test solution

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter.

7.7 Chromatographic conditions (Buffer pH - High):

Column	: *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent
Flow rate	: 1.0 ml / min
Detection wavelength	: 210 nm
Injection volume	: 20 µL
Oven temperature	: 30°C
Mobile phase	: Buffer: methanol (70: 30)

Preparation of buffer:

Dissolve 4.1 g of mono basic potassium dihydrogen ortho phosphate in 1000 ml of water. Adjust the pH to 2.30 ± 0.05 with 10 % orthophosphoric acid. Filter through 0.45 µ membrane filter.

Standard solution:

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water.

Test solution:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter.

7.8 Chromatographic conditions (Column Oven Temperature - Low):

Column	: *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent
Flow rate	: 1.0 ml / min
Detection wavelength	: 210 nm
Injection volume	: 20 µL
Oven temperature	: 28°C
Mobile phase	: Buffer: methanol (70: 30)

Preparation of buffer:

Dissolve 4.1 g of mono basic potassium dihydrogen ortho phosphate in 1000 ml of water. Adjust the pH to 2.20 ± 0.05 with 10 % orthophosphoric acid. Filter through 0.45 µ membrane filter.

Standard solution:

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water.

Test solution:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter.

7.9 Chromatographic conditions (Column Oven Temperature - High):

Column	: *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent
Flow rate	: 1.0 ml / min
Detection wavelength	: 210 nm
Injection volume	: 20 µL
Oven temperature	: 32°C
Mobile phase	: Buffer: methanol (70: 30)

Preparation of buffer:

Dissolve 4.1 g of mono basic potassium dihydrogen ortho phosphate in 1000 ml of water. Adjust the pH to 2.20 ± 0.05 with 10 % orthophosphoric acid. Filter through 0.45 µ membrane filter.

Standard solution:

Weigh accurately about 100.0 mg of calcium pantothenate working standard and 10 mg of biotin working standard into a clean 100 ml volumetric flask. Dissolve in 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water.

Test solution:

Weigh and finely powder 20 tablets. Weigh accurately about 540 mg of powdered tablets into a clean 100 ml volumetric flask. Add 25 ml of water and 0.2 ml of ammonia solution. Sonicate for 5 minute, and make up the volume with the water and filter.

8. Solution Stability

Measure the stability of the Hairgro tablets test solution against 100% of the standard concentration by keeping the solution up to 48 hours at 15°C. Inject the sample at different time intervals (i.e: Initial, 6, 12, 18, 24, 36& 48 hours) and calculate the percentage relative standard deviation of calcium pantothenate and biotin in Hairgro tablets at different interval of time.

Acceptance Criteria:

The overall % RSD should not be more than 2.0%.

CHAPTER 7:

RESULTS &

DISCUSSION

RESULTS

1. Specificity:

Placebo solution was prepared separately at a concentration of 4.3 mg/ml with matrix blend. Calcium pantothenate was prepared at its working concentration and analyzed as per the method. Biotin was prepared at its working concentration and analyzed as per the method. A typical chromatogram for specificity is shown in **Figure- 7.1 - 7.6. Table 7.1** Summarizes the retention time and relative retention time values for *placebo* peaks, calcium pantothenate and biotin.

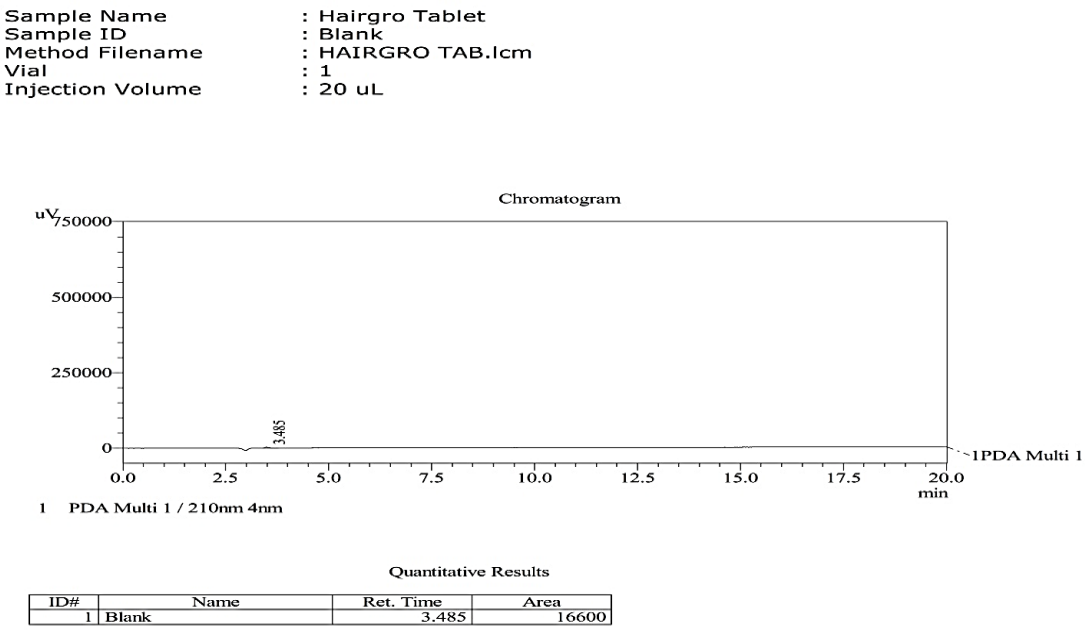
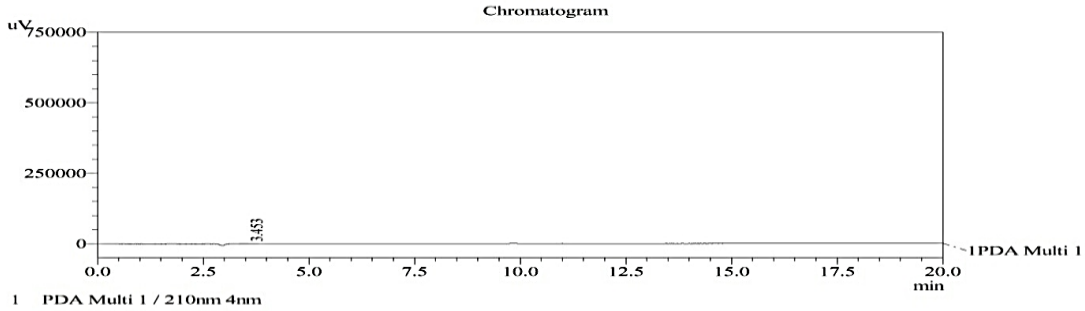


Figure 7.1: Chromatogram of blank

Sample Name : Hairgro Tablet
Sample ID : Placebo
Method Filename : HAIRGRO TAB.lcm
Vial : 2
Injection Volume : 20 uL

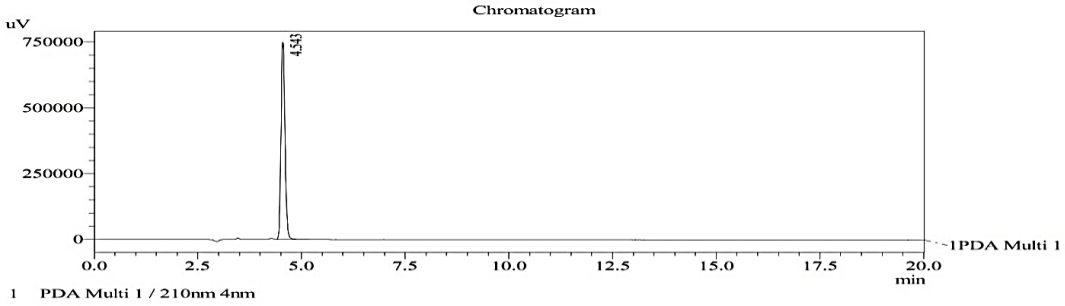


Quantitative Results

ID#	Name	Ret. Time	Area
1	Blank	3.453	2895

Figure 7.2: Chromatogram of placebo

Sample Name : Calcium Pantothenate
Sample ID : Specificity
Method Filename : HAIRGRO TAB.lcm
Vial : 4
Injection Volume : 20 uL

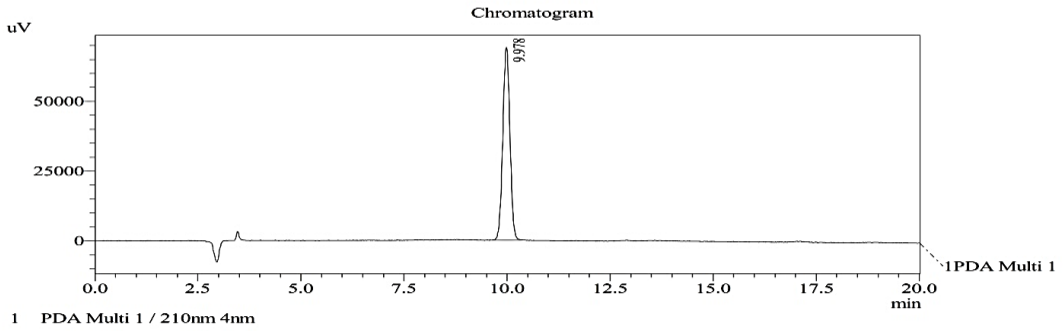


Quantitative Results

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.543	4984635

Figure 7.3: Chromatogram of calcium pantothenate

Sample Name : Biotin
Sample ID : Specificity
Method Filename : HAIRGRO TAB.lcm
Vial : 3
Injection Volume : 20 uL

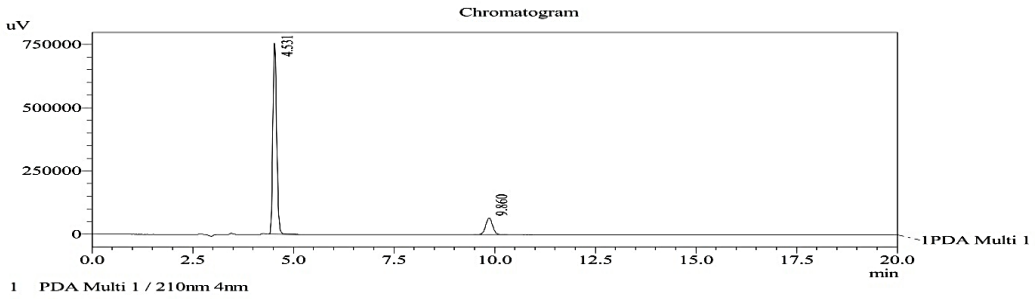


Quantitative Results

ID#	Name	Ret. Time	Area
1	Biotin	9.978	828235

Figure 7.4: Chromatogram of biotin

Sample Name : Standard
Sample ID : Specificity
Method Filename : HAIRGRO TAB.lcm
Vial : 5
Injection Volume : 20 uL

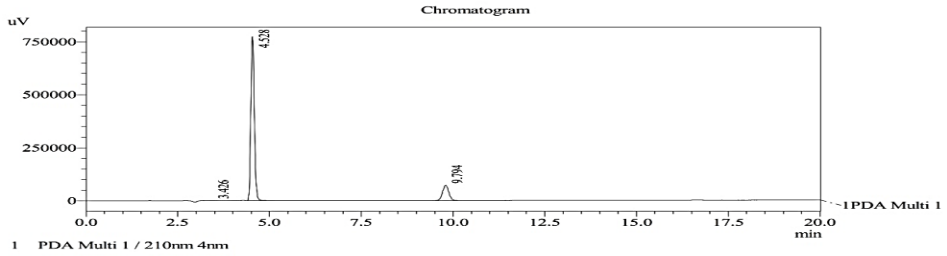


Quantitative Results

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.531	4991449
2	Biotin	9.860	779360

Figure 7.5: Chromatogram of standard

Sample Name : Standard + Placebo
Sample ID : Specificity
Method Filename : HAIRGRO TAB.lcm
Vial : 6
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area
1	Blank Peak	3.426	1648
2	Calcium Pantothenate	4.528	5062816
3	Biotin	9.794	845017

Figure 7.6: Chromatogram of standard + placebo

Table 7.1: Summary of retention time and relative retention time values for placebo peaks, calcium pantothenate and biotin.

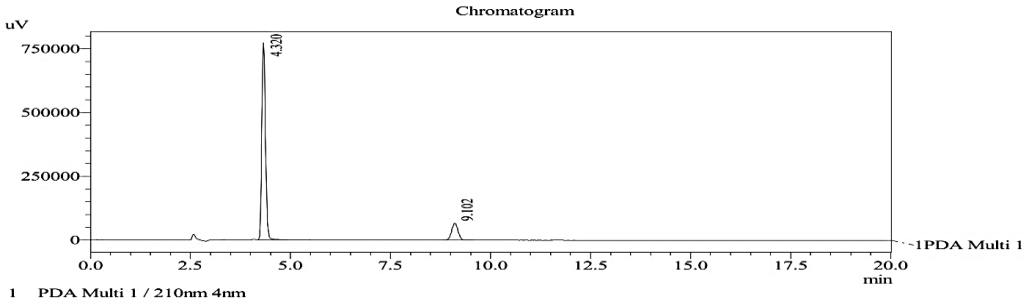
Peak name	Retention time (minutes)	Relative retention time
Blank peak	3.43	0.35
Calcium pantothenate	4.53	0.46
Biotin	9.79	1.00

No peak was observed at the retention time of calcium pantothenate and biotin in the chromatogram of *placebo*.

2. System precision:

A standard solution of 1.00 mg/ml of calcium pantothenate, and 0.10 mg/ml of biotin was prepared and analyzed as per the method. Inject the standard solution (6 injections). Ensure the following system suitability criteria. A typical chromatogram for system precision is shown in **Figure-7.7.** Table **7.2:** Summarizes the Retention time, % RSD of peak area, tailing factor and theoretical plates of the calcium pantothenate peak. Table **3:** Summarizes the retention time, % RSD of peak area, tailing factor and theoretical plates of the biotin Peak

Sample Name : Standard
Sample ID : System Precision
Method Filename : HAIRGRO TAB.lcm
Vial : 2
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.320	4966028	1.23	8638
2	Biotin	9.102	737506	1.05	14392

Figure 7.7: Chromatogram of system precision

Table 7.2: Summary of retention time, % RSD of peak area, tailing factor and theoretical plates of the calcium pantothenate peak

S.No	Retention time (Minutes)	Area	Tailing factor	Theoretical plates
1	4.32	4966028	1.23	8638
2	4.31	4987693		
3	4.40	4988008		
4	4.31	4983644		
5	4.32	4981030		
6	4.30	5003737		
Average		**4985023**		
% RSD		**0.24**		

The percentage relative standard deviation of peak area of calcium pantothenate was 0.24 with the tailing factor and theoretical plates of 1.23 and 8638 respectively.

Table 7.3: Summary of retention time, % RSD of peak area, tailing factor and theoretical plates of the biotin peak

S.No	Retention time (Minutes)	Area	Tailing factor	Theoretical plates
1	9.10	737506	1.05	14392
2	9.11	744892		
3	9.15	740429		
4	9.27	745914		
5	9.09	735393		
6	9.14	736349		
Average		**740081**		
% RSD		**0.60**		

The percentage relative standard deviation of peak area of biotin was 0.60 with the tailing factor and theoretical plates of 1.05 and 14392 respectively.

3. Linearity and Range:

(a) Linearity of calcium pantothenate

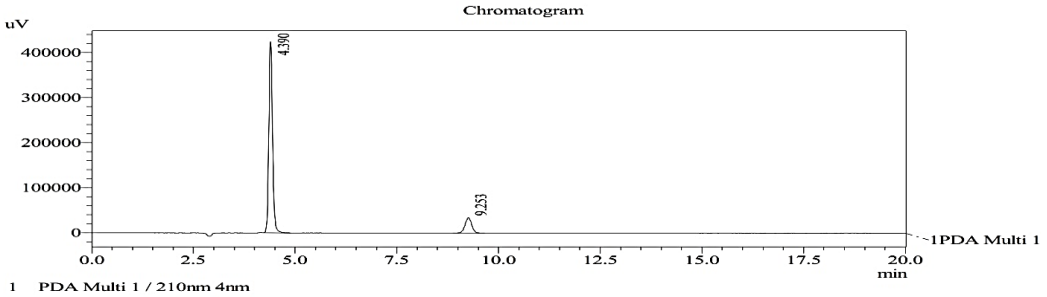
The linearity of the RP-HPLC method was demonstrated for calcium pantothenate ranging from 0.4960 mg/ml to 1.4880 mg/ml, which is equivalent to 50% to 150% of the calcium pantothenate working strength. Five standard solutions at the concentrations within the mentioned range were prepared and analyzed as per the method. The linearity results obtained are shown in **Table 7.4**. Figure 7.13 shows the line of best fit for peak area versus concentration of biotin.

(b) Linearity of biotin

The linearity of the RP-HPLC method was demonstrated for biotin solutions ranging from 0.05200 mg/ml to 0.15000 mg/ml, which is equivalent to 50% to 150% of the biotin working strength. Five standard

solutions at the concentrations within the mentioned range were prepared and analyzed as per the method. The linearity results obtained are shown in **Table 7.5**. Figure 7.14 shows the line of best fit for peak area versus concentration of biotin. A typical chromatogram for linearity is shown in **Figure- 7.8-7.12**

Sample Name : Hairgro Tablets
Sample ID : Linearity - 50%
Method Filename : HAIRGRO TAB.lcm
Vial : 3
Injection Volume : 20 uL

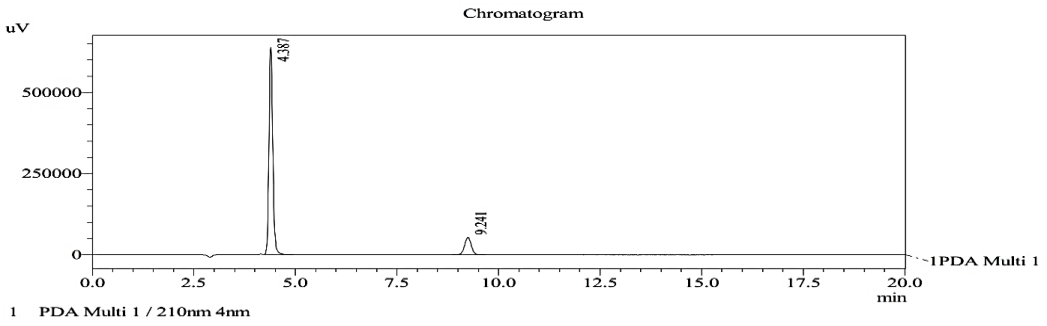


Quantitative Results

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.390	2702601
2	Biotin	9.253	389460

Figure 7.8: Chromatogram of linearity-50%

Sample Name : Hairgro Tablets
Sample ID : Linearity - 80%
Method Filename : HAIRGRO TAB.lcm
Vial : 4
Injection Volume : 20 uL

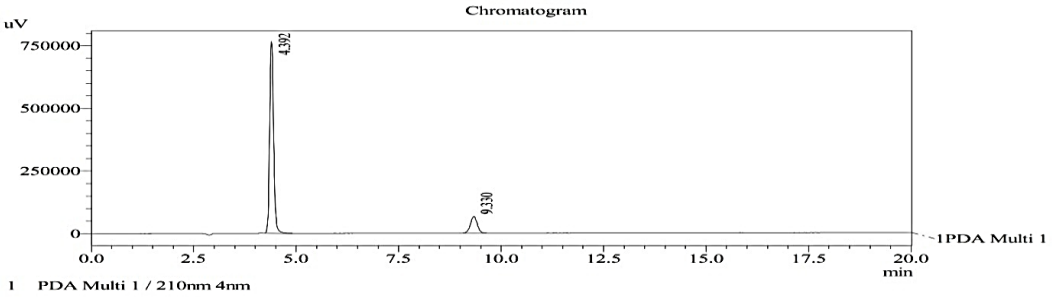


Quantitative Results

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.387	4162205
2	Biotin	9.241	621587

Figure 7.9: Chromatogram of linearity – 80%

Sample Name : Hairgro Tablets
Sample ID : Linearity - 100%
Method Filename : HAIRGRO TAB.lcm
Vial : 5
Injection Volume : 20 uL

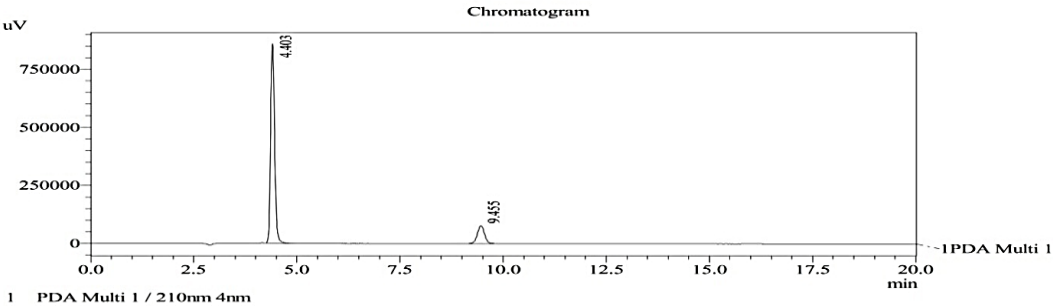


Quantitative Results

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.392	5122685
2	Biotin	9.330	768768

Figure 7.10: Chromatogram of linearity – 100%

Sample Name : Hairgro Tablets
Sample ID : Linearity - 120%
Method Filename : HAIRGRO TAB.lcm
Vial : 6
Injection Volume : 20 uL

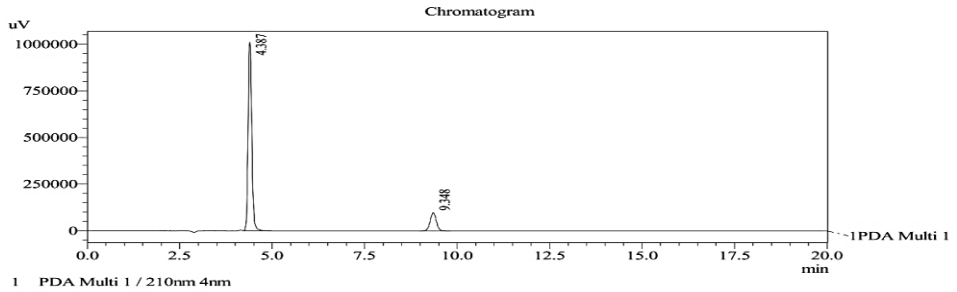


Quantitative Results

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.403	5859779
2	Biotin	9.455	929599

Figure 7.11: Chromatogram of linearity – 120%

Sample Name : Hairgro Tablets
Sample ID : Linearity - 150%
Method Filename : HAIRGRO TAB.lcm
Vial : 7
Injection Volume : 20 uL



Quantitative Results

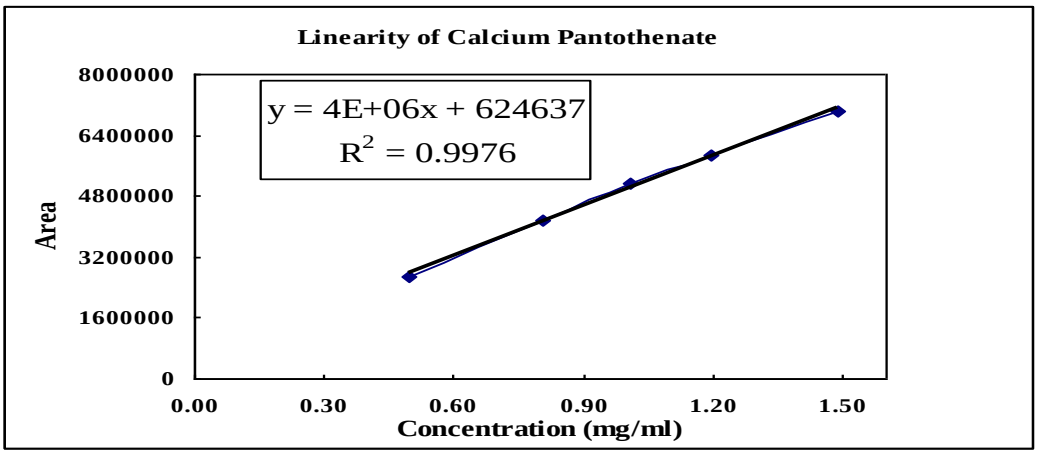
ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.387	7028796
2	Biotin	9.348	1154724

Figure 7.12: Chromatogram of linearity – 150%

Table 7.4: Linearity of calcium pantothenate

Level	% of CP working strength	Conc (mg/ml)	Peak area
50%	49.6	0.4960	2702601
80%	80.3	0.8030	4162205
100%	100.8	1.0090	5122685
120%	119.4	1.1940	5859779
150%	148.8	1.4880	7028796
Correlation coefficient			0.9976

Figure 7.13: Linearity graph for calcium pantothenate

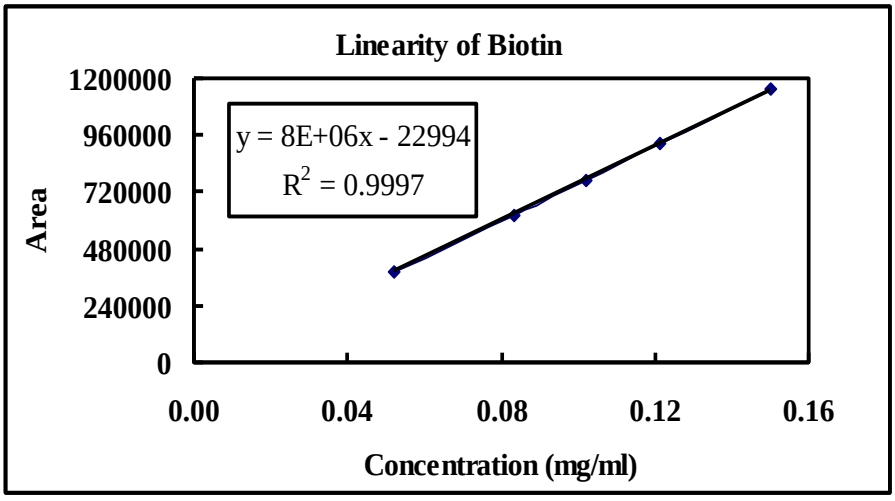


Thus, the RP-HPLC method for the estimation of calcium pantothenate in Hairgro tablets was shown to be linear in the range of 50% to 150% of the working concentration with a correlation coefficient of 0.9976. The range of the RP-HPLC method for determining the assay of calcium pantothenate in Hairgro tablets is 50% to 150% of the working strength.

Table 7.5: Linearity of biotin

Level	% of BI working strength	Conc (mg/ml)	Peak area
50%	52.0	0.05200	389460
80%	83.0	0.08300	621587
100%	102.0	0.10200	768768
120%	121.0	0.12100	929599
150%	150.0	0.15000	1154724
Correlation coefficient			0.9997

Figure7.14: Linearity graph for biotin

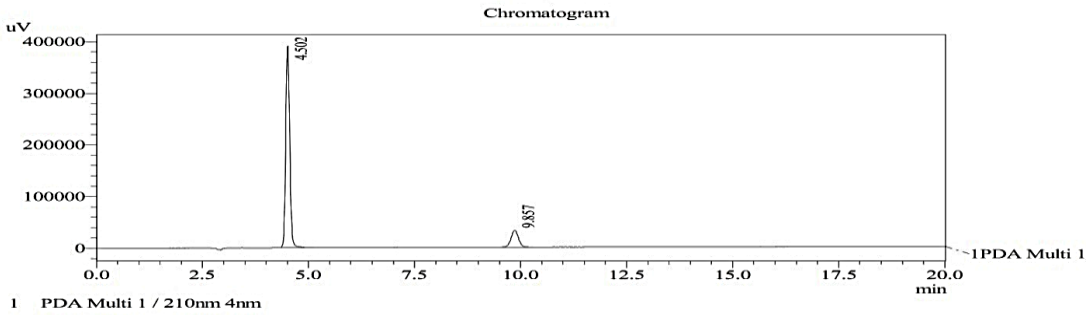


Thus, the RP-HPLC method for the estimation of biotin in Hairgro tablets was shown to be linear in the range of 50% to 150% of the working concentration with a Correlation coefficient of 0.9997. The range of the RP-HPLC method for determining the assay of Biotin in Hairgro tablets is 50% to 150% of the working strength.

4. Accuracy:

The accuracy of the method was determined by using three solutions containing *placebo* spiked with calcium pantothenate and biotin at approximately 50%, 100% and 150% of its working strength. Each level was analyzed as per the method. A typical chromatogram for accuracy is shown in **Figure 7.15 – 7.17**

Sample Name : Hairgro Tablets
Sample ID : Accuracy- 50% _1
Method Filename : HAIRGRO TAB.lcm
Vial : 3
Injection Volume : 20 uL

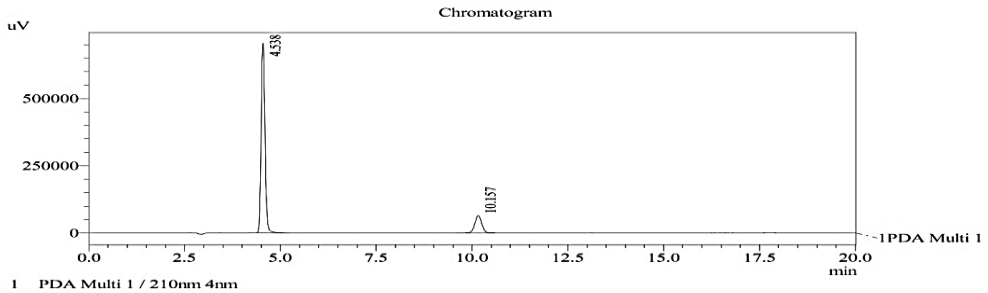


Quantitative Results

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.502	2535470
2	Biotin	9.857	397569

Figure7.15: Chromatogram of accuracy – 50%

Sample Name : Hairgro Tablets
Sample ID : Accuracy- 100% _1
Method Filename : HAIRGRO TAB.lcm
Vial : 6
Injection Volume : 20 uL

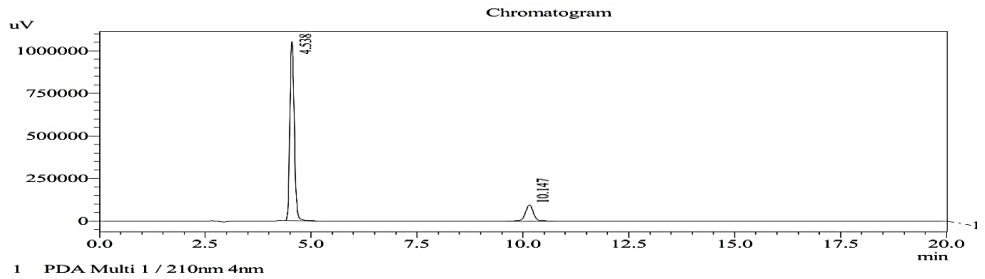


Quantitative Results

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.538	4808669
2	Biotin	10.157	816379

Figure7.16: Chromatogram of accuracy –100%

Sample Name : Hairgro Tablets
Sample ID : Accuracy- 150% _1
Method Filename : HAIRGRO TAB.lcm
Vial : 9
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.538	7594448
2	Biotin	10.147	1205132

Figure7.17: Chromatogram of accuracy –150

Table 7.6: Accuracy of calcium pantothenate

Level	%CP Working strength	Theoretical Conc (mg/ml)	Measured Conc (mg/ml)	%Recovery
50%	50.50	0.50500	0.51221	101.43
	50.80	0.50800	0.51354	101.09
	50.70	0.50700	0.51431	101.44
100%	99.30	0.99300	0.98134	98.83
	99.50	0.99500	0.97858	98.35
	99.50	0.99500	0.98305	98.80
150%	150.70	1.50700	1.53332	101.75
	151.40	1.51400	1.53993	101.71
	151.80	1.51800	1.53902	101.38

The percentage recovery values were in the range of 98.35%- 101.75% which is within the acceptance criteria

Table 7.7: Accuracy of biotin

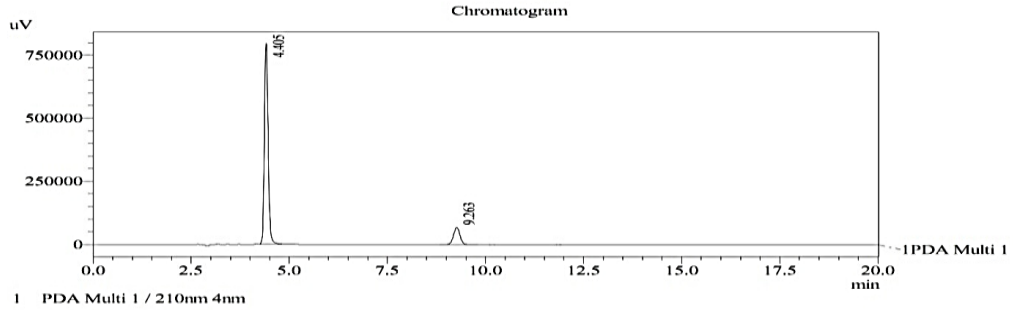
Level	% BI Working strength	Theoretical Conc (mg/ml)	Measured Conc (mg/ml)	% Recovery
50%	49.00	0.04900	0.04941	100.84
	50.00	0.05000	0.05009	100.18
	49.00	0.04900	0.04948	100.98
100%	101.00	0.10100	0.10171	100.70
	102.00	0.10200	0.10201	100.01
	103.00	0.10300	0.10257	99.58
150%	151.00	0.15100	0.14963	99.09
	150.00	0.15000	0.14788	98.59
	149.00	0.14900	0.14817	99.44

The percentage recovery values were in the range of 98.59%-100.98% which is within the acceptance criteria.

5. Method precision:

The method precision was performed by analyzing a sample solution of Hairgro tablets (B.No: RD16047) as per the test method (six replicate sample preparation). A typical chromatogram for method precision is shown in **Figure – 7.18**

Sample Name : Hairgro Tablets
Sample ID : Method Precision-1
Method Filename : HAIRGRO TAB.lcm
Vial : 3
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.405	5328922
2	Biotin	9.263	794338

Figure7.18: Chromatogram of method precision

Table 7.8: Summary of results for precision of the method

Sample	Level	% Assay (CP)	% Assay(BI)
1	100%	104.39	106.83
2	100%	102.99	103.84
3	100%	104.47	106.43
4	100%	105.31	106.55
5	100%	102.53	102.90
6	100%	102.54	104.42
Average		**103.71**	**105.16**
%RSD		**1.13**	**1.58**

The %RSD for the assay values of calcium pantothenate and biotin in Hairgro tablets were 1.13 % and 1.58 % respectively.

6. Ruggedness (Intermediate Precision)

The ruggedness of the method was performed by analyzing a sample solution of Hairgro tablets (B.No: RD16047) as per the test method (six replicate sample preparation) and injected each solution in duplicate using different instrument, column, reagent, and analyst on different days. The results of set I were compared with the results of set II. A typical chromatogram for ruggedness is shown in **Figure-7.19.** Table 7.10 summerizes the results for ruggedness

Table 7.9

Parameter	Set I	Set II
Instrument to instrument	Instrument – 1	Instrument – 2
Column to column	Column – 1	Column – 2
Reagent to reagent	Reagent – 1	Reagent – 2
Analyst to analyst	Analyst – 1	Analyst – 2
Day to day	Day – 1	Day – 2

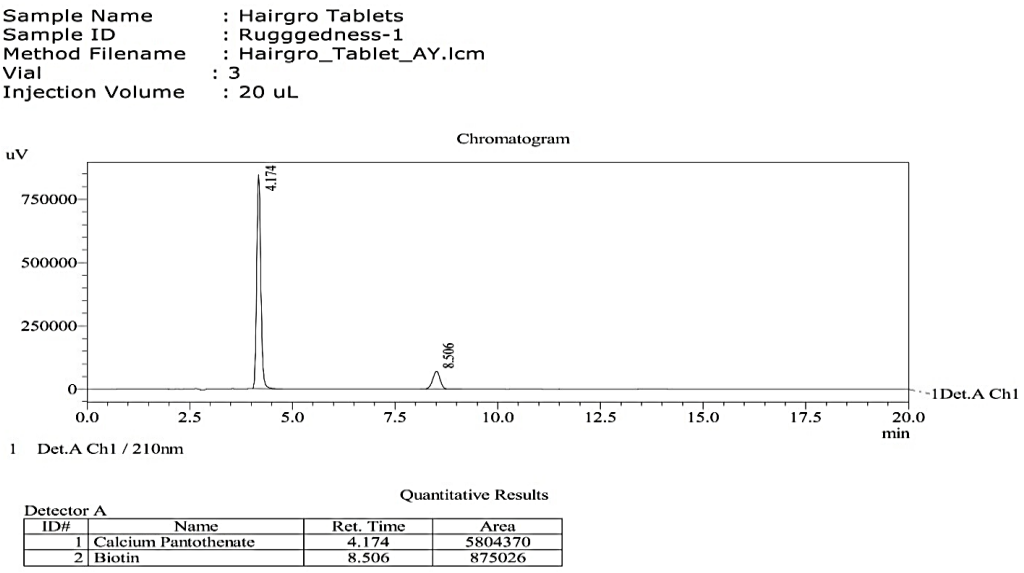


Figure 7.19: Chromatogram of ruggedness

Table 7.10: Summary of results for ruggedness

Sample	Set – I	Set – II	Set – I	Set – II
	CP	CP	BI	BI
1	104.39	106.83	103.68	103.90
2	102.99	103.84	103.16	103.00
3	104.47	106.43	103.82	103.80
4	105.31	106.55	103.82	103.90
5	102.53	102.90	105.09	105.10
6	102.54	104.42	104.52	104.40
Average	**103.71**	**105.16**	**104.02**	**104.02**
% RSD	**1.13**	**1.58**	**0.66**	**0.67**
Overall Avg	**103.84**		**104.59**	
Overall % RSD	**0.89**		**1.29**	
Set	**Set - I**		**Set – II**	
Instrument	I/RD/HPC/02		I/RD/HPC/04	
Column	*Cosmosil* ,5C_{18}-MS-II (250 X 4.6 mm, 5µ), RD/COL/76		*Cosmosil* ,5C_{18}-MS-II (250 X 4.6 mm, 5µ), RD/COL/77	
Reagent	KH_2PO_4 (Rankem) Methanol (Fisher)		KH_2PO_4 (Finar) Methanol (Finar)	
Analyst	Analyst-1		Analyst-2	

The above result indicates that the test method is rugged for instrument to instrument, column to column, reagent to reagent, analyst to analyst and day to day variation. The overall % RSD for the assay value of calcium pantothenate and biotin in Hairgro tablets was 0.89% and 1.29% respectively.

7. Robustness:

The following table (Table 7.11) shows the parameters of the method that were altered to test the robustness of the method. The robustness of the method is to be determined by analyzing the standard solution six times with varying RP-HPLC conditions as described below. A typical chromatogram for robustness is shown in **Figure-7.20 – 7.37**

Table 7.11: Parameters altered for robustness test

Parameter	Actual	Low	High
Flow rate	1.00 ml/min	0.90 ml/min	1.10 ml/min
Mobile phase ratio	70 : 30	72 : 28	68 : 32
Buffer pH	2.20	2.10	2.30
Column oven temperature	30°C	28°C	32°C

7.1 Chromatographic conditions (Actual):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 1.0 ml / min

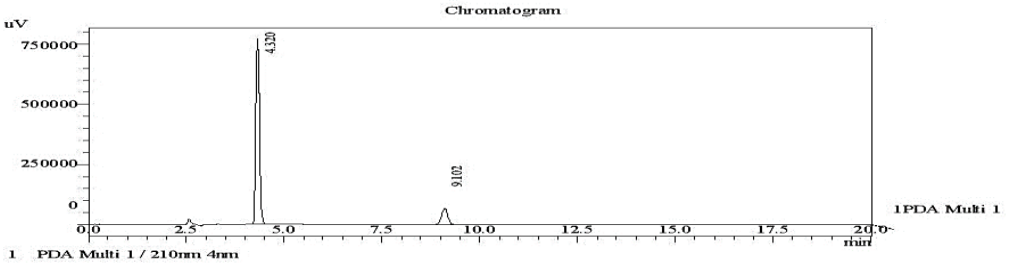
Detection wavelength : 210 nm

Injection volume : 20 µL

Oven temperature : 30°C

Mobile phase : Buffer: methanol (70: 30)

Sample Name : Standard
Sample ID : Robustness (Actual)
Method Filename : HAIRGRO TAB.lcm
Vial : 2
Injection Volume : 20 uL

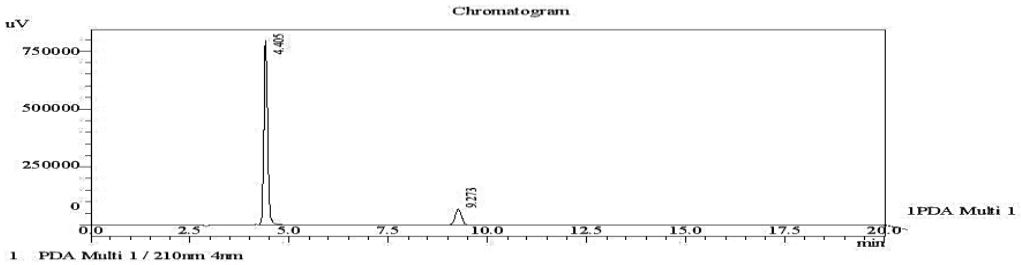


Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.320	4966028	1.23	8638
2	Biotin	9.102	737506	1.05	14392

Figure 7.20: Chromatogram of robustness- standard (Actual)

Sample Name : Hairgro Tablets
Sample ID : Robustness (Actual)
Method Filename : HAIRGRO TAB.lcm
Vial : 3
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.405	5328922
2	Biotin	9.273	794338

Figure 7.21: Chromatogram of robustness- sample (Actual)

7.2 Chromatographic conditions (Flow-Low):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 0.9 ml / min

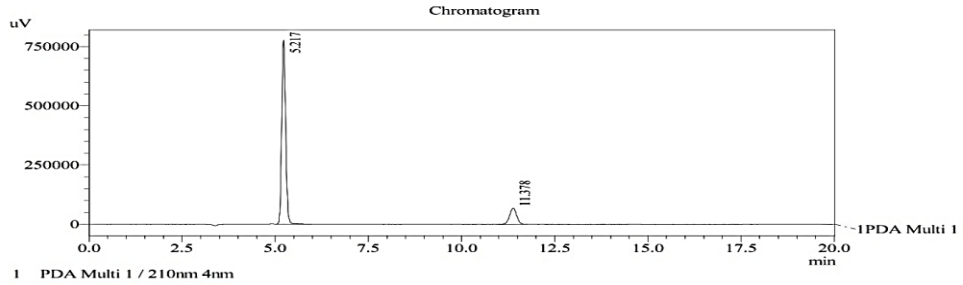
Detection wavelength : 210 nm

Injection volume : 20 µL

Oven temperature : 30°C

Mobile phase : Buffer: methanol (70: 30)

Sample ID : Robustness Flow Low
Method Filename : HAIRGRO TAB_Flow Low.lcm
Vial : 2
Injection Volume : 20 uL

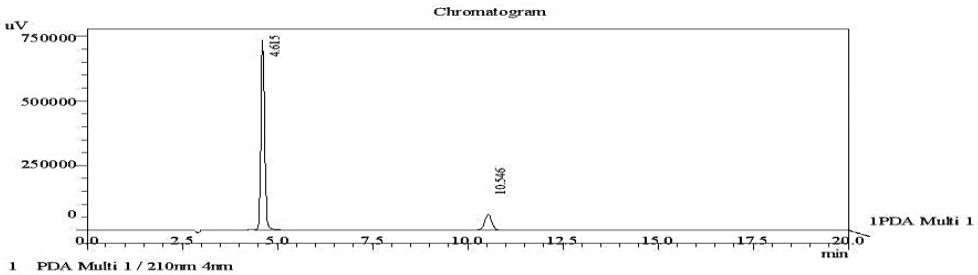


Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	5.217	5719726	1.19	10171
2	Biotin	11.378	890362	1.02	16407

Figure 7.22: Chromatogram of robustness- standard (Flow low)

Sample Name : Hairgro Tablets
Sample ID : Robustness Flow Low
Method Filename : HAIRGRO TAB_Flow Low.lcm
Vial : 3
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.615	4974642	1.20	9123
2	Biotin	10.546	793125	1.00	14763

Figure 7.23: Chromatogram of robustness- sample (Flow low)

7.3 Chromatographic conditions (Flow-High):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 1.1 ml / min

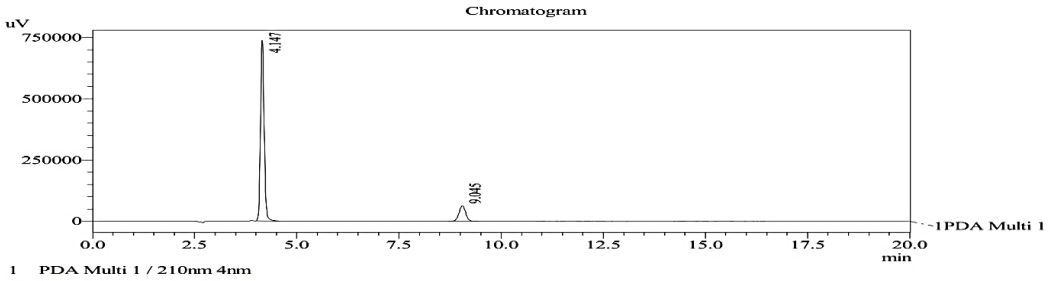
Detection wavelength : 210 nm

Injection volume : 20 µL

Oven temperature : 30°C

Mobile phase : Buffer: methanol (70: 30)

Sample ID : Robustness Flow High
Method Filename : HAIRGRO TAB_Flow High.lcm
Vial : 2
Injection Volume : 20 uL

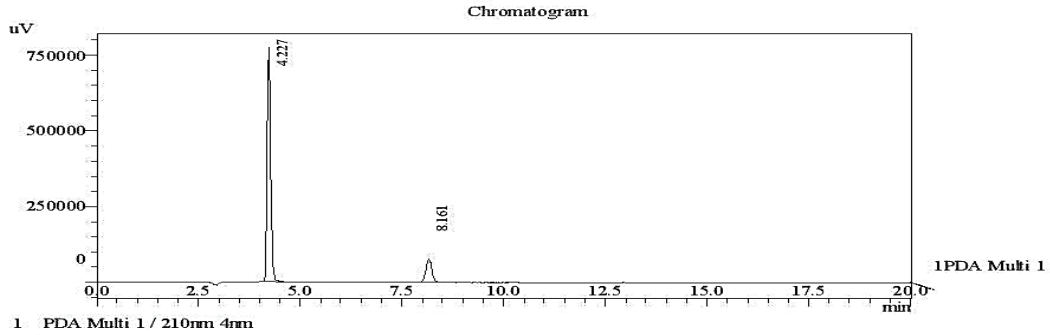


Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.147	4558706	1.20	8501
2	Biotin	9.045	716128	1.03	14020

Figure 7.24: Chromatogram of robustness- standard (Flow high)

Sample Name : Hairgro Tablets
Sample ID : Robustness Flow High
Method Filename : HAIRGRO TAB_Flow High.lcm
Vial : 3
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.227	4872558	1.21	8604
2	Biotin	8.161	771434	1.03	13715

Figure 7.25: Chromatogram of robustness- sample (Flow high)

Table 7.12: Summary of robustness results of standard (Flow)

Flow	Actual		Low		High	
	CP	**BI**	**CP**	**BI**	**CP**	**BI**
RT (min)	4.30-4.53	9.09-9.27	4.22-4.23	8.12-8.16	4.57-4.59	10.16-10.43
Area	4966028-4991449	745914-779360	4836134-4872558	764132-771434	4940974-5020412	782565-791915
Tailing factor	1.19	1.05	1.21	1.03	1.21	1.01
Theoretical plates	8586	14487	8604	13715	8586	14487
Average (Area)	4978738	762637	4850959	772652	4967201	786025
%RSD (Area)	0.27	0.65	0.28	0.73	0.59	0.43

Table 7.13: Summary of robustness results of sample (Flow)

Flow	Actual		Low		High	
	CP	**BI**	**CP**	**BI**	**CP**	**BI**
RT (min)	4.31-4.40	9.09-9.27	5.14-5.22	11.29-11.38	4.13-4.15	8.99-9.05
Area	5003737-4966028	735393-745914	5775729-5640003	873982-890362	4585191-4469026	715423-720801
Tailing factor	1.23	1.05	1.19	1.02	1.20	1.03
Theoretical plates	8638	14392	10171	16407	8501	14020
Average (Area)	4985023	740081	5705806	885693	4519688	718206
%RSD (Area)	0.24	0.60	0.98	0.73	1.14	0.38

7.4 Chromatographic conditions (Mobile Phase – Ratio - Low):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 1.0 ml / min

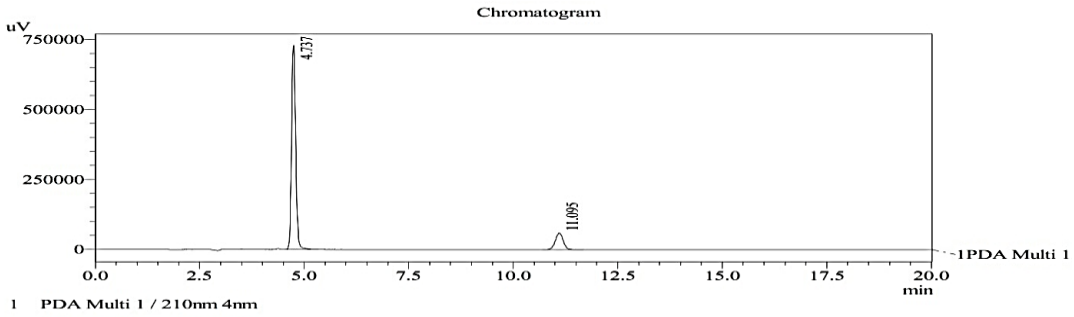
Detection wavelength : 210 nm

Injection volume : 20 µL

Oven temperature : 30°C

Mobile phase : Buffer: methanol (72: 28)

Sample Name : Standard
Sample ID : Robus MP Ratio_Org Low(72:28)
Method Filename : HAIRGRO TAB_MP_ORG_Low.lcm
Vial : 2
Injection Volume : 20 uL

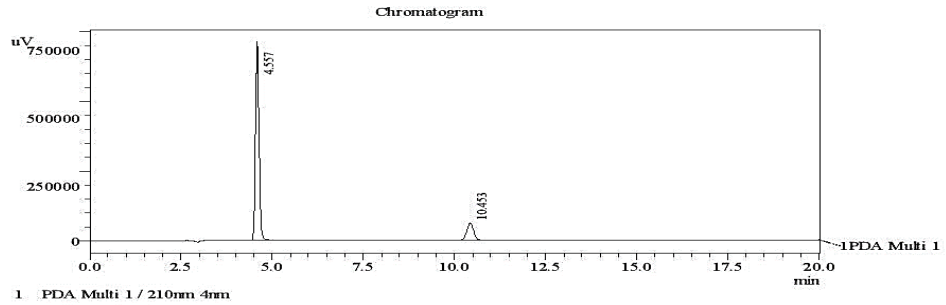


Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.737	4995979	1.20	9434
2	Biotin	11.095	790776	1.03	15471

Figure 7.26: Chromatogram of robustness- standard (MP ratio low)

Sample Name : Hairgro Tablets
Sample ID : Robus MP Ratio_Org Low
Method Filename : HAIRGRO TAB_MP_ORG_Low.lcm
Vial : 3
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.557	4964006	1.21	8586
2	Biotin	10.453	783084	1.01	14487

Figure 7.27: Chromatogram of robustness- sample (MP ratio low)

7.5 Chromatographic conditions (Mobile Phase – Ratio - High):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 1.0 ml / min

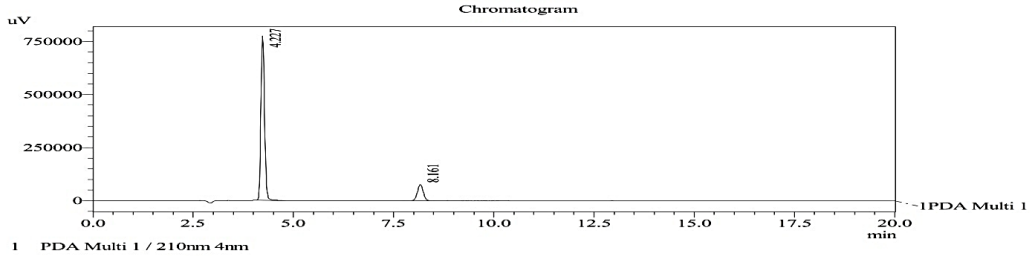
Detection wavelength : 210 nm

Injection volume : 20 µL

Oven temperature : 30°C

Mobile phase : Buffer: methanol (68: 32)

Sample Name : Standard
Sample ID : Robus MP Ratio_Org High (68:32)
Method Filename : HAIRGRO TAB_MP_ORG_HIGH.lcm
Vial : 2
Injection Volume : 20 uL

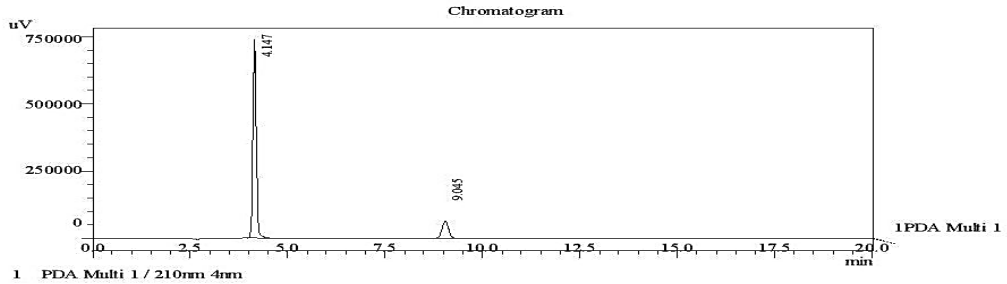


Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.227	4872558	1.21	8604
2	Biotin	8.161	771434	1.03	13715

Figure 7.28: Chromatogram of robustness- standard (MP ratio high)

Sample Name : Hairgro Tablets
Sample ID : Robus MP Ratio_Org High (68:32)
Method Filename : HAIRGRO TAB_MP_ORG_High.lcm
Vial : 3
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.147	4558706	1.20	8501
2	Biotin	9.045	716128	1.03	14020

Figure 7.29: Chromatogram of robustness- sample (MP ratio high)

Table 7.14: Summary of robustness results of standard (Mobile phase ratio)

Flow	Actual		Low		High	
	CP	BI	CP	BI	CP	BI
RT (min)	4.30-4.53	9.09-9.27	4.83-4.95	12.07-12.28	4.73-4.74	11.08-11.10
Area	4966028-4991449	745914-779360	5025626-5106931	791713-798454	4995979-5003793	785982-801047
Tailing factor	1.19	1.05	1.20	1.01	1.20	1.03
Theoretical plates	8586	14487	9139	14853	9434	15471
Average (Area)	4978738	762637	5060695	796461	4999953	792574
%RSD (Area)	0.27	0.65	0.71	0.32	0.06	0.67

Table 7.15: Summary of robustness results of sample (Mobile phase ratio)

Flow	Actual		Low		High	
	CP	**BI**	**CP**	**BI**	**CP**	**BI**
RT (min)	4.31-4.40	9.09-9.27	4.73-4.74	11.08-11.10	4.22-4.23	8.12-8.16
Area	5003737-4966028	735393-745914	4995979-5003793	785982-801047	4836134-4872558	764132-771434
Tailing factor	1.23	1.05	1.20	1.03	1.21	1.03
Theoretical plates	8638	14392	9434	15471	8604	13715
Average (Area)	4985023	740081	4999953	792574	4850959	772652
%RSD (Area)	0.24	0.60	0.06	0.67	0.28	0.73

7.6 Chromatographic conditions (Buffer pH - Low):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 1.0 ml / min

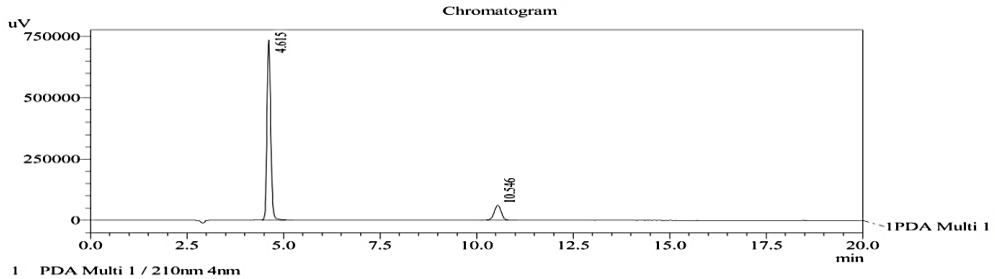
Detection wavelength : 210 nm

Injection volume : 20 µL

Oven temperature : 30°C

Mobile phase : Buffer: methanol (70: 30)

Sample Name : Standard
Sample ID : Robustness pH Low
Method Filename : HAIRGRO TAB.lcm
Vial : 2
Injection Volume : 20 uL

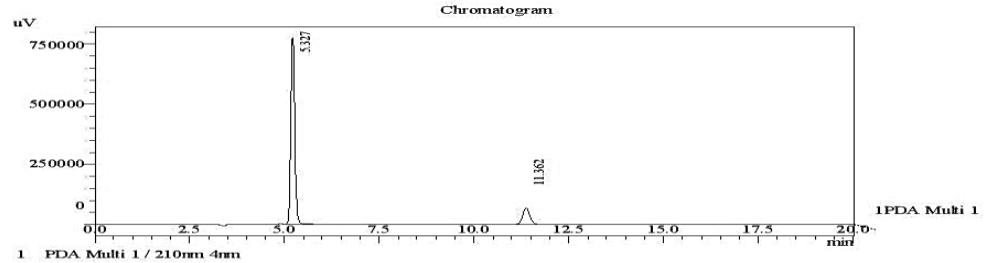


Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.615	4974642	1.20	9123
2	Biotin	10.546	793125	1.00	14763

Figure 7.30: Chromatogram of robustness- standard (Buffer pH low)

Sample Name : Hairgro Tablets
Sample ID : Robustness pH Low
Method Filename : HAIRGRO TAB.lcm
Vial : 3
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	5.327	5719726	1.19	10171
2	Biotin	11.362	890362	1.02	16407

Figure 7.31: Chromatogram of robustness- sample (Buffer pH low)

7.7 Chromatographic conditions (Buffer pH - High):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 1.0 ml / min

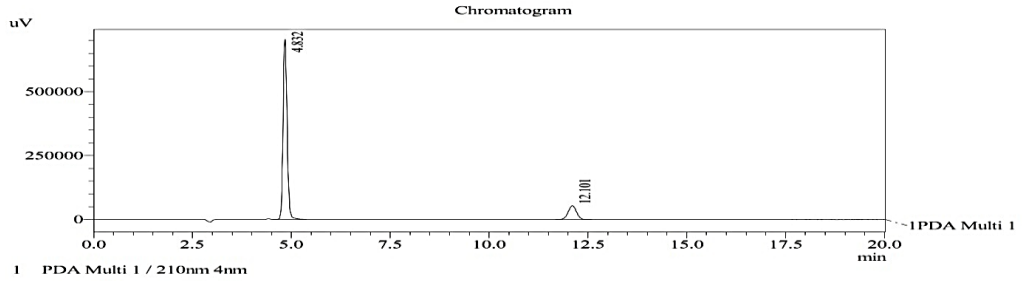
Detection wavelength : 210 nm

Injection volume : 20 µL

Oven Temperature : 30°C

Mobile phase : Buffer: methanol (70: 30)

Sample Name : Standard
Sample ID : Robustness pH High
Method Filename : HAIRGRO TAB.lcm
Vial : 2
Injection Volume : 20 uL

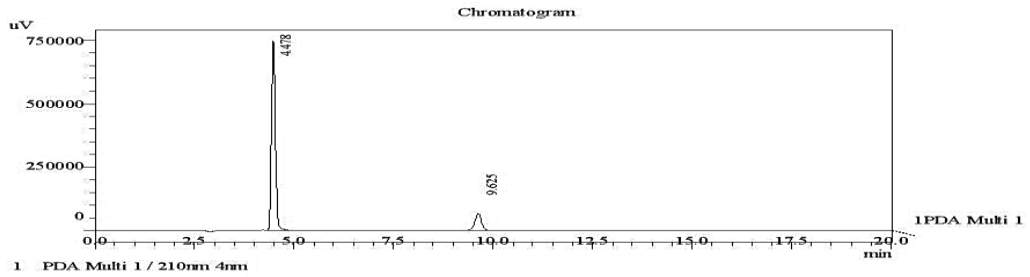


Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.832	5025626	1.20	9139
2	Biotin	12.101	797672	1.01	14853

Figure 7.32: Chromatogram of robustness- standard (Buffer pH high)

Sample Name : Hairgro Tablets
Sample ID : Robustness pH High
Method Filename : HAIRGRO TAB.lcm
Vial : 3
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.478	5003080	1.21	8634
2	Biotin	9.625	785411	1.03	14573

Figure 7.33: Chromatogram of robustness- sample (Buffer pH high)

Table 7.16: Summary of robustness results of standard (Buffer pH)

Flow	Actual		Low		High	
	CP	**BI**	**CP**	**BI**	**CP**	**BI**
RT (min)	4.30-4.53	9.09-9.27	4.48-4.52	9.53-9.73	5.14-5.22	11.29-11.38
Area	4966028-4991449	745914-779360	4911396-5003080	784140-793037	5775729-5640003	873982-890362
Tailing factor	1.19	1.05	1.21	1.03	1.19	1.02
Theoretical plates	8586	14487	8634	14573	10171	16407
Average (Area)	4978738	762637	4937913	789483	5705806	885693
%RSD (Area)	0.27	0.65	0.68	0.63	0.98	0.73

Table 7.17: Summary of robustness results of sample (Buffer pH)

Flow	Actual		Low		High	
	CP	**BI**	**CP**	**BI**	**CP**	**BI**
RT (min)	4.31-4.40	9.09-9.27	4.61-4.63	10.48-10.68	4.83-4.95	12.07-12.28
Area	5003737-4966028	735393-745914	4949496-4988077	790990-797800	5025626-5106931	791713-798454
Tailing factor	1.23	1.05	1.20	1.00	1.20	1.01
Theoretical plates	8638	14392	9123	14763	9139	14853
Average (Area)	4985023	740081	4971249	793773	5060695	796461
%RSD (Area)	0.24	0.60	0.34	0.38	0.71	0.32

7.8 Chromatographic conditions (Column oven temperature - low):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 1.0 ml / min

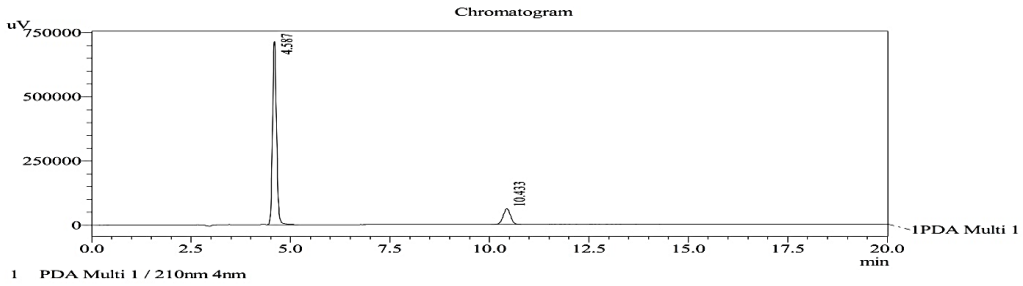
Detection wavelength : 210 nm

Injection volume : 20 µL

Oven Temperature : 28°C

Mobile phase : Buffer: methanol (70: 30)

Sample ID : Robustness Column Temp Low
Method Filename : HAIRGRO TAB_Column Temp Low.lcm
Vial : 2
Injection Volume : 20 uL

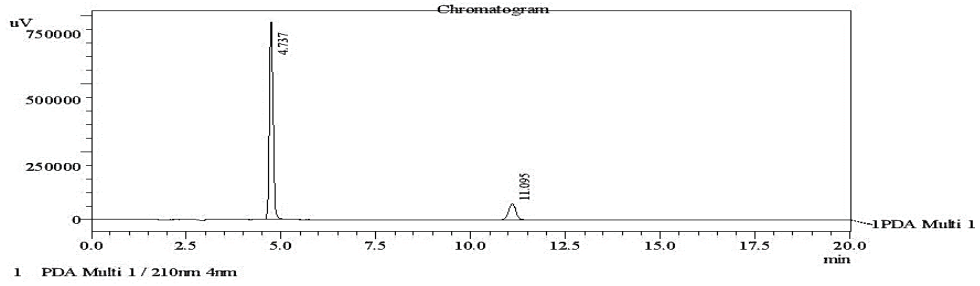


Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.587	4964006	1.21	8586
2	Biotin	10.433	783084	1.01	14487

Figure 7.34: Chromatogram of robustness- standard (column temp low)

Sample Name : Hairgro Tablets
Sample ID : Robustness Column Temp Low
Method Filename : HAIRGRO TAB_Column Temp Low.lcm
Vial : 3
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.737	4995979	1.20	9434
2	Biotin	11.095	790776	1.03	15471

Figure 7.35: Chromatogram of robustness- sample (column temp low)

7.9 Chromatographic conditions (Column oven temperature - High):

Column : *Cosmosil, C_{18},* 250 X 4.6 mm, 5 µm or equivalent

Flow rate : 1.0 ml / min

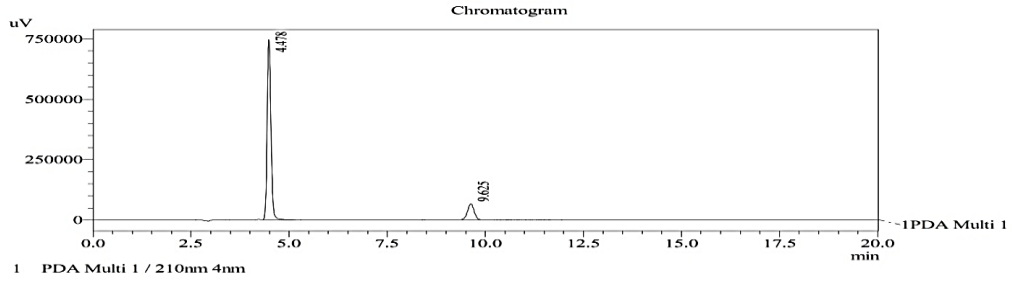
Detection wavelength : 210 nm

Injection volume : 20 µL

Oven temperature : 32°C

Mobile phase : Buffer: methanol (70: 30)

Sample Name : Standard
Sample ID : Robustness Column Temp High
Method Filename : HAIRGRO TAB_Column Temp High.lcm
Vial : 2
Injection Volume : 20 uL

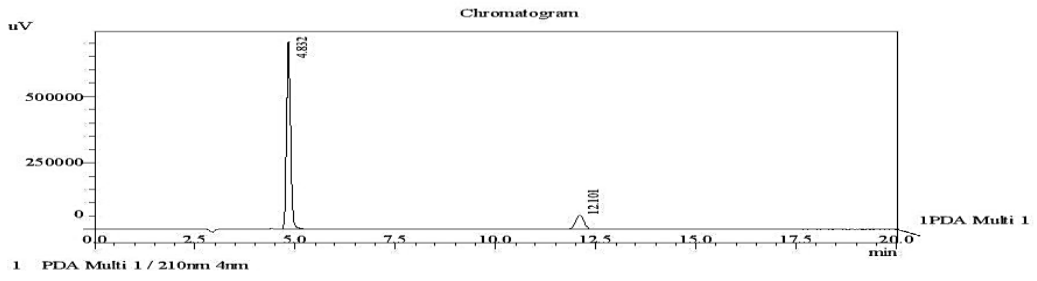


Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.478	5003080	1.21	8634
2	Biotin	9.625	785411	1.03	14573

Figure 7.36: Chromatogram of robustness- standard (column temp high)

Sample Name : Hairgro Tablets
Sample ID : Robustness Column Temp High
Method Filename : HAIRGRO TAB_ Column Temp High.lcm
Vial : 3
Injection Volume : 20 uL



Quantitative Results

ID#	Name	Ret. Time	Area	Tailing Factor	Theoretical Plate
1	Calcium Pantothenate	4.832	5025626	1.20	9139
2	Biotin	12.101	797672	1.01	14853

Figure 7.37: Chromatogram of robustness- sample (column temp high)

Table 7.19: Summary of robustness results of standard (Column temperature)

Flow	Actual		Low		High	
	CP	**BI**	**CP**	**BI**	**CP**	**BI**
RT (min)	4.30-4.53	9.09-9.27	4.13-4.15	8.99-9.05	4.61-4.63	10.48-10.68
Area	4966028-4991449	745914-779360	4585191-4469026	715423-720801	4949496-4988077	790990-797800
Tailing factor	1.19	1.05	1.20	1.03	1.20	1.00
Theoretical plates	8586	14487	8501	14020	9123	14763
Average (Area)	4978738	762637	4519688	718206	4971249	793773
%RSD (Area)	0.27	0.65	1.14	0.38	0.34	0.38

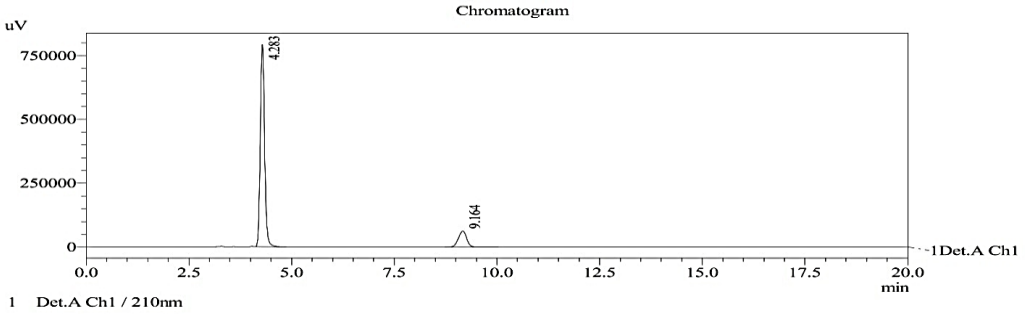
Table 7.20: Summary of robustness results of sample (Column temperature)

Flow	Actual		Low		High	
	CP	**BI**	**CP**	**BI**	**CP**	**BI**
RT (min)	4.31-4.40	9.09-9.27	4.57-4.59	10.16-10.43	4.48-4.52	9.53-9.73
Area	5003737-4966028	735393-745914	4940974-5020412	782565-791915	4911396-5003080	784140-793037
Tailing factor	1.23	1.05	1.21	1.01	1.21	1.03
Theoretical plates	8638	14392	8586	14487	8634	14573
Average (Area)	4985023	740081	4967201	786025	4937913	789483
%RSD (Area)	0.24	0.60	0.59	0.43	0.68	0.63

8. Solution stability

Measure the stability of the Hairgro tablets test solution against 100% of the standard concentration by keeping the solution up to 48 hours at 15°C. Inject the sample at different time intervals (i.e: Initial, 6, 12, 18, 24, 36& 48 hours) as per the method. A typical chromatogram for solution stability is shown in **Figure- 7.38- 7.45**

Sample Name : Hairgro Tablets
Sample ID : Solution Stability (0 Hr)
Method Filename : Hairgro_Tablet_AY.lcm
Vial : 3
Injection Volume : 20 uL



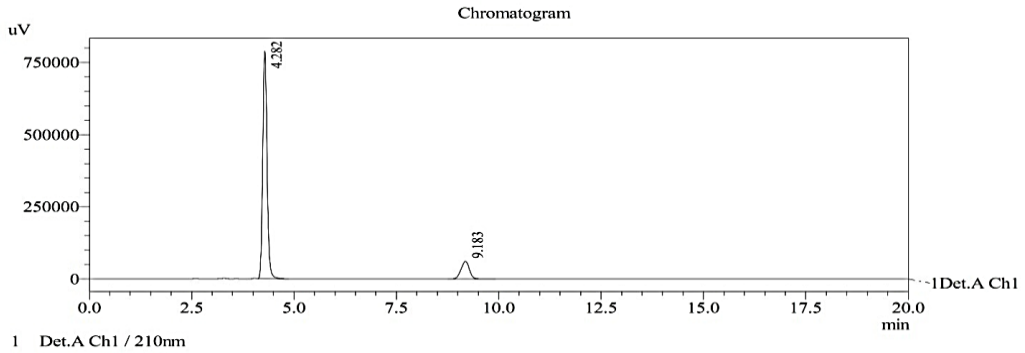
Quantitative Results

Detector A

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.283	5836202
2	Biotin	9.164	869245

Figure 7.38: Chromatogram of solution stability- Initial

Sample Name : Hairgro Tablets
Sample ID : Solution Stability (6 Hr)
Method Filename : Hairgro_Tablet_AY.lcm
Vial : 3
Injection Volume : 20 uL



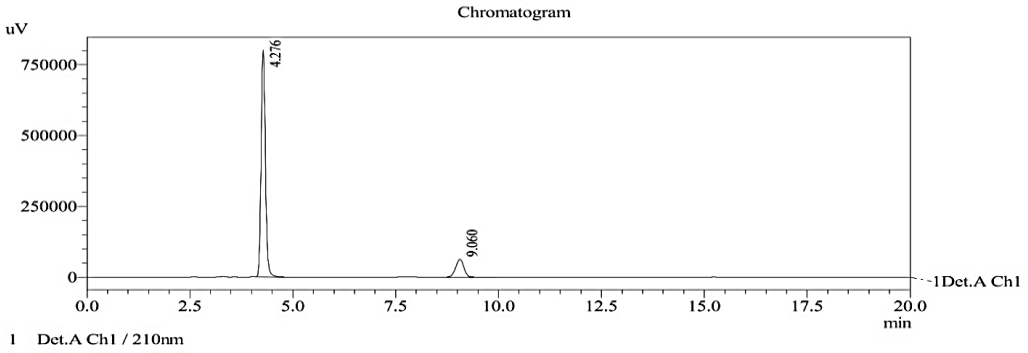
Quantitative Results

Detector A

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.282	5821094
2	Biotin	9.183	866568

Figure 7.39: Chromatogram of solution stability- 6 hours

Sample Name : Hairgro Tablets
Sample ID : Solution Stability (12 Hr)
Method Filename : Hairgro_Tablet_AY.lcm
Vial : 3
Injection Volume : 20 uL



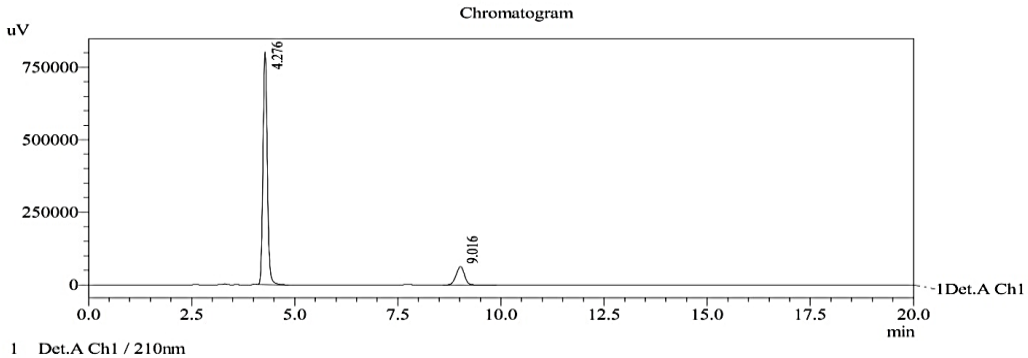
Quantitative Results

Detector A

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.276	5841000
2	Biotin	9.060	872703

Figure 7.40: Chromatogram of solution stability- 12 hours

Sample Name : Hairgro Tablets
Sample ID : Solution Stability (18 Hr)
Method Filename : Hairgro_Tablet_AY.lcm
Vial : 3
Injection Volume : 20 uL



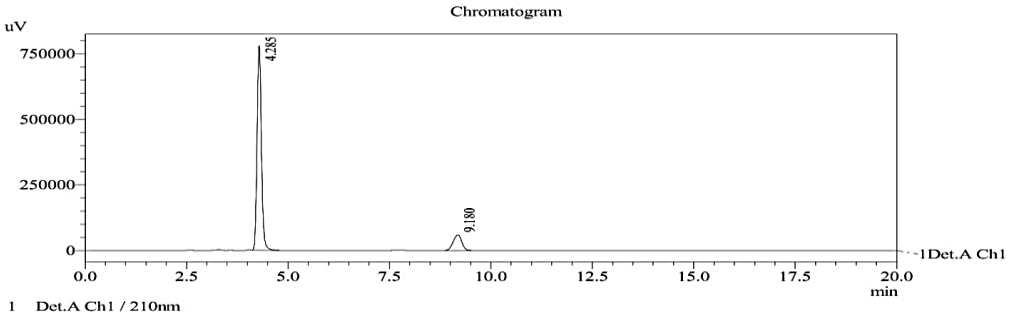
Quantitative Results

Detector A

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.276	5858372
2	Biotin	9.016	875517

Figure 7.41: Chromatogram of solution stability- 18 hours

Sample Name : Hairgro Tablets
Sample ID : Solution Stability (24 Hr)
Method Filename : Hairgro_Tablet_AY.lcm
Vial : 3
Injection Volume : 20 uL



1 Det.A Ch1 / 210nm

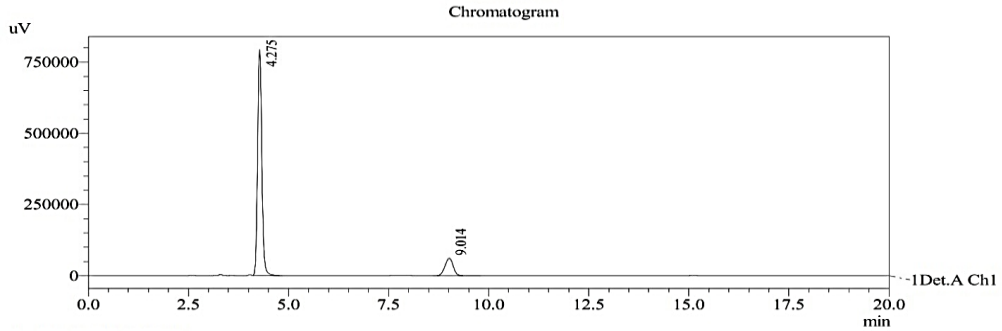
Quantitative Results

Detector A

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.285	5838173
2	Biotin	9.180	869618

Figure 7.42: Chromatogram of solution stability- 24 hours

Sample Name : Hairgro Tablets
Sample ID : Solution Stability (36 Hr)
Method Filename : Hairgro_Tablet_AY.lcm
Vial : 3
Injection Volume : 20 uL



1 Det.A Ch1 / 210nm

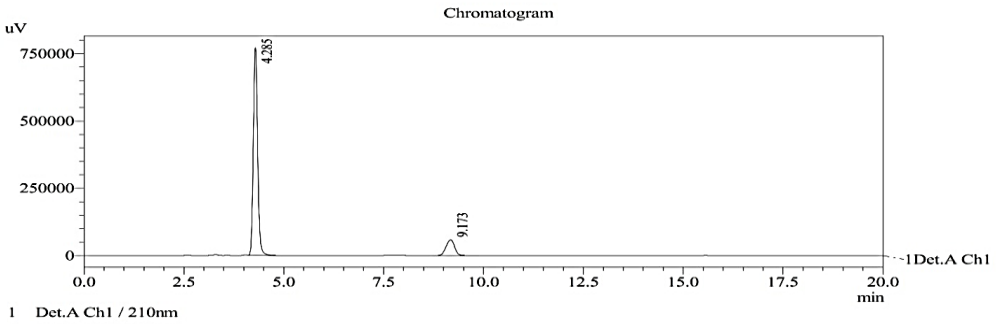
Quantitative Results

Detector A

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.275	5856166
2	Biotin	9.014	874134

Figure 7.43: Chromatogram of solution stability- 36 hours

Sample Name : Hairgro Tablets
Sample ID : Solution Stability (48 Hr)
Method Filename : Hairgro_Tablet_AY.lcm
Vial : 3
Injection Volume : 20 uL



Quantitative Results

Detector A

ID#	Name	Ret. Time	Area
1	Calcium Pantothenate	4.285	5841252
2	Biotin	9.173	869066

Figure 7.44: Chromatogram of solution stability- 48 hours

Table 7.21: Summary of solution stability results

Time intervals	% CP	% BI
Initial	103.64	104.61
6 hours	103.39	104.30
12 hours	103.81	105.01
18 hours	104.08	105.42
24 hours	103.67	104.58
36 hours	104.05	105.26
48 hours	103.69	104.55
% RSD	**0.23**	**0.40**

The assay values of calcium pantothenate in Hairgro tablets were in the range of **103.39% to 104.08%**. The %RSD between the assay results from the initial to 48 hours was 0.23, which is within the acceptance limit of 2.0%. The assay values of biotin in Hairgro tablets was in the range of **104.30% to 105.42%**. The %RSD between the assay results from the initial to 48 hours was 0.40, which is within the acceptance limit of 2.0%. Therefore, the Hairgro tablets sample solutions are stable up to 48 hours at 15°C.

DISCUSSION

The working condition for the RP-HPLC method were established for biotin and calcium pantothenate then applied on pharmaceutical dosage form.

Mobile phase system were prepared and used to provide ratio of Buffer: methanol (70:30) gave a better resolution and sensitivity. The retention time of biotin and calcium pantothenate were found to be **9.79** and **4.53** respectively. The asymmetry factor or tailing factor of biotin and calcium pantothenate is **1.05** and **1.23** respectively which indicates symmetrical nature of peak.

The number of theoretical plates of biotin and calcium pantothenate was **14392** and **8638** respectively which indicates efficient performance of column. The retention time of drugs were found to be within the limits of **0-10** minutes. These parameters represent the specificity of method.

From the linearity studies, specified concentration range was determined. It was observed that biotin and calcium pantothenate were in the range of **50% - 150%** each for the target concentration. The linearity range is **0.5 – 1.5mg/ml** for calcium pantothenate and **0.05 – 0.15mg/ml** for biotin were found to obey linearity with correlation coefficient of **0.9976** for calcium pantothenate and **0.9997** for biotin. The validation for the proposed method was verified by system precision and method precision. There was %RSD of system precision for biotin and calcium pantothenate was **0.60** and **0.24** respectively. The method precision was conducted and the % label claim was obtained as **103.71%** for calcium pantothenate and **105.16%** for biotin. The % recovery value of **98.35% - 101.75%** for calcium pantothenate and **98.58% - 100.98%** for biotin indicates non-interferences of excipient in the formulation.

The validation of the proposed method was verified by recovery studies. The robustness studies were performed by changing the flow-rate, pH, column temperature and mobile phase concentration. The ruggedness studies were performed. All parameters including flow-rate, temperature, detector, wavelength and sensitivity were maintained constant throughout the procedure. The analytical method validation was carried as per the ICH guidelines.

SUMMARY REPORT:

S.No	Parameters	Observation	Acceptance Criteria
1	**Specificity** *Placebo* Interference	No peak was observed at the retention time of calcium pantothenate and biotin in the chromatogram of *placebo*	The *placebo* chromatogram should not show any peak at the retention time of calcium pantothenate and biotin.
2	**System precision** **(**a) Calcium pantothenate	1.23	Tailing factor: NMT 2.0
		8638	Theoretical plates :NLT 2000
		0.24	% RSD: NMT 2.0
	(b) Biotin	1.05	Tailing factor :NMT 2.0
		14392	Theoretical plates: NLT 2000
		0.60	% RSD: NMT 2.0
3	**Linearity & Range** (a) Calcium pantothenate	0.9976	Correlation coefficient: NLT 0.995
	(b) Biotin	0.9997	
4	**Accuracy** (a) Calcium pantothenate	98.35 – 101.75 %	98.0 – 102.0%
	(b) biotin	98.58 – 100.98 %	
5	**Method Precision** (a) Calcium pantothenate	1.13	% RSD : NMT 2.0 (Assay)
	(b) Biotin	1.58	

S. No	Parameters	Observation	Acceptance criteria
6	**Ruggedness** (a) Calcium pantothenate	0.89	Overall % RSD NMT 2.0 (assay)
	(b) Biotin	1.29	
7	**Robustness** (a) Calcium pantothenate	1.19 – 1.23	Tailing factor: NMT 2.0
		8501 – 10171	Theoretical plates: NLT 2000
		0.06 – 1.14	% RSD: NMT 2.0
	(b) Biotin	1.00 – 1.05	Tailing factor: NMT 2.0
		13715 – 16403	Theoretical plates: NLT 2000
		0.32 – 0.73	% RSD: NMT 2.0
8	**Solution stability** (a) Calcium pantothenate	0.23	%RSD NMT : 2.0 (assay)
	(b) Biotin	0.40	

CHAPTER 8:
SUMMARY & CONCLUSION

CONCLUSION

Cosmosil C_{18} column as stationary phase and phosphate buffer: methanol (70: 30) as a mobile phase and **210 nm** is selected for the detection in this RP-HPLC.

The system precision shows RSD value obtained was below 1 which indicate precision and system suitability indicates retention time for calcium pantothenate and biotin were **4.53** and **9.79** respectively.

Co-effecient of correlation for calcium pantothenate and biotin denote sensitivity.

Quantitative estimation of calcium pantothenate and biotin gives accuracy which lies between **98.35% – 101.75%** for calcium pantothenate and **98.58% - 100.98%** for biotin.

By using system to system suitability and analyst to analyst variability all the parameters met the system suitability.

The percentage purity of calcium pantothenate and biotin lies in between **103.71%** and **105.16%** respectively.

The proposed RP-HPLC method was simple, precise, rapid & accurate and involves easy sample preparation. The linearity, reproducibility and recovery data confirms no major interferences in the assay determination. **So, this method can be used for routine quality control analysis of this drug.**

CHAPTER 9:

GLOSSARY

GLOSSARY

%	-	Percentage
µg	-	Microgram
ng	-	Nanogram
AR	-	Analytical Reagent
M	-	Molarity
N	-	Normality
mg	-	Milli gram
mins	-	Minutes
ml	-	Milli liter
mm	-	Milli meter
NA	-	Not Applicable
RP-HPLC	-	Reverse Phase- High Performance Liquid chromatography
ICH	-	International Conference on Harmonization
BI	-	Biotin
CP	-	Calcium Pantothenate
KH_2PO_4	-	Potassium dihydrogen orthophosphate
H_3PO_4	-	Phosphoric acid
NMT	-	Not more than
NLT	-	Not less than
pH	-	Negative logarithm of hydrogen ion concentration

CHAPTER 10:

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