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"DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF METHYLCOBALAMIN, ALPHA LIPOIC ACID, PYRIDOXINE AND FOLIC ACID BY RP-HPLC IN BULK AND MARKETED DOSAGE FORM"

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Abstract:

A simple, specific, precise and accurate Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method was developed and validated for the quantitative estimation of Methylcobalamin, alpha lipoic acid, pyridoxine and folic acid by RP-HPLC in bulk and marketed dosage form". The proposed RP-HPLC method was carried out on 250 mm x 4.6 mm, 5um column with mobile 0.1% o-phosphoric acid: Methanol (50:50). The retention time of torsemide was found at 6.0±0.2min. The method was validated for specificity, precision, accuracy, linearity and robustness. The linearity range was 10-30 ?g/mL and correlation coefficient (r^2) was found to be 0.9980. The mean % recovery for Torsemide was found to be 99.80. The developed method could be employed for the routine analysis of Torsemide from different formulations and for the Torsemide calculations as well.

1. Introduction

HPLC is an abbreviation for High Performance Liquid Chromatography. "Chromatography" is a technique for separation, "chromatogram" is the result of chromatography, and "chromatograph" is the instrument used to conduct chromatography.

Among the various technologies developed for chromatography, devices dedicated for molecular separation called columns and high-performance pumps for delivering solvent at a stable flow rate are some of the key components of chromatographs. As related technologies became more sophisticated, the system commonly referred to as High Performance Liquid Chromatography, simply became referred to as "LC". Nowadays, Ultra

High Performance Liquid Chromatography (UHPLC), capable of high-speed analysis, has also become more wide-spread.

Only compounds dissolved in solvents can be analyzed with HPLC. HPLC separates compounds dissolved in a liquid sample and allows qualitative and quantitative analysis of what components and how much of each component are contained in the sample.

Fig.1 shows a basic overview of the HPLC process. The solvent used to separate components in a liquid sample for HPLC analysis is called the mobile phase. The mobile phase is delivered to a separation column, otherwise known as the stationary phase, and then to the detector at a stable flow rate controlled by the solvent delivery pump. A certain amount of sample is injected into the column and the compounds contained in the sample are separated. The compounds separated in the column are detected by a detector downstream of the column and each compound is identified and quantified. [1,2]

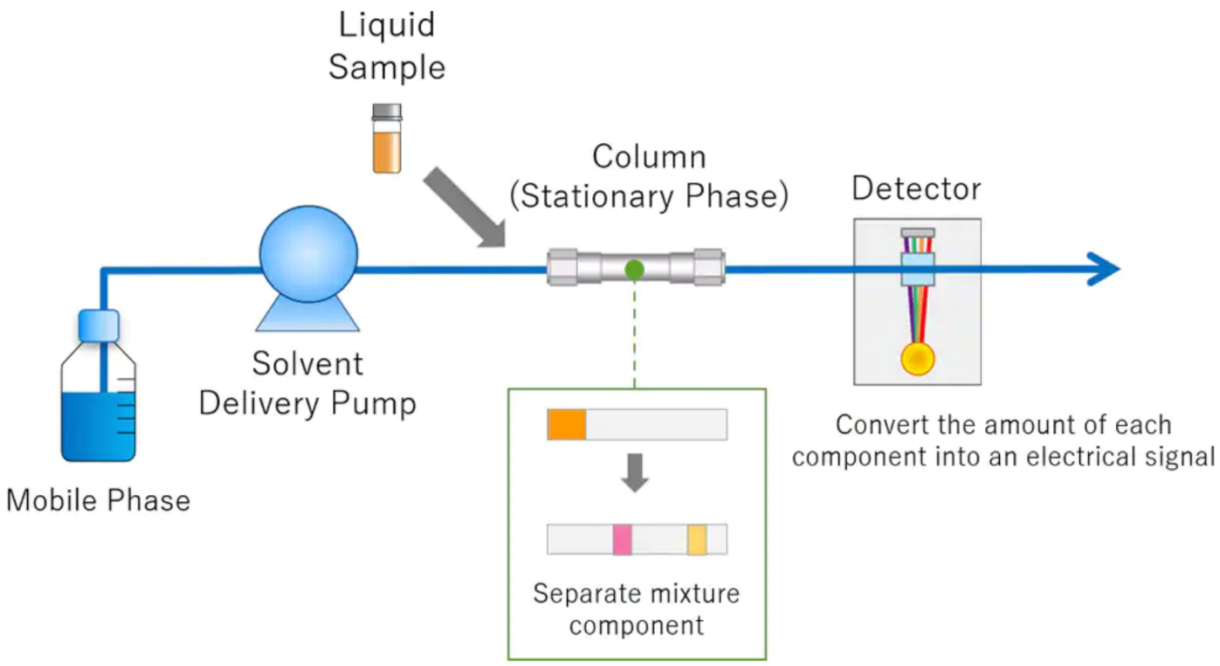


Figure 1. Overview of HPLC

Apparatus of HPLC

The “Basic Overview of the HPLC process"(As shown in Fig.1) and its mechanisms have now been covered. Going into more detail, HPLC consists of a variety of components, including a solvent delivery pump, a degassing unit, a sample injector, a column oven, a detector, and a data processor. Fig.2 shows the HPLC flow diagram and the role of each component. [1,3]

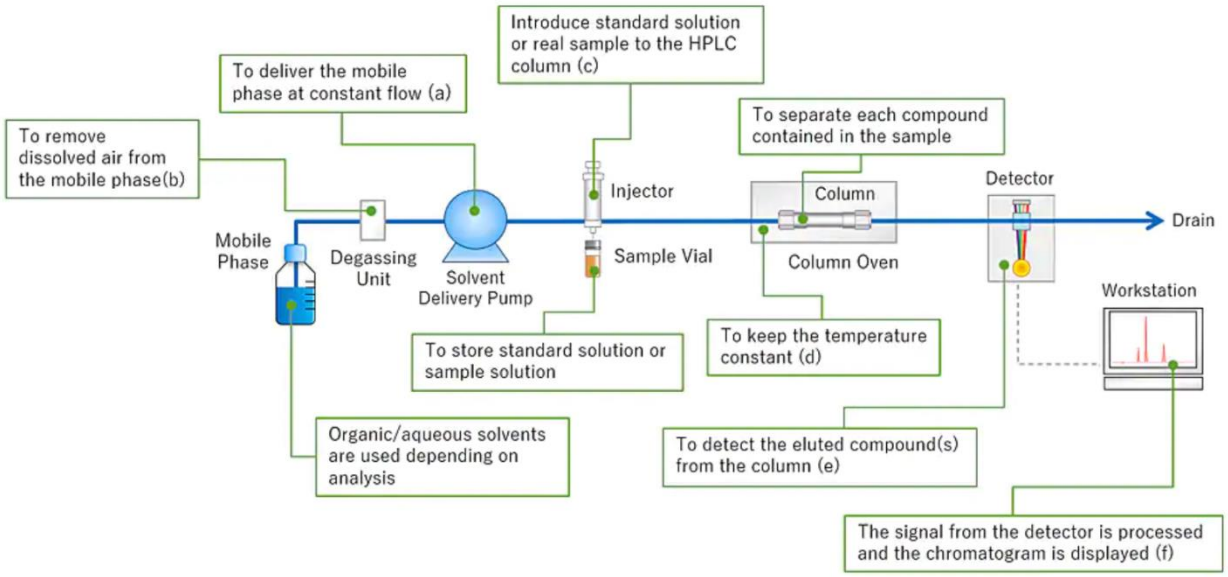


Figure 2. HPLC flow diagram and the role of each component.

As for HPLC, the pump delivers the mobile phase at a controlled flow rate(a). Air can easily dissolve in the mobile phase under the standard atmospheric pressure in which we live in. If the mobile phase contains air bubbles and enters the delivery pump, troubles such as flow rate fluctuations and baseline noise/drift may occur. The degassing unit helps prevent this issue by removing air bubbles in the mobile phase(b). After the dissolved air has been removed, the mobile phase is delivered to the column. The sample injector then introduces a standard solution or sample solution into the mobile phase (c). Temperature fluctuations can affect the separation of compounds in the column. The column is placed in a column oven to keep the temperature constant(d). Compounds eluted from the column are detected by a detector which is placed downstream of the column(e). A workstation processes the signal from the detector to obtain a chromatogram to identify and quantify the compounds(f). [1,4]

HPLC Separation

HPLC can separate and detect each compound by the difference of each compound's speed through the column. Fig.3 shows an example of HPLC separation.

There are two phases for HPLC: the mobile phase and the stationary phase. The mobile phase is the liquid that dissolves the target compound. The stationary phase is the part of a column that interacts with the target compound.

In the column, the stronger the affinity (e.g.; van der waals force) between the component and the mobile phase, the faster the component moves through the column along with the mobile phase. On the other hand, the stronger the affinity with the stationary phase, the slower it moves through the column. Fig. 3 shows an example in which the yellow component has a strong affinity with the mobile phase and moves quickly through the column, while the pink component has a strong affinity with the stationary phase and moves through slowly. The elution speed in the column depends on the affinity between the compound and the stationary phase. [1,5]

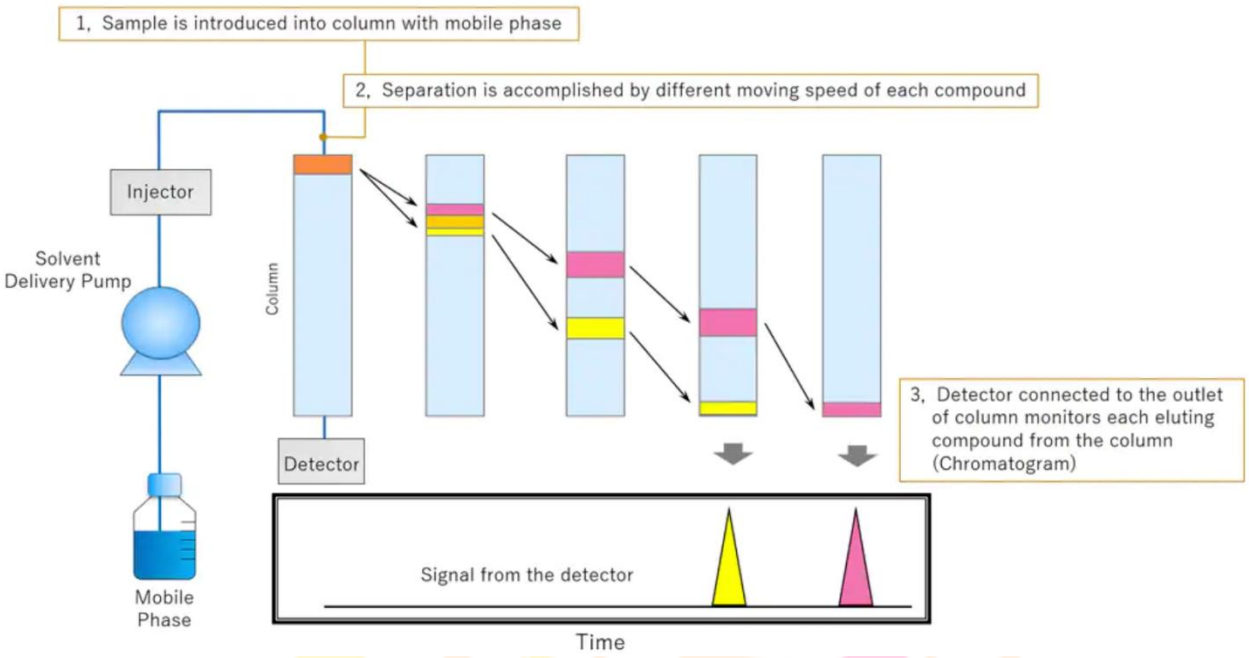


Figure 3. An Example of HPLC Separation

How to Read a Chromatogram

The word "chromatogram" means a plot obtained via chromatography. Fig.4 shows an example of a chromatogram. The chromatogram is a two-dimensional plot with the vertical axis showing concentration in terms of the detector signal intensity and the horizontal axis representing the analysis time. When no compounds are eluted from the column, a line parallel to the horizontal axis is plotted. This is called the baseline. The detector responds based on the concentration of the target compound in the elution band. The obtained plot is more like the shape of a bell rather than a triangle. This shape is called a “peak”. [1,6]

Retention time (tR) is the time interval between sample injection point and the apex of the peak. The required time for non-retained compounds (compounds with no interaction for the stationary phase) to go from the injector to the detector is called the dead time (t0).

The peak height (h) is the vertical distance between a peak's apex and the baseline, and the peak area (A) colored in light blue is the area enclosed by the peak and baseline. These results will be used for the qualitative and quantitative analysis of a sample's components.[1,7]

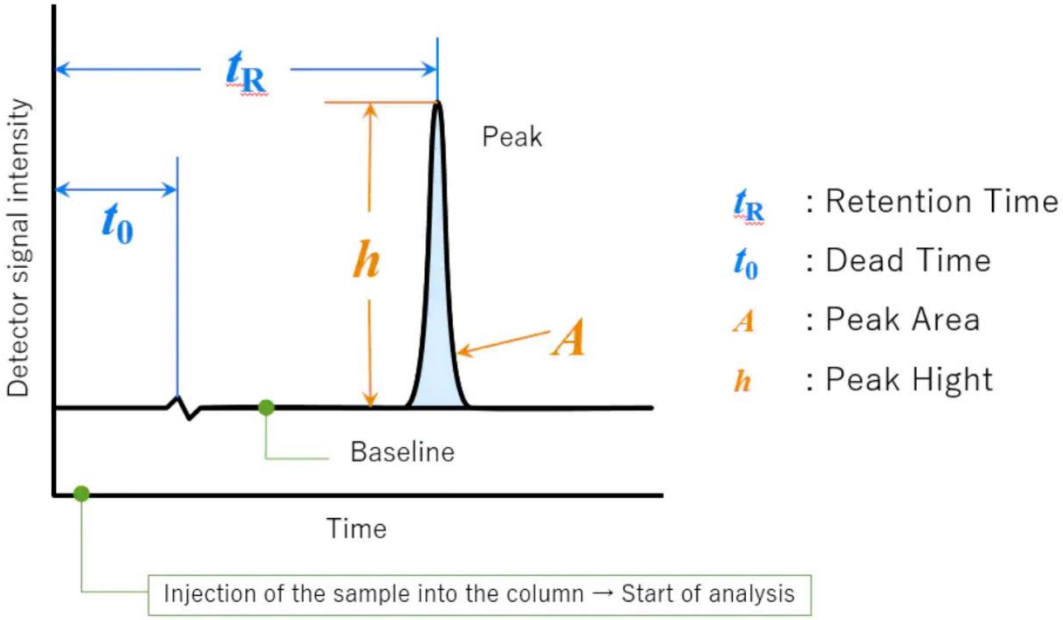


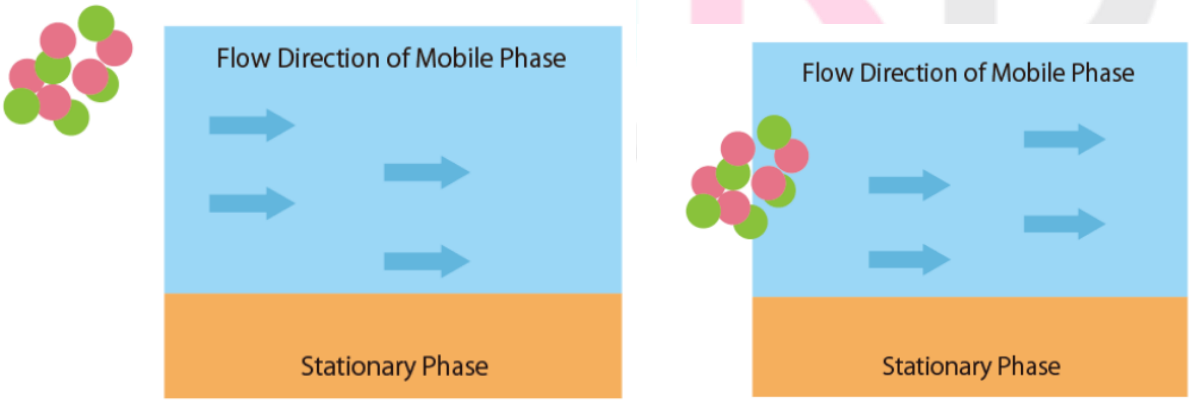
Figure 4. Chromatogram terminologies

Principle of Separation technique

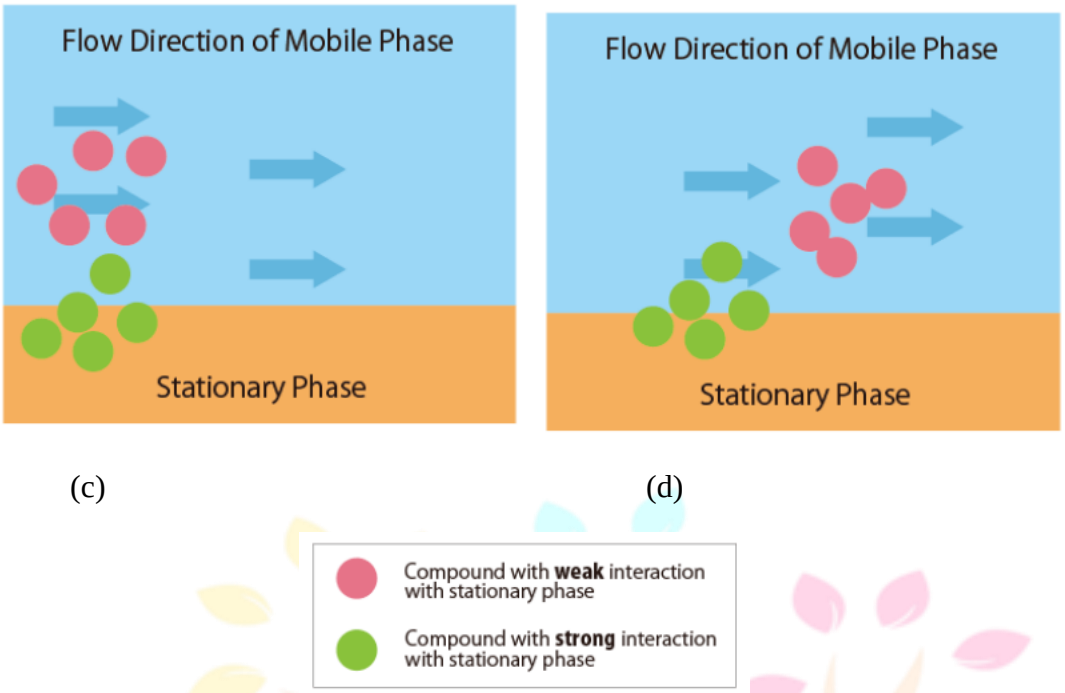
There are 3 factors in the column: Compounds, Mobile Phase, and Stationary Phase. Each definition is shown as follows;

- Compounds : Solutes in the sample solution
- Mobile Phase : Solution delivered using solvent delivery pumps
- Stationary Phase : Functional groups chemically modified in spherical particles packed in the column

Fig.5schematically shows the relationship between the sample compounds and the stationary and mobile phases. Compounds that interact more strongly with the mobile phase elute more faster from the column. In contrast, compounds that interact more strongly with the stationary phase remain in the column longer. [1,8]



(a) (b)



Reference

Figure 5. Mechanism of Separation in HPLC Column.

"Interaction" refers to the chemical attraction between molecules. The selection of the interaction type suitable for the property of the target compound is necessary for the optimization of the separation method. The type of interaction is related to the separation mode.Table 1 shows the list of general HPLC separation modes and basic principle. [1,9]

Separation Mode	**Interaction**	**Components**
Reversed phase chromatography (RP)	Hydrophobicity(Low Polarity)	Small molecule pharmaceuticals, Vitamins, etc.
Normal phase chromatography (NP)	Hydrophilicity (High Polarity)	Saccharides, Nuclear acids, etc.
Ion exchange chromatography (IEX)	Electrostaticity	Inorganic ions, Amino acids, Protein, etc.
Size exclusion chromatography (SEC)	Molecular size	Synthetic polymer, Biopolymer, Polysaccharide, etc.

HPLC Detectors

HPLC analysis requires a detector to monitor the compounds themselves in order to quantify and identify the compounds separated in the column. In other words, detector plays a role of "eyes" for HPLC analysis. Appropriate detectors are selected based on the properties of the target compound and the affinity of the analytical conditions. Table 2 lists the HPLC detectors and their characteristics.

Detector	Target Compounds
Absorbance	UV-absorbing compounds
Fluorescence	Fluorescent compounds
Refractive Index	All
Evaporative Light Scattering	All（Exclude non-volatile compounds）
Conductivity	Cation, Anion
Mass Spectrometry	Ionic compounds

In general, compounds with characteristic structures tend to be highly selective and sensitive for detection. Absorbance detectors such as ultraviolet absorption detectors and photodiode array detectors are commonly used for HPLC analysis because many target compounds have chromophores due to double bonds in their molecular structures.

For non-UV absorbing compounds, they can be detected with other characteristics such as ionicity. Compounds that fluoresce upon irradiation with a specific wavelength can be detected with a fluorescence detector. Detectors that can detect all compounds, such as a refractive index detector and an evaporative light scattering detector, are described as “universal detectors”. [1,10]

Absorbance Detector

Absorbance detection is the most common detection method in HPLC analysis. Light is a type of electromagnetic wave, and electromagnetic waves are given different names depending on their wavelengths. Fig.6 shows the types of electromagnetic waves and their corresponding wavelengths.

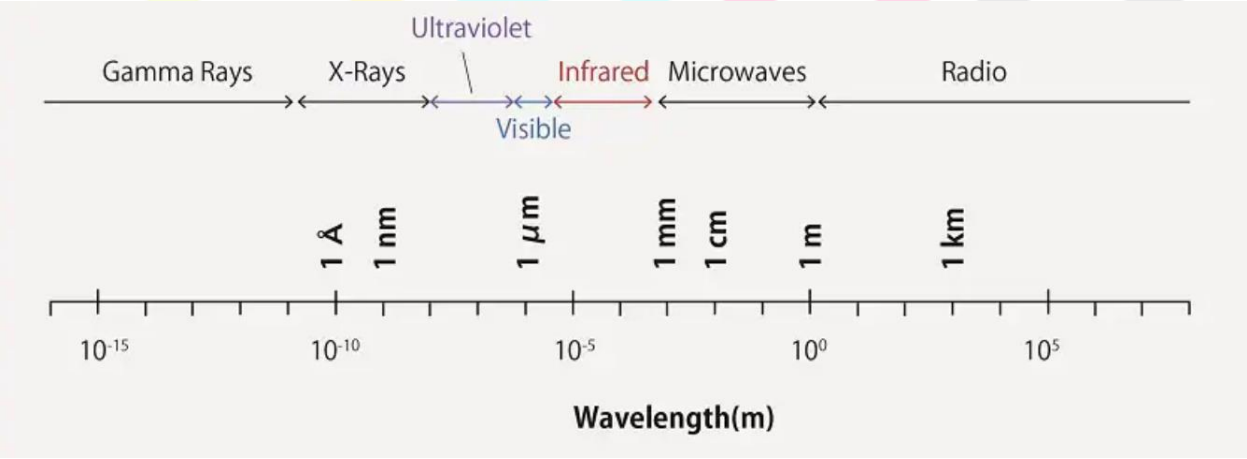


Figure 6. Electromagnetic Spctrum

When a substance is irradiated with light, it absorbs light of a specific wavelength, and the energy of electrons change from their ground state (lowest state) to an excited state (high energy state). The wavelength of light absorbed depends on the structure of the substance. Light in the ultraviolet to visible range is used for absorbance detection.

Fig.7 shows the principle of absorbance detection in HPLC analysis. The absorbance is measured by monitoring the rate of decrease in the amount of light that passes through the cell when irradiating the solution in the flow cell with light of a particular wavelength. The absorbance is proportional to the concentration of the target substance. This can be defined as Lambert-Beer's law. [1,11]

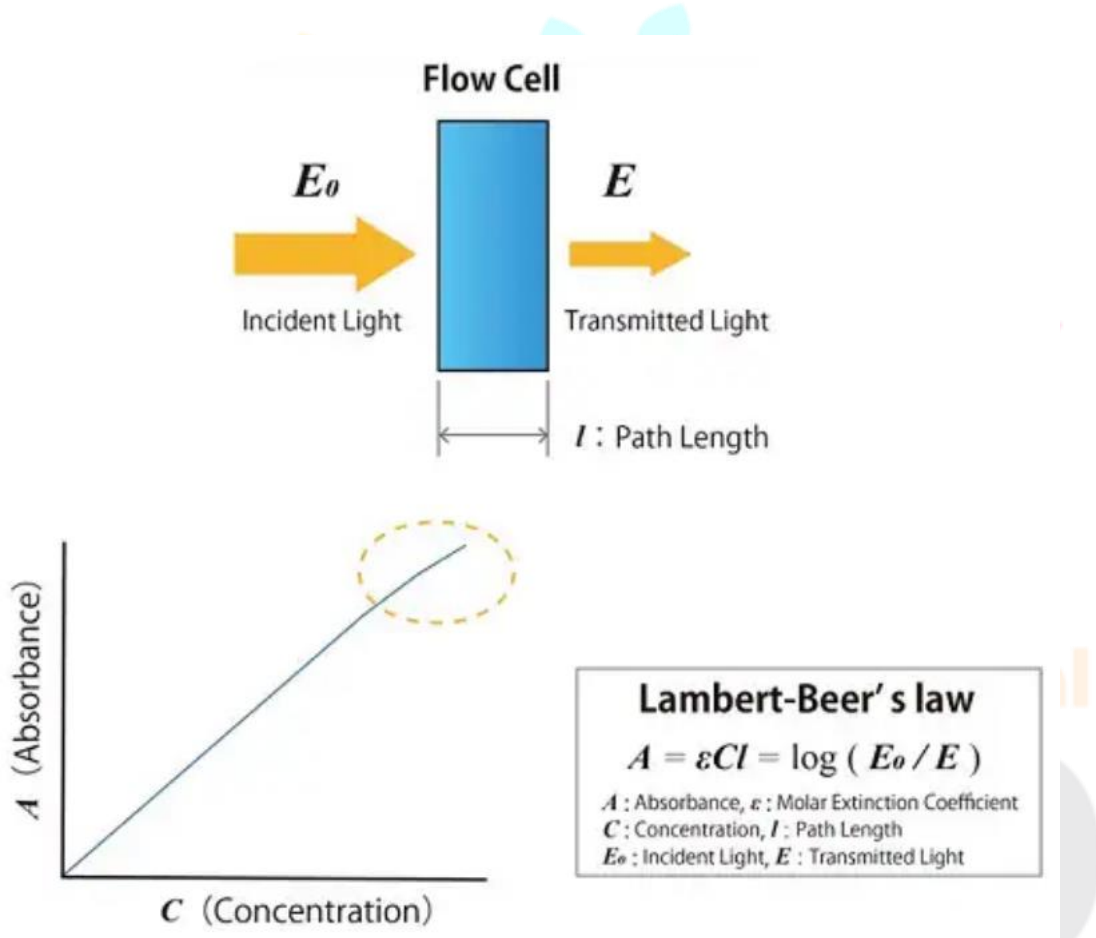


Figure 7. Principle of Absorbance Detection in HPLC Analysis (Above : Measurement of Absorbance, Below :Lambert-Beer's Law) [12]

The concentration of the component can be calculated by measuring the change in absorbance of the mobile phase passing through the flow cell of the detector. According to Lambert-Beer's law, the absorbance is proportional to the component concentration, but in practice, too high a concentration prevents the detector from obtaining an accurate measurement, as shown in Fig.2 . Therefore, it is necessary to quantify within a concentration range that guarantees a linear proportion with the absorbance rate, otherwise known as the dynamic range.

Fig. 8 shows a schematic diagram of an ultraviolet detector (UV). A deuterium (D2) lamp is used as an ultraviolet light source for the UV detector. The light emitted from the lamp is separated into a light beam of a certain wavelength with a diffraction grating, and then passes through the flow cell. The light that passes through the flow cell enters the photodetector (photodiode) and is converted into an electrical signal corresponding to the intensity of the light, which is processed as absorbance. A UV-visible detector with a D2 lamp and a tungsten lamp is suitable for monitoring not only ultraviolet but also visible light. [13]

Fig. 9 shows a schematic diagram of a photodiode array detector (PDA *). Light emitted from a lamp installed in the PDA detector passes through the flow cell and then separated with a diffraction grating. When the separated light beam is received by a photodiode, which is a sequence of 1,024 photodetectors, only the wavelengths in the specified range are converted into electrical signals and processed as absorbance data. * In some cases, this is called a diode array detector (DAD). [14]

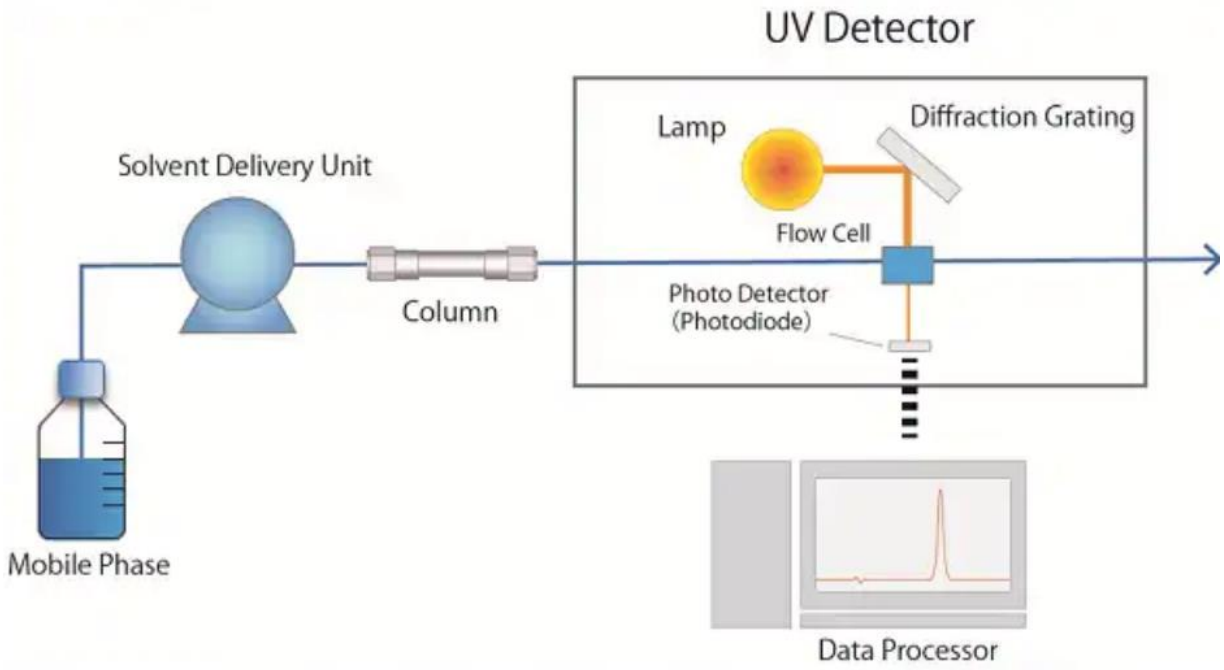


Figure 8. Schematic Diagram of a UV detector

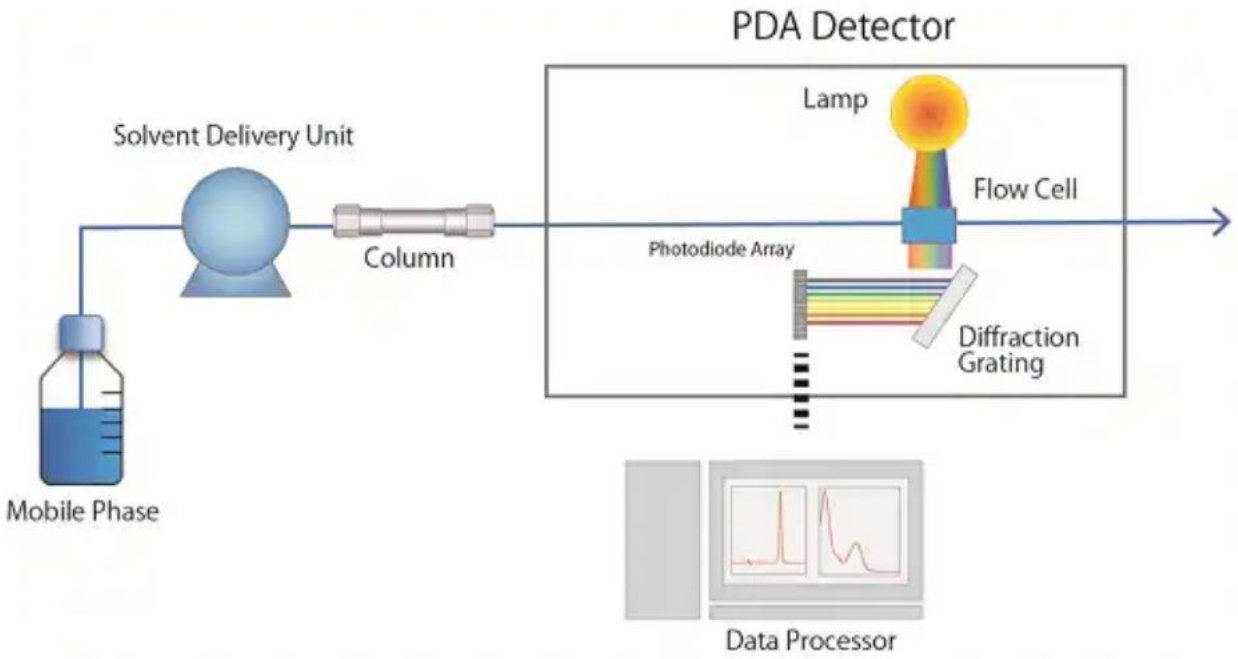


Figure 9. Schematic Diagram of a PDA detector

Using a PDA detector allows you to measure a continuous UV spectrum, resulting in a multi-wavelength chromatogram. The detector provides not only a chromatogram that refers to time on the X axis and absorbance on the Y axis, as obtained with the UV detector, but also three-dimensional data having a wavelength axis on the Z axis [1, 15]

HPLC Apparatus

A HPLC system consist of various of components, including solvent delivery pumps, a sample injector, a column oven, a detector, and a workstation. This page introduces the operating principles of main each components. Figure 10 shows the appearance of HPLC. [1,13,16]

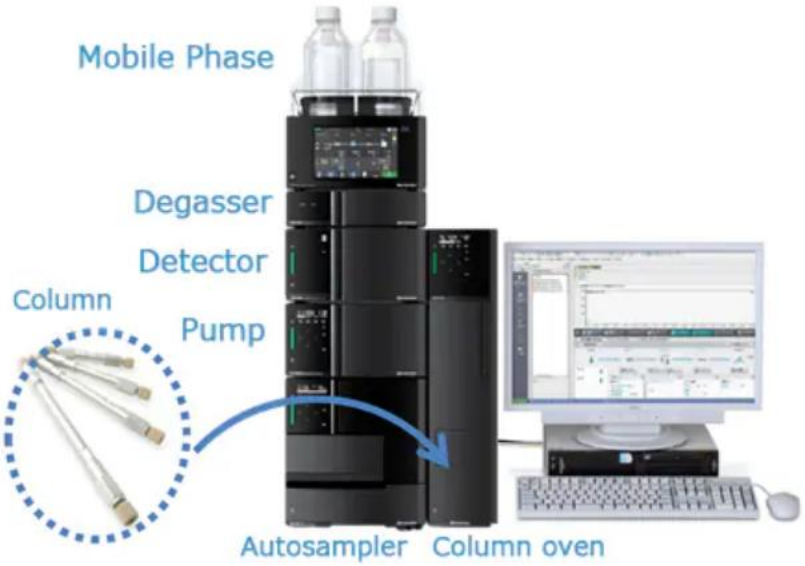


Figure 10. HPLC Apparatus

1. Solvent Delivery Pump

A stability of solvent delivery affects the precision of the retention time of compound and the calculation of peak area. Fig.11 shows the sketch of the inside of solvent delivery pump. Seeing from the front of the solvent delivery pump, it contains two pump heads and a drain valve. The mobile phase is delivered through the pump head. Drain valve is used to replace the mobile phase or remove air from the flow path. By opening the drain valve, the flow path can be switched from the downstream flow path from the pump to the drain (waste container). Each pump head has check valves at the inlet and outlet. [1,11,17]

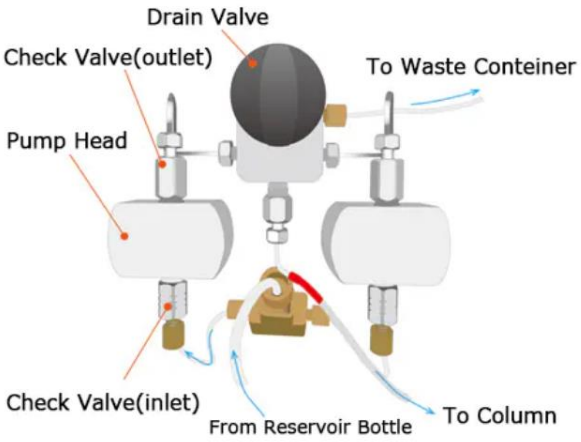


Figure 11. Inside of Solvent Delivery Pump

A typical LC pump is based on the reciprocating piston design. The basic elements of the pump are a cylindrical pump chamber that holds the piston, a motor that operates a driving cam, a plunger seal, and a pair of check valves. Check valves consisting of tapered seats and balls are placed at the chamber inlet and outlet, respectively. When the plunger is pulled to the left in the figure, the inside of the chamber is depressurized. Then, the inside of inlet check valve is depressurized then a ball inside the check valve floats making a gap between the ball and the sheet. This action allows mobile phase to enter the pump chamber. Conversely, when the plunger moves to the right due to the action of the cam connected to the motor, the chamber is pressurized and the inlet check valve closes. Then, the outlet check valve opens and the mobile phase in the chamber flows toward the column. The pump operates these processes continuously to deliver the mobile phase.[1,16,18]

2. Sample Injector

Sample injection devices that can inject sample solutions are classified into two types: manual injectors and autosamplers. A manual injector is a device that introduces a sample into a column with a syringe manually. It is easy to handle the simple mechanism, but the accuracy of sample injection depends on the operator's skill. The

autosampler is a device that affords automatic sample measurement, sample injection, and cleaning of needle. There are total volume injection method and loop injection method in the injection method. Fig.12 shows the sketch of the inside of the autosampler. The autosampler consists of a sample rack for setting sample vials, a sample needle, a sample loop, and a valve for changing the flow path of the mobile phase. [1,4,19]

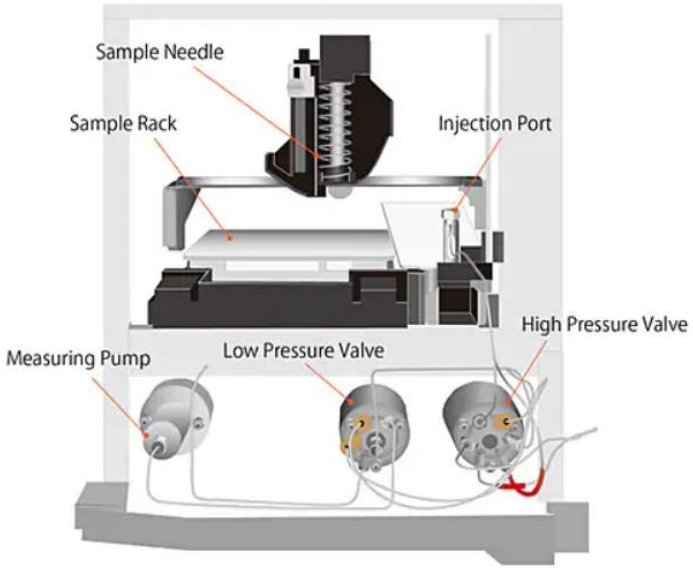


Figure 12. Inside of autosampler

The mobile phase coming from the solvent delivery pumps flows through the high pressure valve, sample loop, needle, injection port, and high pressure valve to the column. The high pressure valve switches the flow path to separate the needle from the flow path of the mobile phase when loading a sample from the sample vial. The needle is inserted into a sample vial and then the measuring pump operates and draws the sample into the needle and the sample loop. The needle is inserted into the injection port and then the high pressure valve switches to the inject position to start analysis. The sample is delivered with the mobile phase through the high pressure valve to the column.. This is a series of flow for the sample injection. [1,2.5,20]

3. Column Oven

The function of a column oven is to keep the temperature of the columns constant. The separation of compounds using chemical interaction between the stationary phase and the target compounds is quite sensitive against temperature change. Moreover, the peak shape and retention time are not stable without the temperature control. For these reasons, a column oven is used.
There are two types of column oven, block heater type and air circulation type. For block heater type, the column is heated on a metal block designed for a couple of assigned column sizes. Since the accuracy of temperature control would be worse if there is a space between the column and the block, installable column size and numbers are limited.

For air circulation type, columns can be installed anywhere in the oven. Therefore, the size and number of columns that can be contained are more flexible than those of the block heater type. On the other hand, the size of oven could be large because of the space for circulation system. This air circulation type can be equipped with a mixer and a manual injector using the empty space of the chamber. It contributes to the stability of analysis in addition to the temperature control. [1,9,20,21]

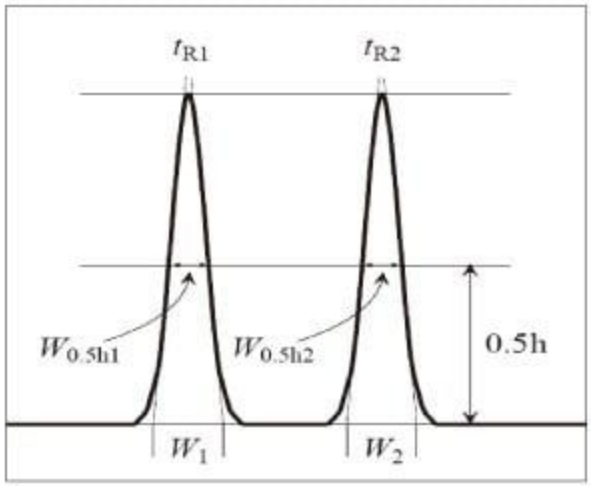
Resolution

Multidimensional chromatography uses a combination of several chromatography techniques, separation modes, and columns to separate multiple components. It achieves significantly higher separation than normal one-dimensional chromatography. Various separation modes and the corresponding mobile phases can be selected for HPLC, and the diverse permutations available suggest the possibility of achieving a degree of selectivity not possible using one-dimensional separation alone. [22-26]

$$R = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(W_1 + W_2)} \quad \cdots (1)$$

$$R = 1.18 \times \left(\frac{t_{R2} - t_{R1}}{W_{0.5h1} + W_{0.5h2}} \right) \quad \cdots (2)$$

Equation 1 and Equation 2



t_{R1}, t_{R2} : Retention time for each peak ($t_{R1} < t_{R2}$)
$W_{0.5h1}$, $W_{0.5h2}$: Full width at half maximum (FWHM) of each peak
W_1, W_2 : Width of each peak
Fig. 1 Two Adjacent Peaks

Figure 13. Two Adjacent peaks.

Equation (1) indicates that the resolution is the difference between peak retention times divided by the average peak width. In a peak with Gaussian distribution, the peak width is W = 4 σ (where σ is the standard deviation) and the

peak FWHM is W0.5h = 2.354σ. Substituting these relationships into equation (1) gives results in equation (2). [1,27-30]

Resolution and Peak separation

The resolution is represented as a numeric value, such as 0.8, 1.0, or 3.0. But what is the relationship between the number representing the resolution and the actual peak separation? At a resolution of 1.0, if the two peaks are assumed to have a Gaussian distribution and have the same peak height and peak width, then the difference in retention time from equation (1) becomes 1.0W, or 1.0 × 4σ = 4 σ. In the case of a Gaussian distribution, 4 σ encompasses 95.4 %, such that the peaks overlap by 2.3 % ((100 % - 95.4 %)/2). This indicates that 2.3 % of the peak intrudes into the other peak from a perpendicular line drawn in the trough. Similarly, a resolution of 1.5 indicates a difference in retention time of 1.5× 4σ = 6σ, which corresponds to an overlap of 0.15 % ((100 % - 99.7 %)/2). See Fig. 14.

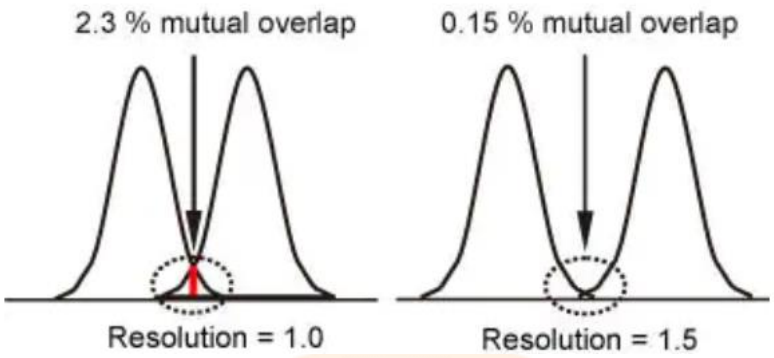


Figure 14. Resolution and Peak separation

Resolution and Separation Factor, Retention Factor, Number of Theoretical Plates

In addition to resolution, the separation factor (α) is also used as an indicator of the separation of two peaks. The separation factor is defined as the ratio of the retention factors (k), as shown in equation (3).

$$\alpha = \frac{k_2}{k_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0} \quad \cdots (3)$$

(t0: Retention time of non-retained component)

Equation 3.

The resolution can be expressed in terms of the number of theoretical plates, separation factor, and retention factor, as shown in equation (4).

$$R = \frac{1}{4}\sqrt{N}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k}{1+k}\right) \quad \cdots (4)$$

Equation 4.

Relational Expression between the Resolution, Number of Theoretical Plates, Separation Factor, and Retention Factor

Equation (1) shows the relationship between the resolution (R), number of theoretical plates (N), separation factor (α), and retention factor (k). (This assumes that the two peak widths are equal and k is the retention factor of the rear peak. [1,31-34]

$$R = \frac{1}{4}\sqrt{N}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k}{1+k}\right) \quad \cdots (1)$$

Equation 5.

When improving the resolution, what contribution do the number of theoretical plates, separation factor, and retention factor make? The explanations below are based on equation (5).

Number of Theoretical Plates

As the resolution is proportional to the square root of the number of theoretical plates in equation (4), doubling the number of theoretical plates increases the resolution by a factor of $\sqrt{2}$ = 1.41; increasing the number of theoretical plates four times increases the resolution by a factor of $\sqrt{4}$ = 2.

Assume that the column used has 10,000 theoretical plates and the resolution between two peaks is 0.8. If the column is replaced to achieve 1.5 resolution (complete separation), what number of theoretical plates is required?

In this case, the resolution must be improved by a factor of 1.5/0.8 = 1.9. Assuming that the separation factor and retention factor remain unchanged, it can be seen that the number of theoretical plates must be increased by a factor of 1.92=3.6, that is, to 36,000. (Fig. 15).

If the same column packing is used, the number of theoretical plates can be increased by extending the column length. In this example, the column length must be increased 3.6 times. [1,35-38]

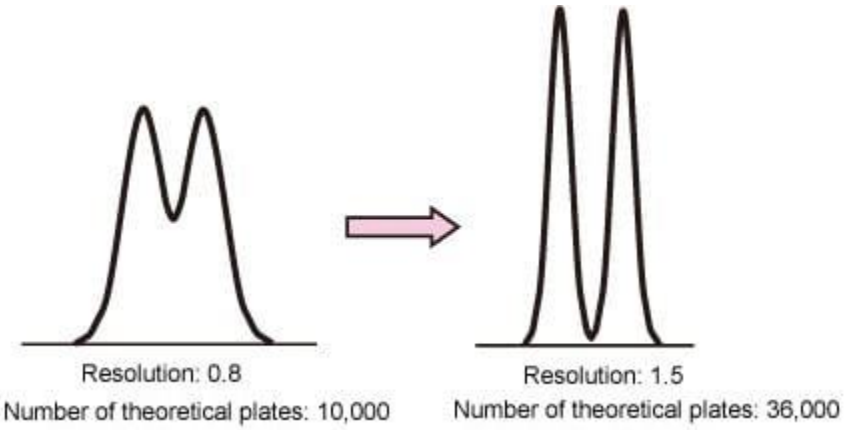


Figure 15. Number of Theoretical Plates and Separation

However, new UHPLC (ultra high performance liquid chromatography) columns with 2 µm packing particle size now offer a higher number of theoretical plates without increasing the column length. Such columns require an HPLC instrument with a high withstand pressure to handle the increased column pressures.

Separation Factor

The diagram at the left of Fig. 2 shows the effect of the separation factor on the resolution from equation (5), that is, the relationship between α and (α-1)/α. As the separation factor is the ratio of two peak retention factors, it varies due to various conditions, such as the packing stationary phase, mobile phase pH (for analysis of ionic species), type of mobile phase organic solvent, and column temperature. It is apparent from Fig. 15 that, up to about 1.2, the separation factor has an approximately linear relationship to the increase in resolution. If the separation factor was 1.1 at 0.8 resolution in the example introduced above, then the resolution can be increased to 1.5 by setting the separation factor to about 1.2. This is an effective means of improving the resolution. However, it takes some time to optimize the conditions. [1,39-40]

Retention Factor

Like the separation factor above, the relationship between k and k/(1+k) is shown in the diagram at the right of Fig. 16. The retention factor increases when a mobile phase with lower elution strength is used. Therefore, the proportion of organic solvent can be reduced in reverse-phase chromatography, for example. However, the diagram at the right of Fig. 16 shows that the retention factor contributes to greater resolution only for peaks that elute quickly. Consequently, increasing the retention factor from 3 to 9, for example, only improves the resolution by a factor of 1.2 but results in longer analysis times.

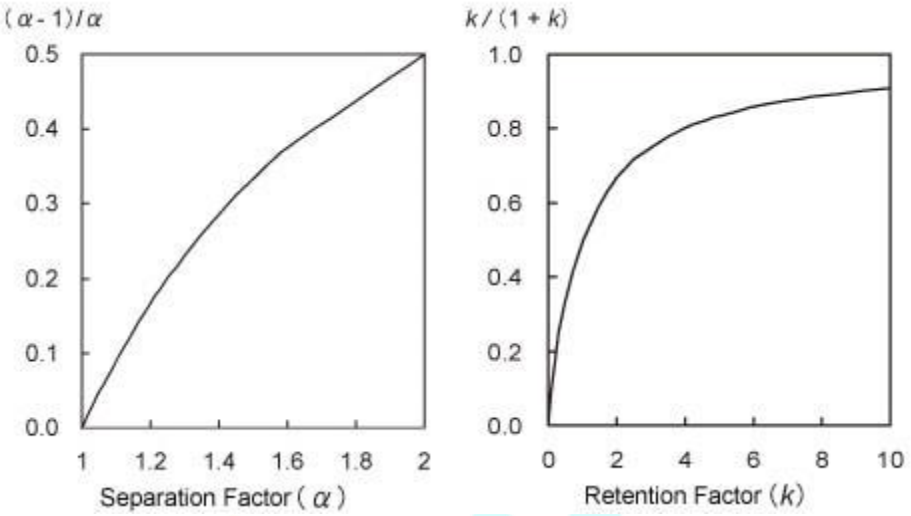


Figure 16. Relationship of Separation Factor and Retention Factor to Separation

The example above shows that equation (5) gives an understanding of the fundamental concepts required to improve resolution. [41-43]

HPLC Method Validation Parameters [44-47]

Quantitative test for formulation or drug product. A typical Validation includes:

1. **Specificity** – Assessment of analyte based on its retention time (polarity). These are usually impurities, matrix and degradant etc. It is to be analyzed that there in interference in the analyte peak due to diluent or impurities. The analyte peak should have a purity of 1. Specificity is performed to confirm the identity of Analyte peak, to ensure accurate content of impurities and to provide exact assay of the analyte to determine the potency or strength.
2. **Accuracy** – Assessment of the method that produces value which is in closeness to true value or spiked value. It is also referred as trueness. This validation parameter determines the recovery of analyte when spiked in a placebo or as such, upon running the HPLC method produces results that are close to the spiked value. To determine the accuracy of method, a range of ± 20% of spiked concentration is covered to ensure that the method is accurate within the range. As per ICH Guidelines, accuracy should be performed for atleast 3 replicates for each range covered. Total of nine data point for 3 percentage range is covered upon which relative standard deviation is calculated which needs to be under 2%.

3. **Precision** – Precision is defined as under same condition of analysis, the closeness of measurement obtained from multiple replicates of same samples or different producing relative standards deviation within 2%. Precision could be Method precision, Intermediate precision, and instrument precision.

Method Precision – This is used to check if the method used for analysis and samples preparation for HPLC analysis is precise or not. 5-6 different samples preparation is done with same condition and techniques and analyzed on HPLC. Following results of pear area or Retention time or percentage area are calculated. Results obtained are later calculated for relative standard deviation which as per ICH guidelines should be within 2%.

Intermediate Precision – This is performed by another analyst using same model of column but with different ID, different instrument and different day. This data produced gives authenticity and precision of method as two different analyst have performed under different conditions. The Relative standard deviation obtained from Method precision and Intermediate precision should be not more than 3%.

Instrument Precision – This implies the instruments capability to analyze same sample under same condition multiple time. The obtained relative standard deviation should be not more than 2% for 5-6 replicate injection of same vial.

4. **Detection Limit** – The least or lowest amount of analyte in a sample that can be detected using the analytical procedure. The calculated value need not be the exact value as the Limit of Detection is determined from Linearity and Method precision.
5. **Quantitation Limit** – The least or lowest amount of analyte in a sample that can be quantified with accuracy and precision is Limit of Quantification. This parameter is used to calculate assay of low amount of impurities, API and Unknown impurities in samples. This parameter is most important for degradants and ppm level impurities.
6. **Linearity** – It's the ability of the analyte in sample i.e. Impurities, APIs or degradant to be linear when injected at different concentration. The determined area from HPLC is directly proportional to the concentration analyte spiked or injected. The R squared value of the linearity proportion should be more than 0.98 for all linearity.
7. **Range** – The Interval between the upper and lower level of linearity if the range of concentration of analyte in sample which gives precise and accurate results.
8. **Robustness** – It's the precision of method to remain unaffected by small change in method validation parameter giving robust and accurate results.
9. **System Suitability** - This is predetermined quality targeted profile for any method validation process which is determined during Method development by Quality-by-design method. Each parameter including Retention Time, Theoretical plate, Peak Purity, Asymmetry and Resolution is defined with an acceptance criteria. The specified acceptance criteria is set throughout the validation for all parameters.

MODERN ANALYTICAL CHEMISTRY: [23-25, 32-35]

Modern analytical chemistry is dominated by instrumental analysis. There are so many different types of

instruments today that it can seem like a confusing array of acronyms rather than a unified field of study. Most modern analytical chemistry is categorized by different analytical methods.

Analytical methods:

• Spectrophotometry and colorimetry. UV-visible spectroscopy.

• Chromatography and Electrophoresis.

Commonly used methods are,

- High Performance Liquid Chromatography (HPLC).
- High Performance Thin Layer Chromatography (HPTLC).
- Gas chromatography (GC).
- Gas chromatography-Mass spectroscopy (GC-MS).
- Liquid chromatography-Mass spectroscopy (LC-MS).

SPECTROPHOTOMETRIC METHODS:

Spectrophotometry is generally preferred by industries as the cost of the equipment is less and the maintenance problems are minimal. The method of analysis based on measuring the absorption of a monochromatic light by colourless compounds in the near ultraviolet path of spectrum (200-380nm).[3] The photometric methods of analysis are based on the Bouger- Lambert Beer's Law, which establishes that the absorbance of a solution is directly proportional to the concentration of the analyte .The fundamental principle of operation of spectrophotometer covering UV region consists in that light of definite interval of wavelength passes through a cell with solvent and falls on to photoelectric cell that transforms the radiant energy into electrical energy measured by galvanometer. The important applications are

- Identification of much type of organic, inorganic molecules and ions.
- Quantitative determination of many biological, organic and inorganic species.

- Quantitative determination of mixtures of analytes.
- Monitoring and identification of chromatographic of effluents.
- Determination of equilibrium constants.
- Determination of stoichiometry and chemical reaction.
- Monitoring of environmental and industrial process.
- Monitoring of reaction rates.

2. AIMS & OBJECTIVES

Development and Validation for Simultaneous Estimation of Methylcobalamin, Alpha Lipoic Acid, Pyridoxine and Folic Acid by RP-HPLC in Bulk and Marketed Dosage Form.

To achieve this Aim, following objectives were listed

- Method development as per ICH Guidelines
- Development to be performed on calibrated instrument.
- Preformulation studies on drugs characteristics to be reviewed
- Physio-chemical properties of the drug be studied.
- HPLC instrument understanding and analysis
- To perform method validation as per ICH guidelines.
- Developed method to be studied fo4 system suitability
- All data collected to be analysed for its respective quality targeted profile

2.2. PLAN OF WORK

2.2.1. Selection of drug and formulation.

By Literature and Market survey

2.2.2. Selection of analytical technique

I. High Performance Liquid Chromatography

2.2.3. DEVELOPMENT OF ANALYTICAL METHOD BY HPLC DAD

- Selection of marketed formulation.
- Study of physicochemical properties of standard drugs.
- Literature survey for method development.
- Solubility study for of Methylcobalamin, Alpha Lipoic Acid, Pyridoxine and Folic Acid
- Selection of λ_{max} for drugs.
- Study of beer lamberts law at selected wavelength.
- Development and Validation for Simultaneous Estimation of Methylcobalamin, Alpha Lipoic Acid, Pyridoxine and Folic Acid by RP-HPLC in Bulk and Marketed Dosage Form.

2.2.4. DEVELOPMENT OF ANALYTICAL METHOD BY HPLC

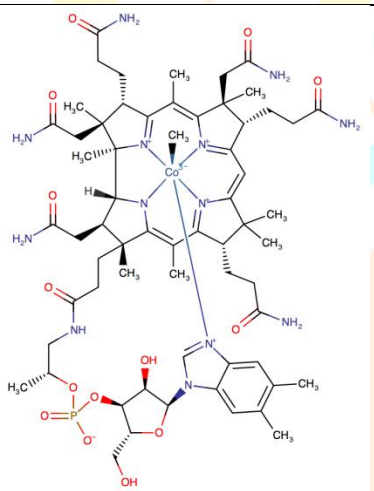
Steps for method development

1. Characterization of sample
2. Analytical wavelength selection
3. Special procedure requirement, sample pretreatment if any
4. Detector selection and setting
5. Chromatographic condition optimization
6. Checking for problem or special procedure requirements

7. Validation of analytical method as per the ICH Guidelines

3. Drug Profile.

Methylcobalamine	
Chemical Name	carbanide;cobalt(2+);[(2*R*,3*S*,4*R*,5*S*)-5-(5,6-dimethylbenzimidazol-

	1-yl)-4-hydroxy-2-(hydroxymethyl)oxolan-3-yl] 1-[3-[(1*R*,2*R*,3*R*,5*Z*,7*S*,10*Z*,12*S*,13*S*,15*Z*,17*S*,18*S*,19*R*)-2,13,18-tris(2-amino-2-oxoethyl)-7,12,17-tris(3-amino-3-oxopropyl)-3,5,8,8,13,15,18,19-octamethyl-2,7,12,17-tetrahydro-1*H*-corrin-24-id-3-yl]propanoylamino]propan-2-yl hydrogen phosphate
Molecular Formula	$C_{63}H_{91}CoN_{13}O_{14}P$
Molecular Mass	1344.3
Cas No.	13422-55-4
Structure	
Boiling Point	> 300 °C
Solubility	Soluble in alcohol; insoluble in acetone, chloroform, ether. In water, 1,25X10+4 mg/l @ 25 °C
Description	**Methylcobalamin (MeCbl), the activated form of vitamin B12, has been used to treat some nutritional diseases and other diseases in clinic, such as Alzheimer's disease and rheumatoid arthritis. As an auxiliary agent, it exerts neuronal protection by promoting regeneration of injured nerves and antagonizing glutamate-induced neurotoxicity.**
Pharmacokinetics	Adsorption: In the duodenum, digestive enzymes free the vitamin B12 from

	haptocorrin, and this freed vitamin B12 combines with intrinsic factor, a transport and delivery binding protein secreted by the stomach's parietal cells. The resulting complex is absorbed in the distal ileum by **receptor-mediated endocytosis**
Mechanism of Action	It works by functioning in the production of a compound called myelin, which covers and protect nerve fibers. Methylcobalamin rejuvenates the damaged neuron. Without enough methylcobalamin, myelin sheath does not form properly due to which nerve fibers suffers and people experience irreversible nerve damage. An intrinsic factor made in the stomach, must be present in the intestinal tract to allow its proper absorption. People lacking this factor show vitamin B_{12} deficiencies such as pernicious anemia (a slow and insidious process that can end in death. Pernicious anemia in fact means 'leading to death'). Methylcobalamin is used as a cofactor in methionine transferase enzyme, an enzyme which converts aminoacid homocysteine to methionine via folate cycle.

- Brouwer M, Chamulitrat W, Ferruzzi G, Sauls DL, Weinberg JB. Nitric oxide interactions with cobalamins: biochemical and functional consequences. Blood. 1996; 88: 1857-1864.
- Randaccio L, Furlan M, Geremia S, Slouf M, Srnova I, Toffoli D. Similarities and differences between cobalamins and cobaloximes, Accurate structural determination of Methylcobalamin and LiCl and KCL containing cynocobalamins by Synchrotron Radiation. Inorganic chemistry journal. 2000; 39: 3403-3413.
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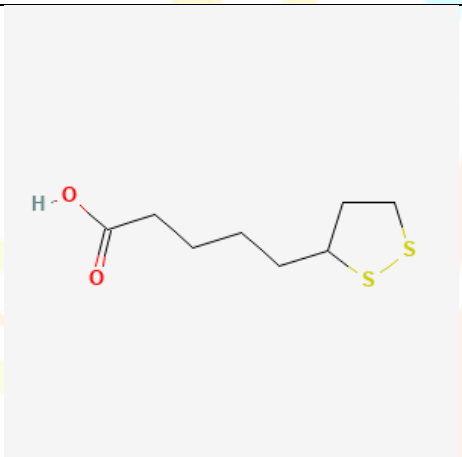
- Ghosh SK, Rawal N, Syed SK, Paik WK, Kim SD. Enzymic methylation of myelin basic protein in myelin. Biochem J.

	1991; 275: 381-387. • Koyama K, Ito A, Yamamoto J, Nishio T, Kajikuri J, Dohi Y, et al. Randomized controlled trail of the effect of short term co-administration of methylcobalamin and folate on serum ADMA concentration in patients receiving long term hemodialysis. American journal of kidney diseases. 2010; 55: 1069-1078.
Toxicity	Absorption of vitamine b12 from the gi tract may be decr by aminoglycoside antibiotics, colchicine, extented-release potassium prepn, aminosalicylic acid & its salts, anticonvulsants (eg, phenytoin, phenobarbital, primadone), cobalt irradiation of the small bowel, & by excessive alcohol intake lasting longer than 2 wk.
Adverse Effects	At a very high dose, methylcobalamin causes blood clots, diarrhea, paresthesia, rhinitis, ataxia, pruritis and allergic reactions. People with polycythemia should consult with a physician before taking this therapy [26-29]. This drug can be applied as a topical paste on the skin without any adverse reaction. Sometimes intravenous injection of this drug leads to hypersensitivity reactions and end up to anaphylactic shock. In some cases, hypokalamia and thrombocytosis has occurred in the patient while treating megaloblastic anemia with methylcobalamin
Uses	Methylcobalamin is used to treat vitamin B12 deficiency. Vitamin B12 is important for the brain and nerves, and for the production of red blood cells.

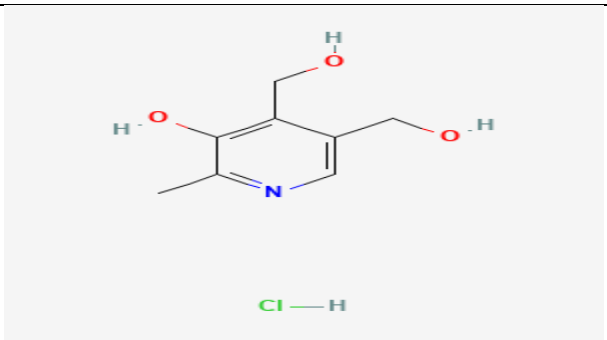
Alpha Lipoic Acid	
Chemical Name	5-(dithiolan-3-yl)pentanoic acid
Synonyms	dl-Thioctic acid

	thioctic acid alpha-Lipoic acid 1077-28-7 5-(1,2-Dithiolan-3-yl)pentanoic acid
Molecular Formula	C8H14O2S2
Molecular Mass	206.3
Cas No.	1077-28-7
Structure	
Melting Point	60.5 °C
Solubility	Yellow powder; solubility in ethanol: 50 mg/mL /Synthetic, oxidized form/ Soluble in methanol, ethanol, diethyl ether and chloroform
Description	Alpha-lipoic acid is an antioxidant also known as Acetate Replacing Factor, ALA, Biletan, Lipoicin, Thioctan. lpha-lipoic acid is a naturally occurring fatty acid that can be found in many foods such as yeast, spinach, broccoli, potatoes, and organ meats such as liver or kidney. Alpha-lipoic acid has been used in alternative medicine as a possibly effective aid in weight loss, treating diabetic nerve pain, healing wounds, lowering blood sugar, improving skin discoloration caused by vitiligo, and decreasing complications of coronary artery bypass

	graft (CABG) surgery. Alpha-lipoic acid has also been used to treat rheumatoid arthritis, Alzheimer's disease, alcoholic liver problems, altitude sickness, heart-related nerve problems, HIV-related brain problems, or eye problems caused by diabetes.
Pharmacokinetics	It is known that ALA is poorly soluble; therefore to increase the solubility was reticulated in an amphiphilic matrix like lecithin. The goal of the present study was to characterize the bioavailability of new formulation and to compare the human pharmacokinetics profiles of two different pharmaceutical form: tablets and soft gel capsules following single oral administration of a ALA 600 mg. Blood samples were collected up to 8 h post dosing, and plasma α-lipoic acid concentrations were determined by Liquid Chromatography Mass Spectrometry (LC/MS/MS) detection. The results revealed that after rapid dissolution there is a good solubilisation by lecithin and that the two formulations show the same human pharmacokinetic profile.
Mechanism of Action	Alpha-lipoic acid can significantly increase the cellular capacity of GSH synthesis by **inducing of nuclear factor erythroid 2-related factor 2 (Nrf-2)-mediated antioxidant gene expression**. ALA activates 5′-AMP-activated protein kinase (AMPK) and inhibits NF-κB, which in turn have a plethora of metabolic consequences
Toxicity	The intoxication is rare because a daily dose of 200–2400 mg/day of ALA is considered to be safe without side effects in adults but there is no reported dose of safety in children. ALA is able to decrease oxidative stress caused by high levels of reactive oxygen and other free radicals. Furthermore, ALA has some activities that provide a cofactor for glucose metabolism in mitochondria. These effects are supposed to be beneficial for cellular damage and hence its usage in diabetic patients, especially for whom neuropathy is common.

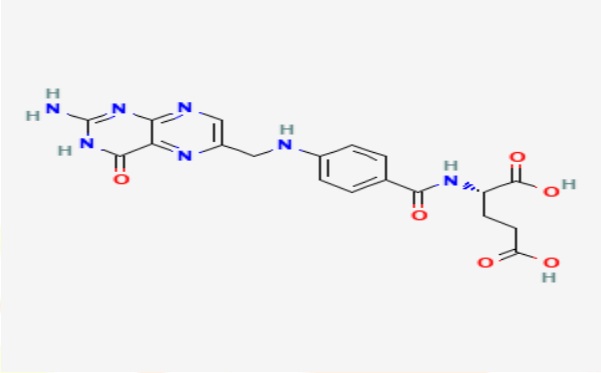
Side Effects	Alpha-lipoic acid is possibly safe for most adults when taken for up to 4 years. It is usually well-tolerated. The most common side effects are headache, heartburn, nausea, and vomiting. Alpha-lipoic acid is possibly safe for most adults when used as a cream for up to 12 weeks. It might cause a rash in some people.
Uses	• Nerve pain in people with diabetes (diabetic neuropathy). Taking 600-1800 mg of alpha-lipoic acid by mouth or by IV seems to improve symptoms such as burning, pain, and numbness in the legs and arms of people with diabetes. Lower doses of alpha-lipoic acid don't seem to work. IV products can only be given by a healthcare provider. • High levels of cholesterol or other fats (lipids) in the blood (hyperlipidemia). Taking alpha-lipoic acid by mouth for up to 4 years seems to lower total cholesterol and low-density lipoprotein (LDL or "bad") cholesterol in people with or without hyperlipidemia. • Obesity. Taking alpha-lipoic acid by mouth can slightly reduce body weight in adults who are overweight.

Pyridoxine Hydrochloride	
Chemical Name	4,5-bis(hydroxymethyl)-2-methylpyridin-3-ol;hydrochloride
Molecular Formula	C8H12ClNO3
Molecular Mass	205.64
Cas No.	58-56-0

Structure	
Boiling Point	Sublimes
Solubility	Dissolves in about 4.5 ml water, 90 ml alcohol; soluble in propylene glycol; sparingly soluble in acetone; insoluble in ether, chloroform
Description	Pyridoxine Hydrochloride is the hydrochloride salt form of pyridoxine, a water-soluble vitamin B. Pyridoxine hydrochloride is converted into the active form, pyridoxal 5'-phosphate (PLP), an essential cofactor in many enzymatic activities including synthesis of amino acids, neurotransmitters, and sphingolipids. This vitamin is essential to red blood cell, nervous system, and immune systems functions and helps maintain normal blood glucose levels.
Pharmacokinetics	Absorption The B vitamins are readily absorbed from the gastrointestinal tract, except in malabsorption syndromes. Pyridoxine is absorbed mainly in the jejunum. The Cmax of pyridoxine is achieved within 5.5 hours. Metabolism Pyridoxine is a prodrug primarily metabolized in the liver. The metabolic scheme for pyridoxine is complex, with formation of primary and secondary metabolites along with interconversion back to pyridoxine. Pyridoxine's major metabolite is 4-pyridoxic acid. **https://go.drugbank.com/drugs/DB00165**
Mechanism of Action	Pyridoxine Hydrochloride/Vitamin B6 is the collective term for a group of three related compounds, pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM), and their phosphorylated derivatives, pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP). Although all six of these

	compounds should technically be referred to as vitamin B6, the term vitamin B6 is commonly used interchangeably with just one of them, pyridoxine. Vitamin B6, principally in its biologically active coenzyme form pyridoxal 5'-phosphate, is involved in a wide range of biochemical reactions, including the metabolism of amino acids and glycogen, the synthesis of nucleic acids, hemoglobin, sphingomyelin and other sphingolipids, and the synthesis of the neurotransmitters serotonin, dopamine, norepinephrine and gamma-aminobutyric acid (GABA) **https://go.drugbank.com/drugs/DB00165**
Toxicity	Oral Rat LD50 = 4 gm/kg. Toxic effects include convulsions, dyspnea, hypermotility, diarrhea, ataxia and muscle weakness. https://go.drugbank.com/drugs/DB00165
Adverse Effects	Pyridoxine usually has no side effects when used in recommended doses. It can cause side effects when taken in large doses for a long time. headache, nausea, drowsiness, numbness/tingling of arms/legs. A very serious allergic reaction to this drug is rare. **https://www.webmd.com/drugs/2/drug-5427/pyridoxine-vitamin-b6-oral/details**
Uses	Pyridoxine (vitamin B6) is used to prevent or treat low levels of vitamin B6 in people who do not get enough of the vitamin from their diets. Most people who eat a normal diet do not need extra vitamin B6. However, some conditions (such as alcoholism, liver disease, overactive thyroid, heart failure) or medications (such as isoniazid, cycloserine, hydralazine, penicillamine) can cause low levels of vitamin B6. Vitamin B6 plays an important role in the body. It is needed to maintain the health of nerves, skin, and red blood cells.Pyridoxine has been used to prevent or treat a certain nerve disorder (peripheral neuropathy) caused by certain medications (such as isoniazid). It has also been used to treat certain hereditary disorders (such as xanthurenic aciduria, hyperoxaluria, homocystinuria). **https://www.webmd.com/drugs/2/drug-5427/pyridoxine-**

	vitamin-b6-oral/details

Folic Acid	
Chemical Name	(2S)-2-[[4-[(2-amino-4-oxo-3H-pteridin-6-yl)methylamino]benzoyl]amino]pentanedioic acid
Molecular Formula	C19H19N7O6
Molecular Mass	441.4
Cas No.	59-30-3
Structure	
Boiling Point	552.4 °C
Solubility	Slightly sol in methanol, less in ethanol and butanol; insol in acetone, chloroform, ether, benzene; relatively sol in acetic acid, phenol, pyridine, and in soln of alkali hydroxides and carbonates. Soluble in hot dil HCl and H2SO4. In water, 1.6 mg/L at 25 °C; soluble up to about 1% in boiling water https://pubchem.ncbi.nlm.nih.gov/compound/135398658#section=Solubility
Description	Folic Acid is a collective term for pteroylglutamic acids and their oligoglutamic acid conjugates. As a natural water-soluble substance, folic acid is involved in carbon transfer reactions of amino acid metabolism, in addition to purine and pyrimidine synthesis, and is essential for hematopoiesis and red blood cell production. **https://pubchem.ncbi.nlm.nih.gov/compound/135398658#section=Solubility**
Pharmacokinetics	Absorption Folic acid is absorbed rapidly from the small intestine, primarily from the proximal portion. Naturally occurring conjugated folates are reduced enzymatically to folic acid in the gastrointestinal tract prior to absorption. Folic acid appears in the plasma approximately 15 to 30 minutes after an oral dose; peak

	levels are generally reached within 1 hour. Route of Elimination After a single oral dose of 100 mcg of folic acid in a limited number of normal adults, only a trace amount of the drug appeared in the urine. **https://pubchem.ncbi.nlm.nih.gov/compound/135398658#section=Absorption-Distribution-and-Excretion**
Mechanism of Action	Folic acid, as it is biochemically inactive, is converted to tetrahydrofolic acid and methyltetrahydrofolate bydihydrofolate reductase (DHFR). These folic acid congeners are transported across cells by receptor-mediated endocytosis where they are needed to maintain normal erythropoiesis, synthesize purine and thymidylate nucleic acids, interconvert amino acids, methylate tRNA, and generate and use formate. Using vitamin B12 as a cofactor, folic acid can normalize high homocysteine levels by remethylation of homocysteine to methionine via methionine synthetase. **https://pubchem.ncbi.nlm.nih.gov/compound/135398658#section=Absorption-Distribution-and-Excretion**
Toxicity	Oral folic acid (pteroylglutamic acid) is generally regarded as not toxic for normal humans but it may cause neurological injury when given to patients with undiagnosed pernicious anemia. The vitamin should be given with caution to drug-treated epileptic patients because seizure control may be affected
Adverse Effects	It is likely safe for most people to take folic acid in doses of no more than 1 mg daily. Doses higher than 1 mg daily may be unsafe. These doses might cause stomach upset, nausea, diarrhea, irritability, confusion, behavior changes, skin reactions, seizures, and other side effects. **https://www.webmd.com/vitamins/ai/ingredientmono-1017/folic-acid**
Uses	Taking folic acid improves folate deficiency. High levels of homocysteine in the blood (hyperhomocysteinemia). This condition has been linked to heart disease and stroke. Taking folic acid by mouth lowers homocysteine levels in people with normal or high homocysteine levels and in people with kidney failure. Toxicity caused by the drug methotrexate. Taking folic acid by mouth seems to reduce nausea and vomiting from methotrexate treatment. Birth defects of the brain and spine (neural tube birth defects). Consuming folic

	acid 600-800 mcg by mouth daily during pregnancy reduces the risk of these birth defects. Folic acid can come from the diet or supplements. Some people who are at high risk should get 4000-5000 mcg daily. **https://www.webmd.com/vitamins/ai/ingredientmono-1017/folic-acid**

5. Review of Literature

5.1. Padmaja V. et. al. developed a RP-HPLC Method for determination Methylcobalamin, Alpha-Lipoic Acid, Pyridoxine Hydrochloride, and Folic acid drug product using Inertsil C18, 250 mm length x 4.6 mm diameter, 5 micron particle size at ambient temperature. The mobile phase used was buffer (5.05 g hexane-1-sulfonic acid is 1000 mL of distilled water) : acetonitrile in the ratio of 10:90% v/v respectively. The flow rate was set at 1 mL/min and UV at 285 nm. retention time for methylcobalamin, alpha-lipoic acid, pyridoxine hydrochloride, and folic acid was found to be 3.5, 6.7, 8.5, and 9.3, respectively.The assay methods were found to be linear from 0 to 2130 ug/mL for methylcobalamin, 0 to 142.5 ug/mL for alpha-lipoic acid, 0 to 4.54 ug/mL for pyridoxine hydrochloride, and 0 to 2 ug/mL for folic acid. The correlation coefficient was 0.999 for all drugs, respectively. The mean percentage values for the developed method were found to be within the range of 98% to 100.6%. The Theoritical plates reported as follow, 43409 - Methylcobalamine, 19674 for Alpha-Lipoic Acid, 117890 - Pyridoxine HCl and 2951 - Folic Acid which are in the range of as per ICH guidance. The Method Precison %RSD ranged between 0.156 to 0.534. Prepared solution for analysis were found to be stable for 24 hours for all 4 drugs. [48]

5.2. S. Poongothai et. al. developed a RP-HPLC Method for Benfotiamine (B1) 100 mg, Pyridoxine hydrochloride (B6) 100 mg, Mecobalamin (B12) 1000 mcg and Alpha–lipoic acid 100 mg in multivitamin capsules using X-Terra reverse phase (RP 18, 250 x 4.6 mm, 5 µm) column and mobile phase contained 0.05 M phosphate buffer adjusted to pH 2.5 and acetonitrile in gradient manner as below:

Time Min	Solvent A%	Solvent B %	Gradient Type
0	100	0	Linear
5	100	0	
20	30	70	
25	100	0	
Post Run time 5 mins		total Run time: 25mins	

Wavelength was set at 320 nm, column temperature set at 20^0C and Injection volume 20 ul and flow rate at 1.5 ml /ml. Results obtained were as below:

Parameter	Benfotiamine (B1)	Pyridoxine hydrochloride (B6)	Mecobalamin (B12)	Alpha–lipoic acid
Retention Time	10.79	2.27	12.19	17.04
Tailing Factor	0.5	1.1	1.2	1.1
Theoretical Plate	35270	9998	105347	221827
Resolution	47.8	105347	6.7	32.3
System precision %RSD	0.6%	1.2%	0.5%	0.2%
Linearity Range	489.9 - 1469.6	499.4 - 1498.1	4.6 - 13.9	497.7 - 1469.6
R^2	0.9997	0.9990	0.9995	0.9998
80% Accuracy %RSD	0.5%	0.6%	1.2%	1.2%
100% Accuracy %RSD	0.3%	1.4%	1.2%	0.5%
80% Accuracy %RSD	0.6%	0.6%	1.7%	0.3%
Capsule Assay %	99.1%	98.0%	136.1%	103.9%

The Method reported was found to be in accordance with ICH Guidelines. [49]

5.3. Nandini R. Pai et. al.developed a HPLC Method for determination of Methylcobalamin 1500 mcg and Alphalipoic acid 300 mg in soft gelatine capsule formulation. Two separate chromatographic conditions were used for estimation of Methylcobalamin[1] & Alphalipoic acid [2] using Phenomenox Luna (C-18, 250 x 4.6 mm, 5 µm) column and gradient elution for both the methods. Mobile phase used was 0.02 M phosphate buffer adjusted to pH 3.5 with hexane-1-sulphonic acid, sodium salt as ion pairing reagent and acetonitrile for both the methods. Wavelength was at 240 nm for alphalipoic acid and at 266 nm for Methylcobalamin.

Gradient Programs as below:

Methylcobalamine:

Time	Buffer (Solvent A)	Acetonitrile (Solvent B)	Gradient
0.00	80	20	Linear
13.00	80	20	
13.01	50	50	
23.00	50	50	
23.01	80	20	
32.00	STOP		

Alpha Lipoic Acid

Time	Buffer (Solvent A)	Acetonitrile (Solvent B)	Gradient
0.00	60	40	Linear
10.00	60	40	
10.01	35	65	
16.00	35	65	
16.01	60	40	
28.00	STOP		

Column Oven temperature was at 40^0C and flow rate at 1.0ml per min. The Injection Volume was at 50 ul for Methylcobalamine and 10 ul for Alphalipoic Acid.

Results reported are as follows:

	Methylcobalamin	Alphalipoic acid
Retention Time in minute	8.355	6.545
Tailing Factor (NMT 2.0)	0.973	1.675
Theoretical plates (More than 2000)	7789.55	9755.88
Peak Purity	1.0000	1.0000
Concentration range	15 - 40 µg/ml	400 - 600 µg/ml
Correlation coefficient	0.99995	0.99941
Slope	8674.92	4072.12
Y – Intercept	-2498.98	10136.83
R-square	0.99990	0.99882
Recovery %RSD	0.31%	1.06%
Precision	0.76	0.46

The method was reported to be robust with change in Column oven temperature and moble phase concentration. [50]

5.4. B. Suhagia et al. used UV-Visible Spectrophotometry and liquid chromatography to create simultaneous estimation of epalrestat and MeCbl in bulk and tablet formulation. The stationary phase was hyper chrome ODS (250mm4.6mm, 5 particle size), while the mobile phase was methanol: water (80:20) percent v/v. The flow rate was 0.8 mL/min, and the detecting wavelength was 210 nm. The retention time of epalrestat and MeCbl, as well as the impurity of MeCbl, were all less than 10 minutes. The percentage assays were 99.38 and 97.77, respectively. The % recovery of epalrestat and MeCbl was determined to be 99.19-103.4 and 98.8-101.2, respectively. [51]

5.5. K. Chaudhari and D. Maheshwari published a technique for estimating epalrestat and MeCbl in their combination dose form using a simple, precise RP-HPLC approach. The epalrestat and MeCbl concentrations were determined using a Phenomenex - C18 (4.6 mm250 mm, 5 m) column using a gradient reverse phase method. epalrestat and MeCbl had retention times of 5.432 0.0085 min and 2.145 0.0027 min, respectively. epalrestat and

MeCbl have linearity ranges of 1 to 5 g/ml and 5 to 25 g/ml, respectively. ICH Guidelines Q2 were used to validate the approach (R1). The percentage recovery was determined to be greater than 98.0. For MeCbl, the percent RSD for intra-day and inter-day accuracy was determined to be 1.05 percent. [52]

5.6. S. Singh and colleagues devised and validated a technique for quantifying citicoline sodium and MeCbl in bulk medication and pharmaceutical dose form. The mobile phase was acetonitrile: 0.02M KH2 PO4 (60:40) percent v/v, and the column was Phenomenex Luna, C18 (250 mm4.6 mm, 5 m particle size). The flow rate was 1 mL/min, and effluents were measured at a wavelength of 254 nm. The peak for citicoline sodium was 3.8 minutes and for MeCbl was 2.3 minutes on the chromatogram. The % recovery of citicoline sodium and MeCbl, respectively, was determined to be 99.76 - 101.79 and 110.92 - 111.79. For the estimation of citicoline sodium and MeCbl, the LOD and LOQ were determined to be 0.003 g/ml, 0.001 g/ml, and 0.01 g/ml, 0.05 g/ml, respectively. [53]

5.7. P. Palnisamy et al. published an RP-HPLC technique for determining diclofenac sodium, vitamin B1, vitamin B6, and vitamin B12 in a soft gelatin capsule formulation. This approach was effectively utilised to estimate diclofenac sodium, vitamin B1, vitamin B6, and vitamin B12 in soft gelatin capsule dose form in regular lab analysis. The separation was performed on a Phenomenex ODS C18 column (150 mm4.6 mm; 5 m particle size) using a mobile phase of 400 mL potassium hydrogen phosphate pH 3.0 and 600 mL acetonitrile. The detection wavelength was 254 nm and the flow rate was 1.0 ml/min. Diclofenac sodium had a retention time of 5.4 minutes. For diclofenac sodium, the percentage recovery was determined to be 100.00 - 101.86. For diclofenac Sodium, the linearity range was determined to be 20–400 g/ml, with a correlation value (r2) of 1. The separation of vitamin B1 and vitamin B6 was done on an Inertsil ODS C18 column (250 4.6 mm; 5 m) using a mobile phase of 1000 mL sodium 1-hexane sulfonic acid solution, 730 mL water, 270 mL glacial acetic acid, and 10 mL methanol. The detection wavelength was 280 nm and the flow rate was 1.5 ml/min. Vitamins B1 and B6 had retention times of 3.70 and 7.74 minutes, respectively. For vitamin B1 and vitamin B6, the percentage recovery was between 99.46 and 101.03 and 99.35 and 101.78, respectively. The linearity range for vitamin B1 was determined to be 10 - 200 g/ml with a correlation value of 1, whereas the linearity range for vitamin B6 was 10.5 - 210 g/ml. The separation of vitamin B12 was done in a Phenomenex ODS C18 column (150x4.6 mm; 5 m) using a mobile phase of 25 mL trifluroacetic acid, 870 mL water, and 130 mL acetonitrile. The detection wavelength was 361 nm and the flow rate was 1.2 ml/min. Vitamin B12 has a 6.75 minute retention time. For Vitamin B12, the percentage recovery was between 98.86 percent and 101.94 percent. For Vitamin B12, the linearity range was determined to be 1-20 g/ml (r2=0.9999). For vitamin B12, the percent recovery ranged between 98.86 and 101.94. [54]

5.8. R. Pai and S. Sawant developed and validated an RPHPLC technique for determining MeCbl and alphalipoic acid in soft gelatine capsule formulations. MeCbl and alphalipoic acid were measured using two different chromatographic settings. The stationary phase of the Phenomenex Luna C18 column (250 mm4.6 mm, 5 m

particle size). For all procedures, the aqueous mobile phase was 0.02 M phosphate buffer pH 3.5 adjusted with hexane-1-sulphonic acid, sodium salt as an ion pairing reagent, and acetonitrile. Following sample preparation, the sample was immediately placed in an amber vial and stored at a constant temperature of 20°C. The temperature in the column oven was set at 40°C. MeCbl flowed at 1.0 ml/min, while alphalipoic acid flowed at 1.2 ml/min. Photodiode array detectors with wavelengths of 266 nm for MeCbl and 240 nm for alphalipoic acid were used for detection. RSD of 0.78 percent for MeCbl and 0.53 percent for alphalipoic acid demonstrate the accuracy. The correlation coefficients (r2) for MeCbl and alphalipoic acid were reported to be 0.99995 and 0.99941, respectively. The average % recovery was 101.43 and 99.43, respectively. MeCbl had an 8.3 minute retention duration while alphalipoic acid had a 6.5 minute retention time. [55]

5.9. Using the RP-HPLC approach, K. Bhatt et al. established simultaneous quantification of pregabalin and MeCbl in formulation. The chromatographic column was a Phenomenex C18 (250mm4.6mm, 3 m particle size) equilibrated with mobile phase water: methanol (60:40) percent v/v adjusted to pH 6.5 with triethylamine (1 percent v/v). The flow rate was kept constant at 1 mL/min, and the effluents were measured at 218 nm. Pregabalin and MeCbl had calibration curves of 50–300 g/ml and 0.5–2.0 g/ml, respectively. Pregabalin had a quantification limit of 24.10 g/ml, whereas MeCbl had a limit of 0.40 g/ml. Pregabalin and MeCbl had retention times of 6.4 and 7.9 minutes, respectively. For pregabalin and MeCbl estimates, the LOD and LOQ were determined to be 8.10 g/ml, 24.50 g/ml, and 0.12 g/ml, 0.40 g/ml, respectively. Pregabalin and MeCbl were found to have percent RSD values of 0.56 and 0.86, respectively. The advantages include a fast run time and the ability to analyse a large number of samples, both of which cut analysis time per sample greatly. [56]

5.10. P. Narmada et al. published a validated RP-HPLC technique for measuring MeCbl and pregabalin in a combination capsule dose form. The stationary phase was Inertsil ODS 3 C-18 (250mm4.6mm, particle size 3 m). The mobile phase was 0.01M potassium dihydrogen and dipotassium hydrogen phosphate with 60:40 v/v methanol as the solvent. The flow rate was 0.6 ml/min, with a 210 nm detecting wavelength. For pregabalin and MeCbl estimates, the LOD and LOQ were determined to be 75 - 1125 g/ml and 0.75 - 11.25 g/ml, respectively. According to ICH criteria, the approach was validated. [57]

6. MATERIALS AND METHODS

6.1 Materials

5.1.1 Drug
5.1.2 Chemicals
5.1.3 Instruments

6.2 Methods

5.2.1 Preliminary Analysis of Alpha Lipoic acid, Mecobalamine, Folic acid and Pyridoxine
5.2.2 HPLC Method Development for Alpha Lipoic acid, Mecobalamine, Folic acid and Pyridoxine
5.2.3 Validation of RP-HPLC Method for Alpha Lipoic acid, Mecobalamine, Folic acid and Pyridoxine

6.1 Materials

6.1.1 Drug

Table 6.1: List of Drugs

1.	**Alpha Lipoic acid**	Aadhaar Life Sciences Pvt. Ltd., Solapur, India.
2.	**Mecobalamine**	Aadhaar Life Sciences Pvt. Ltd., Solapur, India.
3.	**Folic acid**	Aadhaar Life Sciences Pvt. Ltd., Solapur, India.
4.	**Pyridoxine**	Aadhaar Life Sciences Pvt. Ltd., Solapur, India.

6.1.2 Chemicals

Table 6.2: List of Chemical

1.	Methanol	Merck Specialities Pvt. Ltd., Mumbai
2.	Water LiChrosolv®	Merck Specialities Pvt. Ltd., Mumbai
3.	O-phosphoric acid®	Merck Specialities Pvt. Ltd., Mumbai

6.1.3 Instruments

Table 6.3: List of Instruments

1.	HPLC	Agilent 1260 Infinity II
	Software	Open Lab ezchrom version 4.8
	Channel vacuum Degasser & Mixer	Infinity Standard Degasser G1322A
	Gradient Pump	Quaternary Pump G1311B
	Injector	Auto injector G7129A
	UV-VIS Detector	Diode Array Detector- G4212B (DAD)

2.	Column	Agilent Zorbax Bonus RP 250 mm x 4.6 mm, 5um
3.	Analytical Balance	Aczet CY224C
4.	Vortex machine	Remi CM 101 plus
5.	Nylon 6,6 membrane 0. 45µm 47mm Filters	Pall Pvt. Ltd
6.	All Glass Filter Holder- 47mm (1L flask, 300ml funnel)	Borosil Glass works Ltd., Mumbai
7.	Melting Point Apparatus	Veego
8.	RC membrane 0.45µm 15mm Syringe Filters	Qualisil
9.	Ultra Sonicator/ water bath	Labman

6.2 Methods

6.2.1 Preliminary Analysis of Drug:

A. Alpha Lipoic acid

a) Description: The sample ofAlpha Lipoic acid was observed for its color and texture.

b) Solubility: The sample ofAlpha Lipoic acid was taken in test tubes and observed for solubility in water, acetonitrile, and methanol.

c) Melting Point: The sample ofAlpha Lipoic acid was taken in capillary tube and kept in melting point apparatus.

B. Mecobalamine

a) Description: The sample of**Mecobalamine** was observed for its color and texture.

b) Solubility: The sample of**Mecobalamine** was taken in test tubes and observed for solubility in water, acetonitrile, and methanol.

c) Melting Point: The sample of**Mecobalamine** was taken in capillary tube and kept in melting point apparatus.

C. Folic acid

a) Description: The sample of**Folic acid** was observed for its color and texture.

b) Solubility: The sample of**Folic acid** was taken in test tubes and observed for solubility in water, acetonitrile, and methanol.

c) Melting Point: The sample of**Folic acid** was taken in capillary tube and kept in melting point apparatus.

D. Pyridoxine

a) Description: The sample of**Pyridoxine** was observed for its color and texture.

b) Solubility: The sample of**Pyridoxine**was taken in test tubes and observed for solubility in water, acetonitrile, and methanol.

c) Melting Point: The sample of**Pyridoxine**was taken in capillary tube and kept in melting point apparatus.

6.2.2 High Performance Liquid Chromatographic Method

I. Selection of Mobile Phase and its Strength:

Methanol and 0.1% O-phosphoric acid were selected as mobile phase. Different Trials with Methanol and 0.1% Acetic were performed and changes in the elutions were recorded. Trials were performed by using different ratios of Methanol and 0.1 % O-phosphoric acid solutoion like 50:50, 40:60, 70:30 and 60:40 respectively.

II. Diluent:

Methanol and 0.1% O-phosphoric acidin the ratio of 50:50 % v/v was used as diluent.

III. Preparation of Standard Stock Solution

A. Alpha Lipoic acid Standard Stock Solution (SSS-I)

10 mg of standard Alpha Lipoic acid was weighed and transferred to a 10ml volumetric flask, containing 5 ml of Diluent, dissolve and the volume was made up to the mark with diluent to obtain Concentration of 1000 µg/ml of Alpha Lipoic acid and labeled as 'Std Stock Alpha Lipoic acid'(SSS-I).

1.0 ml of SSS-I was pipetted out in 10 ml volumetric flask. 5 ml diluent was added and vortexed; volume was marked up with diluent. (Conc. = 100 µg/ml) (Working Standard Solution (WSS) -1)

B. Mecobalamine Standard Stock Solution(SSS-II)

10 mg of standard Mecobalaminewas weighed and transferred to a 10ml volumetric flask, containing 5 ml of Diluent, dissolve and the volume was made up to the mark with diluent to obtain Concentration of 1000 µg/ml of Mecobalamine. Further pipette out 1.5 ml of above solution in 10 ml volumetric flask and mix with 5 ml of diluent and make up the volume with diluent to obtain concentration of 150µg/ml of Mecobalamineand labeled as 'Std Stock Mecobalamine'(SSS-II).

1.0 ml of SSS-II was pipetted out in 10 ml volumetric flask. 5 ml diluent was added and vortexed; volume was marked up with diluent. (Conc. = 15 µg/ml)(Working Standard Solution (WSS)-II).

C. Folic acid Standard Stock Solution (SSS-III)

10 mg of standard Folic acid was weighed and transferred to a 10ml volumetric flask, containing 5 ml of Diluent, dissolve and the volume was made up to the mark with diluent to obtain Concentration of 1000 µg/ml of Folic acid. Further pipette out 1.5 ml of above solution in 10 ml volumetric flask and mix with 5 ml of diluent and make up the volume with diluent to obtain concentration of 150 µg/ml of Folic acid and labeled as 'Std Stock Folic acid (SSS-III).

1.0 ml of SSS-III was pipetted out in 10 ml volumetric flask. 5 ml diluent was added and vortexed; volume was marked up with diluent. (Conc. = 15 µg/ml)(Working Standard Solution (WSS)-III).

D. Pyridoxine Standard Stock Solution (SSS-IV)

10 mg of standard Pyridoxinewas weighed and transferred to a 10ml volumetric flask, containing 5 ml of Diluent, dissolve and the volume was made up to the mark with diluent to obtain Concentration of 1000 µg/ml of

Pyridoxine. Further pipette out 3.0 ml of above solution in 10 ml volumetric flask and mix with 5 ml of diluent and make up the volume with diluent to obtain concentration of 300 µg/ml of Folic acid and labeled as 'Std Stock Pyridoxine' (SSS-IV).

1.0 ml of SSS-IV was pipetted out in 10 ml volumetric flask. 5 ml diluent was added and vortexed; volume was marked up with diluent. (Conc. = 30 µg/ml)(Working Standard Solution (WSS)-IV).

IV. Selection of Analytical Wavelength

To investigate the appropriate wavelength for determination of Alpha Lipoic acid, Mecobalamine,Folic acidand Pyridoxinesolution in the mobile phase were scanned HPLC- DAD in the range of 200-400nm.

V. Selection of column (stationary phase)

To get well resolved, symmetric peak with highest number of theoretical plates the solution of the Alpha Lipoic acid, Mecobalamine,Folic acidand Pyridoxinewere analyzed using Zorbax Bonus RP column as a stationary phase.

VI. Chromatographic Conditions

- ✓ **Analytical Column:** : Zorbax Bonus RP (250 x 4.6 mm, 5µ)
- ✓ **Mobile Phase:** 0.1% o-phosphoric acid: Methanol (50:50)
- ✓ **Flow Rate:** 0.8 ml/min
- ✓ **Injection Volume:** 10 µl
- ✓ **Detection Wavelength:** 270 nm

VII. Preparation of Mobile Phase

Mobile phase was prepared by mixing 500 ml of 0.1 % O-phosphoric acid and 500 ml of Methanol and filtered through 0.45µm nylon filter using vacuum pump and ultra sonicate for 30min for degassing.

VIII. Preparation of 0.1 % O-phosphoric Acid

1 ml of O-phosphoric Acid was added to 500 ml of Type I water in a 1000 ml beaker and mixed. Volume was made upto the mark using Type I water.

6.2.3 Validation of RP-HPLC Method

I. Specificity

The chromatogram of blank, standard, test sample were compared to justify the specificity of the target analyte.

II. Linearity

Linearity of Alpha Lipoic acid, Mecobalamine, Folic acid and Pyridoxine was performed by preparing a series of dilution from stock solutions (SSS-I,SSS-II, SSS-III and SSS-IV).

A ml, B ml, C mland D ml of SSS-I, SSS-II, SSS-III and SSS-IV was diluted to 10 ml respectively to prepare solutions of 80%, 90%, 100%, 110% and 120% Concentrations in a 10ml Volumetric Flask.

Table 5.4: Concentration of Linearity

% Level	A ml of SSS-II	B ml of SSS-II	C ml of SSS-III	D ml of SSS-IV	Diluted to	Final concentration of Alpha Lipoicacid in ug/ml	Final concentration of Mecobalamine in ug/ml	Final concentration of Folic acid in ug/ml	Final concentration of Pyridoxine in ug/ml
0.80	0.8	0.8	0.8	0.8	10 ml	80	12	12	24
90	0.9	0.9	0.9	0.9	10 ml	90	13.5	13.5	27
100	1.0	1.0	1.0	1.0	10 ml	100	15	15	30
110	1.1	1.1	1.1	1.1	10 ml	110	16.5	16.5	33
120	1.2	1.2	1.2	1.2	10 ml	120	18	18	36

III. Range

The range of analytical method was decided from the interval between upper and lower level of calibration curves by plotting the curve.

IV. Accuracy

80%, 100% and 120% solutions were prepared by adding A ml,B ml, C ml and D ml of SSS-I, SSS-II, SSS-IIIand SSS-IVfrom stock solution to Four different 10 ml volumetric flasks and volume adjusted up to 10 ml with Diluent. All the solutions were filtered through 0.45 um Millipore syringe filter and injected into the HPLC system and chromatograms were recorded under the same chromatographic conditions after getting a stable baseline. Peak area was recorded for all the peaks. From above data percentage recoveries were calculated. The sample solutions were injected in two reps in order to calculate % Relative Standard Deviation.

Table 5.5: Concentration of Accuracy

% Level	A ml of SSS-I	B ml of SSS-II	C ml of SSS-III	D ml of SSS-IV	Diluted to	Final concentration of Alpha Lipoic acid ug/ml	Final concentration of Mecobalamine ug/ml	Final concentration of Folic acid ug/ml	Final concentration of Pyridoxine ug/ml
80	0.80	0.80	0.80	0.80	10 ml	80	12	12	24
100	1.0	1.0	1.0	1.0	10 ml	100	15	15	30
120	1.20	1.20	1.20	1.20	10 ml	120	18	18	36

V. Precision

The precision of an analytical method was studied by performing Repeatability.

a) **Repeatability:**

Working Standard solution was filtered through syringe filter and 10µl injected into the HPLC system and its chromatogram was recorded under the same chromatographic conditions after getting a stable baseline. Peak area was recorded. The procedure was repeated for five times and the % RSD was calculated.

VI. Limit of Detection

LOD calculated by the following formulae.

$$LOD = \frac{3.3 \times SD}{S}$$

Where, SD- Standard deviation; S- Slope of Curve.

VII. Limit of Quantitation

LOQ calculated by the following formulae.

$$LOQ = \frac{10 \times SD}{S}$$

Where, SD- Standard deviation; S- Slope of Curve.

VIII. System Suitability

Chromatograms were studied for different parameters such as tailing factor, retention time and theoretical plates to see that whether they comply with the recommended limit or not.

IX. Robustness

Change the parameter as below;

Column Oven Temperature 28^{o}C: Run Working Standard and Drug Product

Column Oven Temperature 30^{o}C: Run Working Standard and Drug Product

Column Oven Temperature 32^{o}C: Run Working Standard and Drug Product

Decrease Mobile Phase A to 48%: Run Working Standard and Drug Product

Normal Mobile Phase A to 50 %: Run Working Standard and Drug Product

Increase Mobile Phase A to 52%: Run Working Standard and Drug Product

X. Intraday and Interday Precision.

Run Working standard and Drug product in Morning and Evening on the same day.

Run Working standard and Drug product on day 1 and day 2.

7. Results and Discussion

7.1. HPLC Method Development

Initially based on solubility of each API, the diluent selected was Methanol and 0.1% O-phosphoric Acid buffer. Therefore, working standard solutions were prepared and stored at room temperature.

Table No. 1. Method Development Trial 1

MP	Ratio	Diluent	Column	Flowrate	Wavelength
0.1% OPA-MeOH	50-50	50 0.1% OPA -50 MeOH	PhenomenexKinetex XB-C18 (150 x 4.6 mm, 5µ)	1 ml/min	250

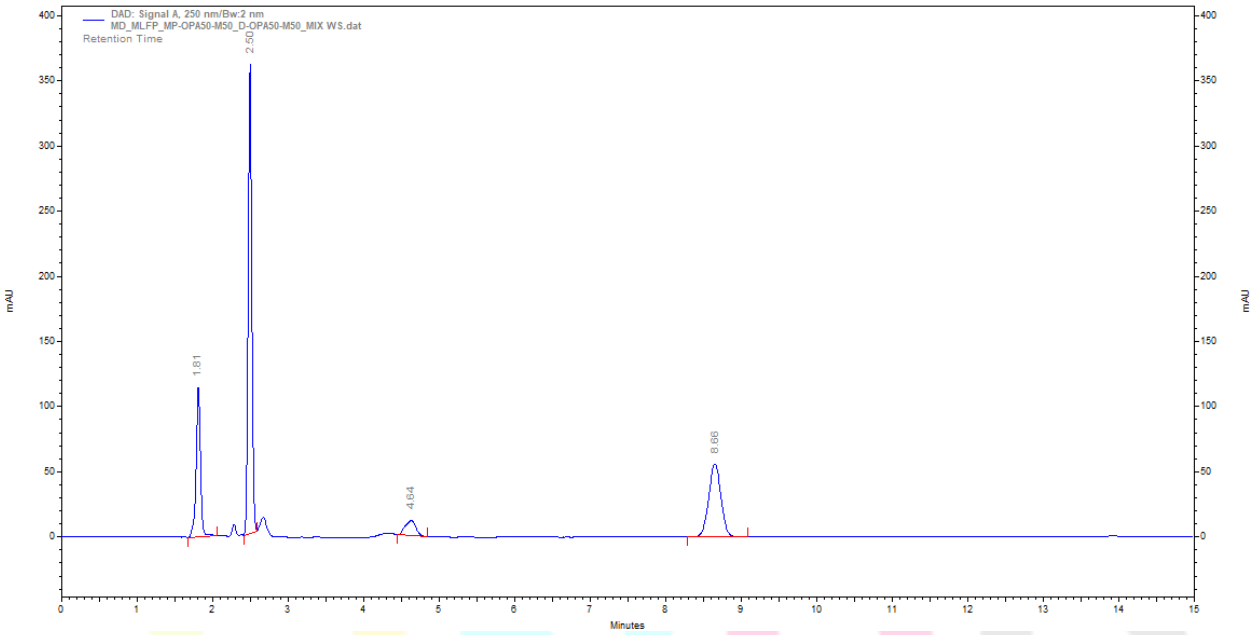


Figure No. Method Development Trial 1

Four peaks were observed and system suitability was calculated and listed as below:

Table No. 2. Results for method Development Trial 1

Alpha lipoic acid				Mecobalamine				Folic acid				Pyridoxine			
RT	Asymmetry	TP	Resolution	RT	Asymmetry	TP	Resolution	RT	Asymmetry	TP	Resolution	RT	Asymmetry	TP	Resolution
1.81	0.92	48865	0.00	2.5	1.06	11639	6.99	4.64	0.92	4596	11.68	8.66	0.99	13324	14.01

Four peaks of Mecobalamine, Alpha Lipoic acid, Folic acid and Pyridoxine were observed. There were multiple diluent peaks besidesMecobalamine. So,the method was changedand reanalyzed.

Table No. Method Development Trial 2

MP	Ratio	Diluent	Column	Flowrate	Wavelength
0.1% OPA-MeOH	70-30	50 0.1% OPA -50 MeOH	Agilent Zorbax Bonus RP (250 x 4.6 mm, 5µ)	1 ml/min	270

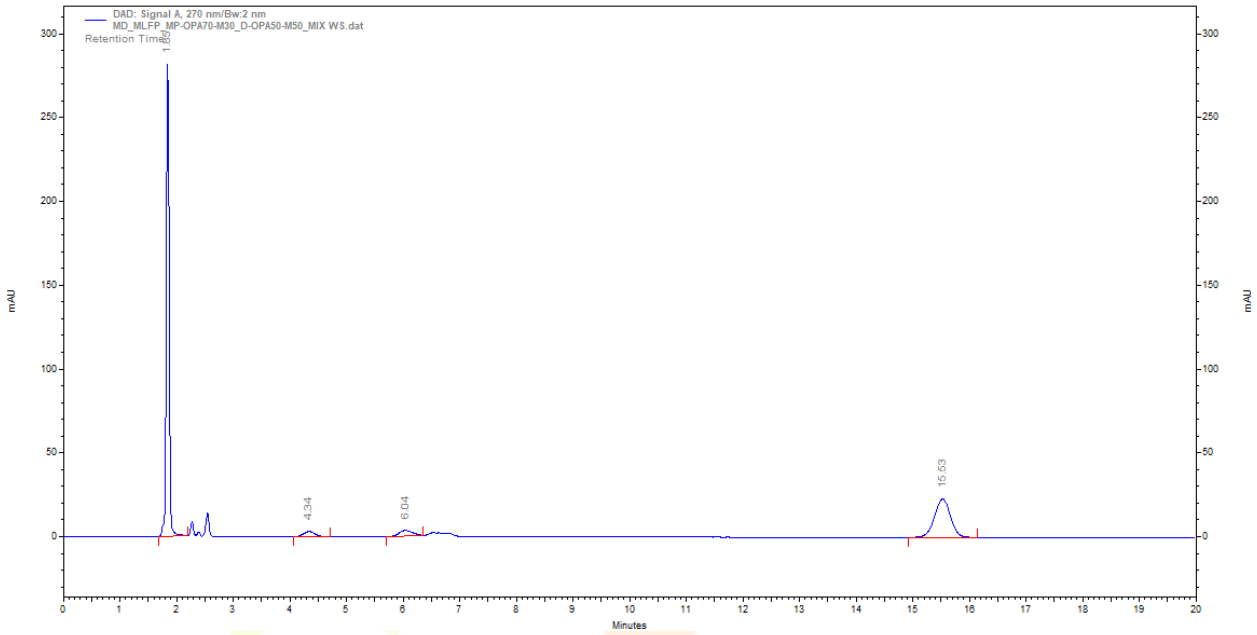


Figure No. Method Development Trial 2

The Mobile phase was changed to 70% o-phosphoric acid and 30% Methanol.The diluent was same. Wavelength for analysis was changed to 270 nm.

Table No. . Results for method Development Trial 2

Alpha lipoic acid				Mecobalamine				Folic acid				Pyridoxine			
RT	Asymmetry	TP	Resolution	RT	Asymmetry	TP	Resolution	RT	Asymmetry	TP	Resolution	RT	Asymmetry	TP	Resolution
1.85	1.00	73204	0.00	4.34	1.09	21371	10.79	6.04	1.08	2732	4.06	15.53	0.99	14347	19.35

The pyridoxine retained for longer time and the run time was 20 min. The theoretical plates were above 2000 for all the peaks and all the peaks were well resolved. The asymmetry for each peak was below 2. To make the runtime shorter, the method was changed and samples were reanalyzed.

Table No. . Method Development Trial 3

MP	Ratio	Diluent	Column	Flowrate	Wavelength
0.1% OPA-MeOH	60-40	50 0.1% OPA -50 MeOH	Agilent Zorbax Bonus RP (250 x 4.6 mm, 5µ)	1 ml/min	270

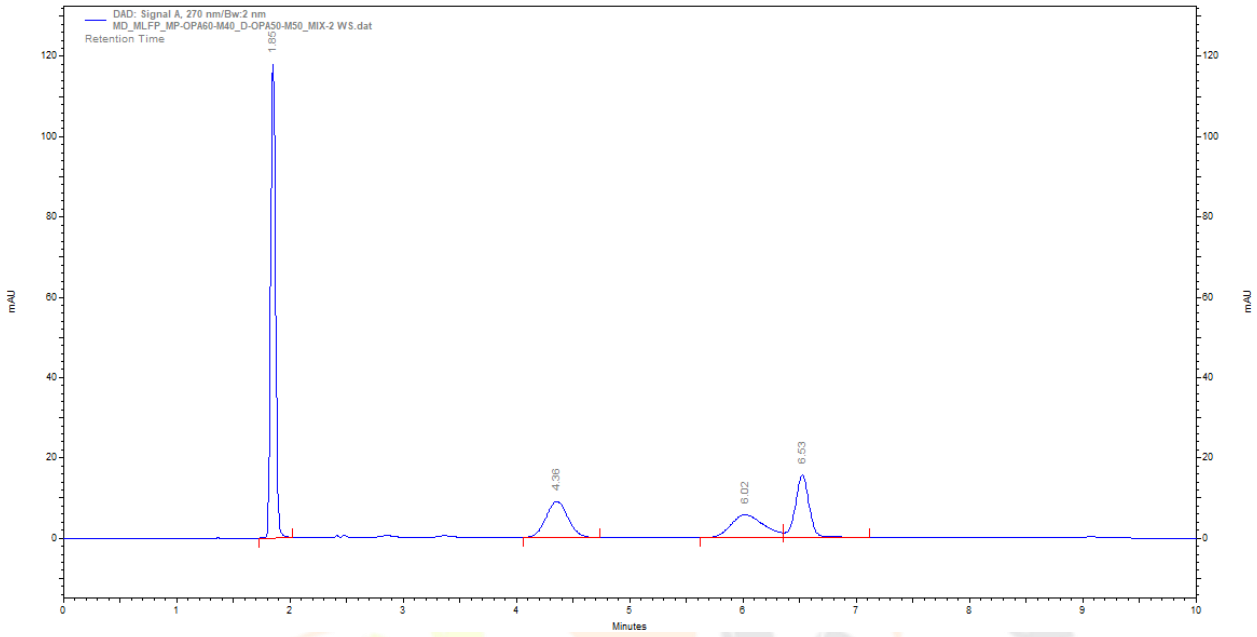


Figure No Method Development chromatogram Trial 3

Table No. . Results for Method Development Trial 3

Alpha lipoic acid				Mecobalamine				Folic acid				Pyridoxine			
RT	Asymmetry	TP	Resolution	RT	Asymmetry	TP	Resolution	RT	Asymmetry	TP	Resolution	RT	Asymmetry	TP	Resolution
1.85	1.05	87261	0.00	4.36	1.04	22831	11.28	6.02	0.00	1876	3.60	6.53	0.00	14547	1.31

Method development trial 3, same sample was used for analysis and the detection wavelength was also same. And all 4 peaks were observed, but the Folic acid and Pyridoxine peaks were merged. To well separate the peaks further method was changed and sample were reanalyzed.

Table No. . Method Development Trial 4

MP	Ratio	Diluent	Column	Flowrate	Wavelength
0.1% OPA-MeOH	50-50	50 0.1% OPA -50 MeOH	Agilent Zorbax Bonus RP (250 x 4.6 mm, 5µ)	0.8 ml/min	270

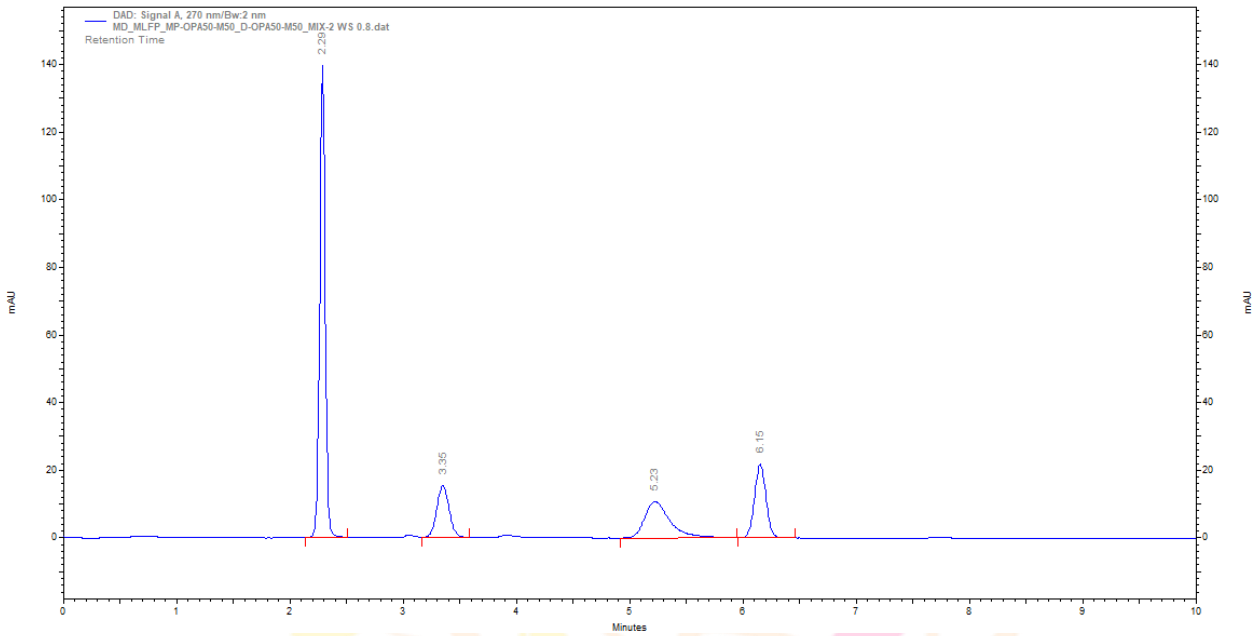


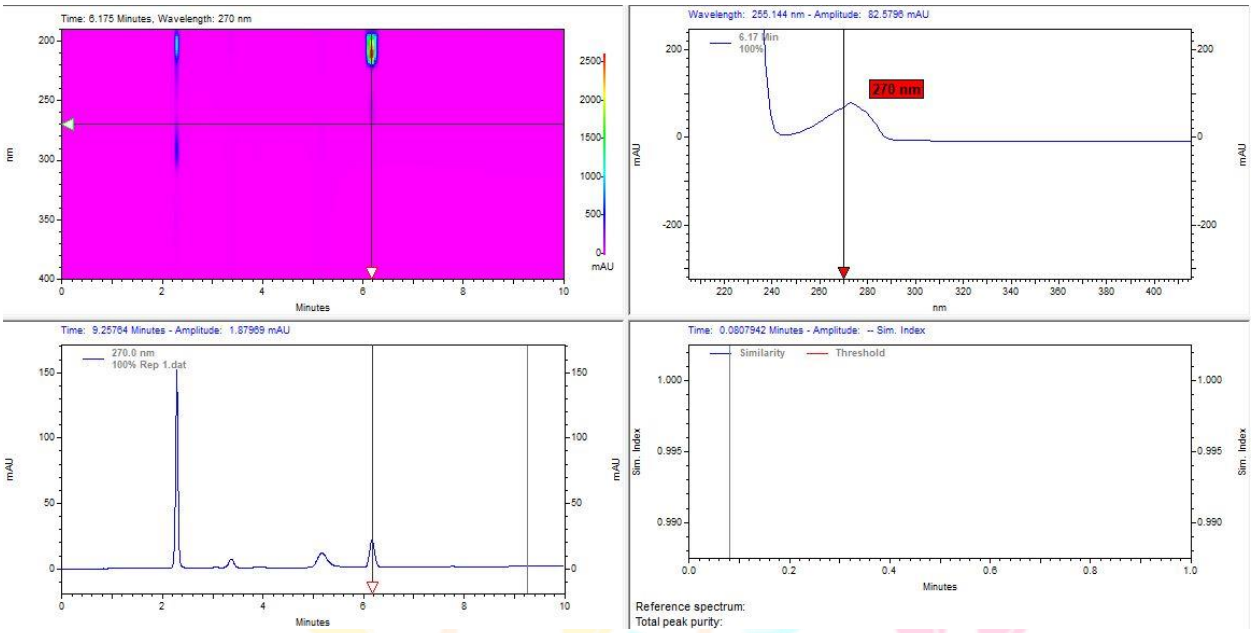
Figure no. Method Development Chromatogram Trial 4

Table No. . Results for Method Development Trial 4

Alpha lipoic acid				Mecobalamine				Folic acid				Pyridoxine			
RT	**Asym metry**	**TP**	**Resol ution**	**RT**	**Asym metry**	**TP**	**Resol ution**	**RT**	**Asym metry**	**TP**	**Resol ution**	**RT**	**Asym metry**	**TP**	**Resol ution**
2. 29	1.04	108 293	0.00	3. 35	1.04	452 74	7.43	5. 23	1.51	29 03	6.38	6. 15	1.04	375 73	3.23

The Method was changed to mobile phase of 50% o-phosphoric acid and 50% methanol at 0.8 ml/min flowrate. All the peaks were well-resolved under 10 min runtime.

Selection of Wavelength:



Wavelength selected was 270 nm.

7.2. Method Validation

7.2.1. Specificity

All individual working standards and combinations were run on HPLC and peaks were identified.

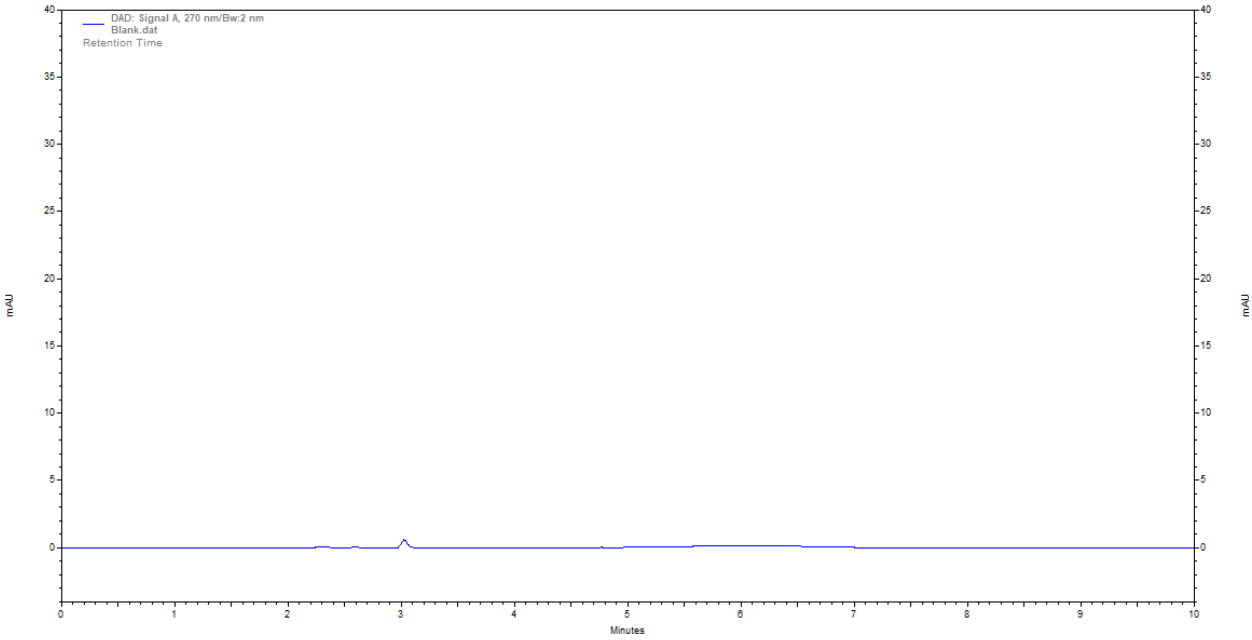


Figure No. Specificity Blank - Diluent Chromatogram

No Significant peaks of blanks were seen in the chromatogram.

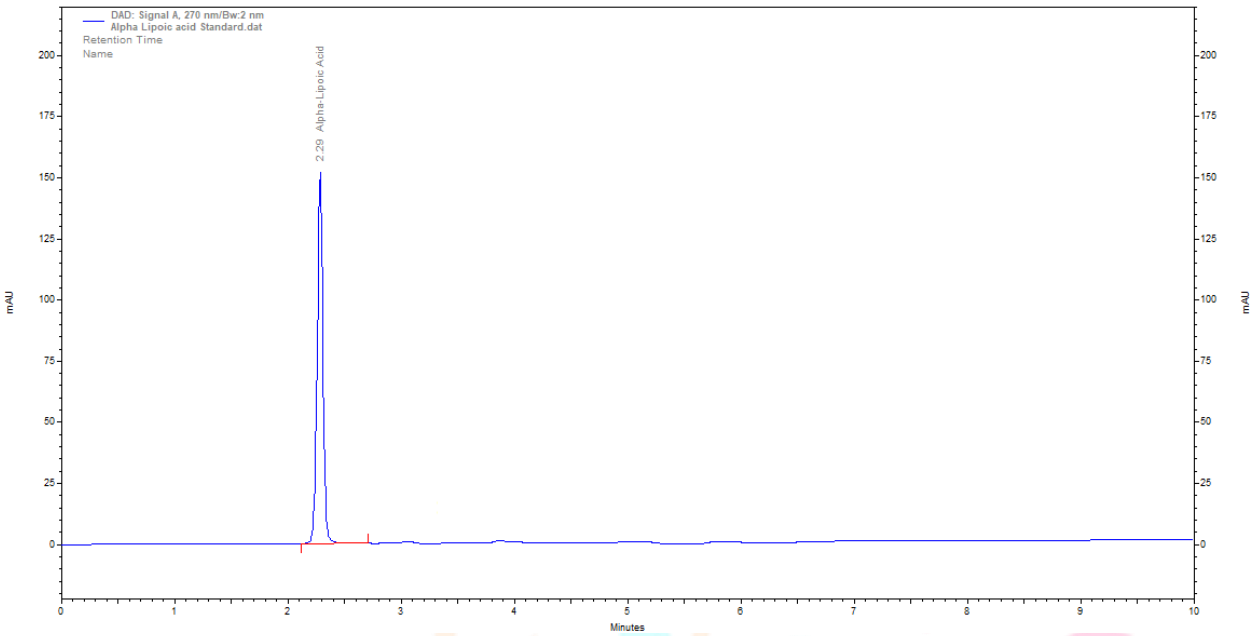


Figure no. Specificity Working Standard alpha Lipoic acid Chromatogram

Significant peak of Alpha Lipoic acid can be seen at RT 2.29 mins.

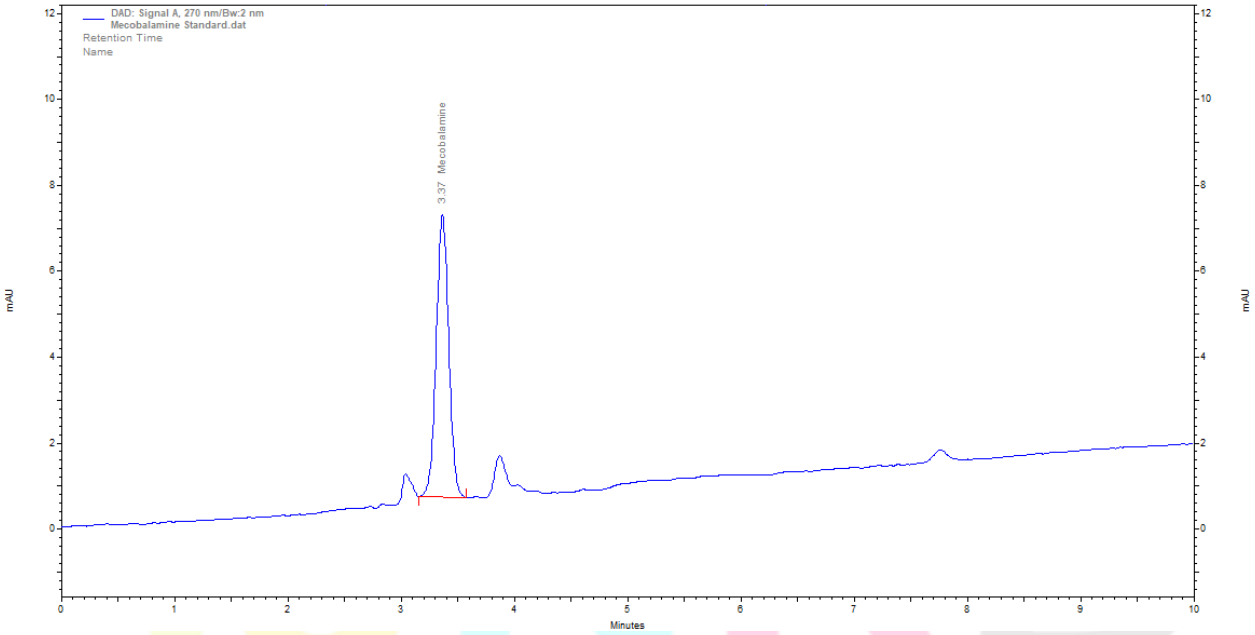


figure No. : Specificity Working Standard Mecobalamine Chromatogram

Sharp peak at RT 3.37mins can be seen in the chromatogram representing Mecobalamine

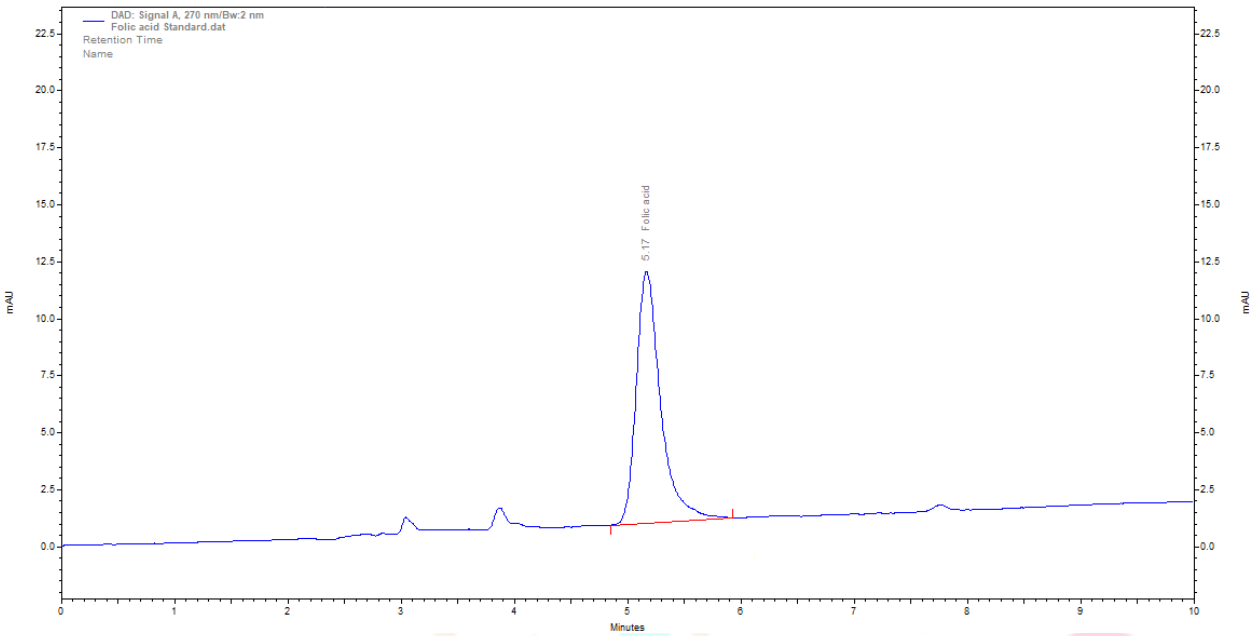


figure No. : Specificity Working Standard Folic acid Chromatogram

Sharp peak at RT 5.17mins can be seen in the chromatogram representing Folic acid

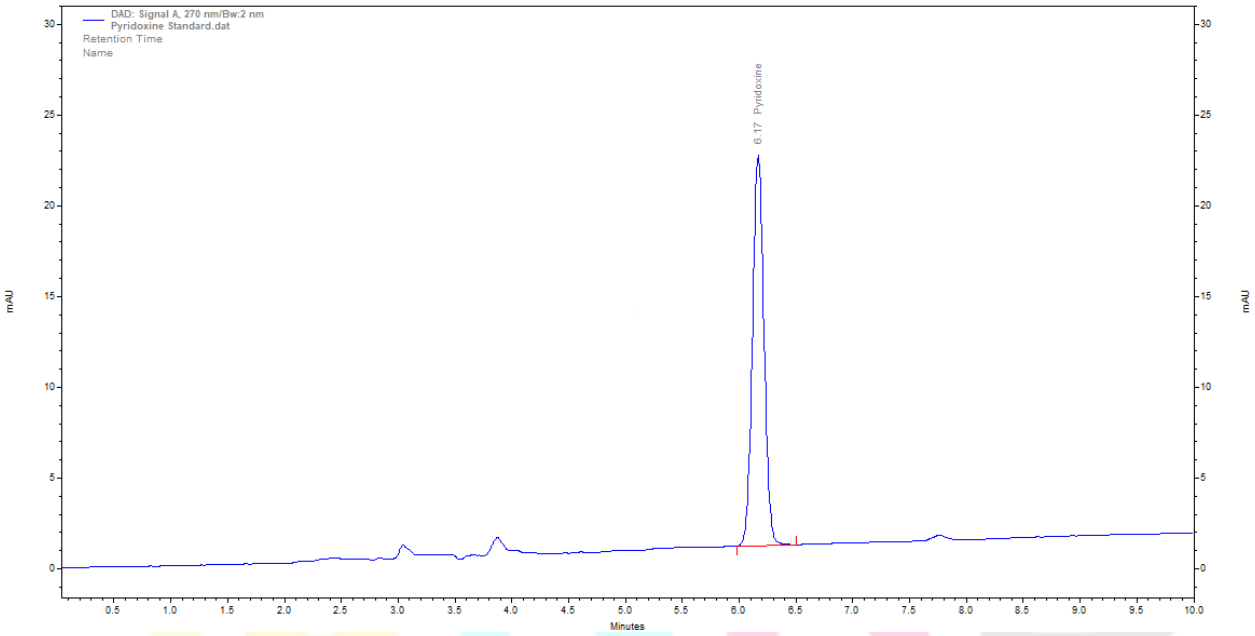


figure No. : Specificity Working Standard Pyridoxine Chromatogram

Sharp peak at RT 6.17 mins can be seen in the chromatogram representing Pyridoxine

A Combination of all Four Working Standard was injected to check if there was any interaction between four APIs.

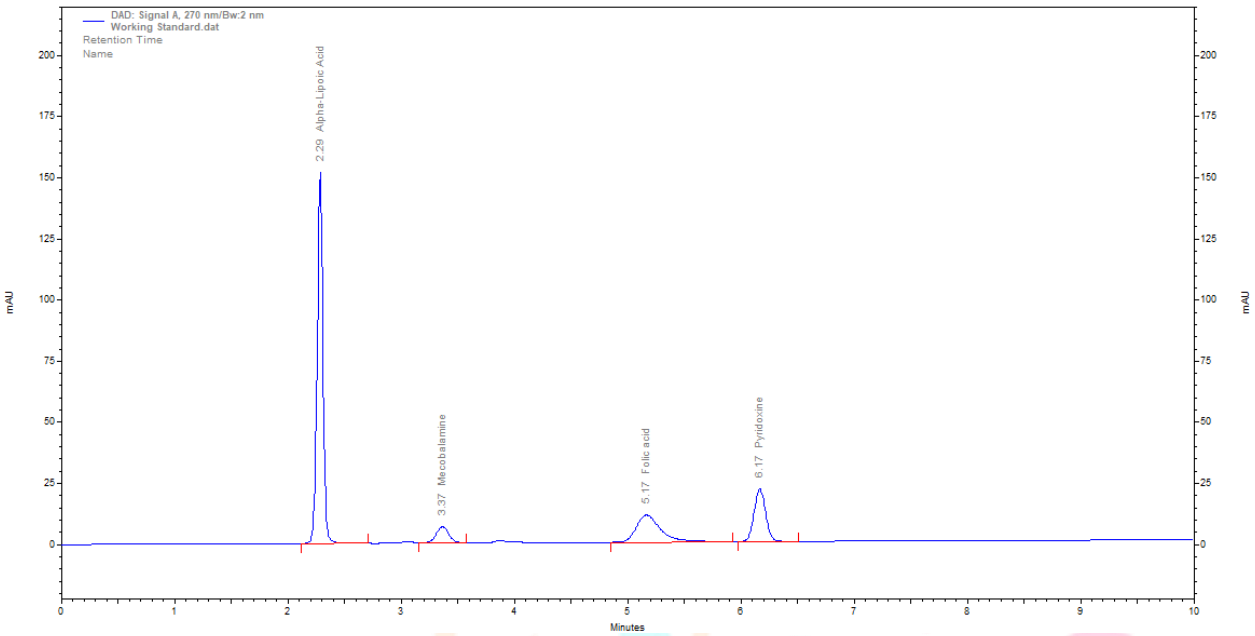


Figure No. Specificity Working Standard combination Chromatogram

It can be seen that there is no interaction between the 4 API peaks. Also, there is no inteference of diluent with any of the APIs peaks.

7.2.2. Assay

Drug product tablet was injected and compared with Working standards chromatogram. Assay was calculated based on area of each identified drug peak.

Sample	Alpha-lipoic acid			Mecobalamine			Folic acid			Pyridoxine		
	RT	Area	% Assay	RT	Area	% Assay	RT	Area	% Assay	RT	Area	% Assay
a-Lipoic acid Std	2.29	1132147	-	-	-	-	-	-	-	-	-	-
Mecobalamines td	-	-	-	3.37	101145	-	-	-	-	-	-	-
Folic acid Std	-	-	-	-	-	-	5.17	345214	-	-	-	-
Pyridoxine Std	-	-	-	-	-	-	-	-	-	6.17	317521	-
MIX WS	2.29	1134214	-	3.37	105887	-	5.17	345564	-	6.17	318638	-
Drug Product	2.29	1133785	99.96	3.37	105714	99.84	5.17	339872	98.35	6.17	312556	98.09

The % Assay for Alpha-Lipoic, Mecobalamine, Folic acidand Pyridoxine was found to be 99.96%, 99.84%, 98.35% and 98.09% respectively.

7.2.3. System Suitability

System suitability was performed as per instruction in Material and Method and results were recorded as below:

Table No. System Suitability for Alpha Lipoic acid

a-Lipoic acid				
Reps	**RT**	**Asymmetry**	**Theoretical Plates**	**Resolution**
Rep 1	2.29	1.04	108293	0.00
Rep 2	2.29	1.06	108545	0.00
Rep 3	2.29	1.01	108547	0.00
Rep 4	2.29	1.10	108668	0.00
Rep 5	2.29	1.08	108994	0.00
Avg	2.29			
STDEV	0			
RSD	0.00			

Table No. System Suitability for Mecobalamine

Mecobalamine				
Reps	**RT**	**Asymmetry**	**Theoretical Plates**	**Resolution**
Rep 1	3.35	1.04	45274	7.43
Rep 2	3.35	1.06	45147	7.43
Rep 3	3.35	1.03	45474	7.43
Rep 4	3.35	1.05	45547	7.43
Rep 5	3.35	1.04	45656	7.43
Avg	3.35			
STDEV	0			
%RSD	0.00			

Table No. System Suitability for Folic acid

Folic acid				
Reps	**RT**	**Asymmetry**	**Theoretical Plates**	**Resolution**
Rep 1	5.23	1.51	2903	6.38
Rep 2	5.23	1.52	2892	6.38
Rep 3	5.23	1.48	2951	6.38
Rep 4	5.23	1.44	2447	6.38
Rep 5	5.23	1.52	2147	6.38
Avg	5.23			
STDEV	0			
%RSD	0.00			

Table No. System Suitability for Pyridoxine

Pyridoxine				
Reps	**RT**	**Asymmetry**	**Theoretical Plates**	**Resolution**
Rep 1	6.15	1.04	37573	3.23
Rep 2	6.15	1.05	37214	3.23

Rep 3	6.15	1.02	37684	3.23
Rep 4	6.15	1.01	37957	3.23
Rep 5	6.15	1.06	37584	3.23
Avg	6.15			
STDEV	0			
%RSD	0.00			

It can be inferred that the % Relative Standard Deviation of each drug is 0.00 which means that there is no change in Retention time of any of the drug for 5 repetitions.

The Asymmetry of all 4 drugs were within the ICH guidelines which is less than 2.

The theoretical plates for each API for all reps are above 2000 as per ICH Guidelines.

Resolution is above 2 for Mecobalamine, Folic acidand Pyridoxine which is as per the specifications.

7.2.4. Precision (Repeatability)

A total of 5 replicate injections were done to check if the method is precise or not.

Table No. Precision results.

	A-LA	**MeCbl**	**FA**	**PDX**
Reps	**Area**	**Area**	**Area**	**Area**
Rep 1	1134214	105887	345564	318638
Rep 2	1132247	105587	341145	317541
Rep 3	1132488	105564	342254	317441
Rep 4	1149821	103574	342987	318243
Rep 5	1143214	105741	349877	318760
Avg	1138397	105271	344365	318125
STDEV	7809.41	957.323	3483.653	610.1699
RSD	0.69	0.91	1.01	0.19

With respect to the areas, all four drugs have %RSD less than 2 which is in accordance with ICH Guidelines.

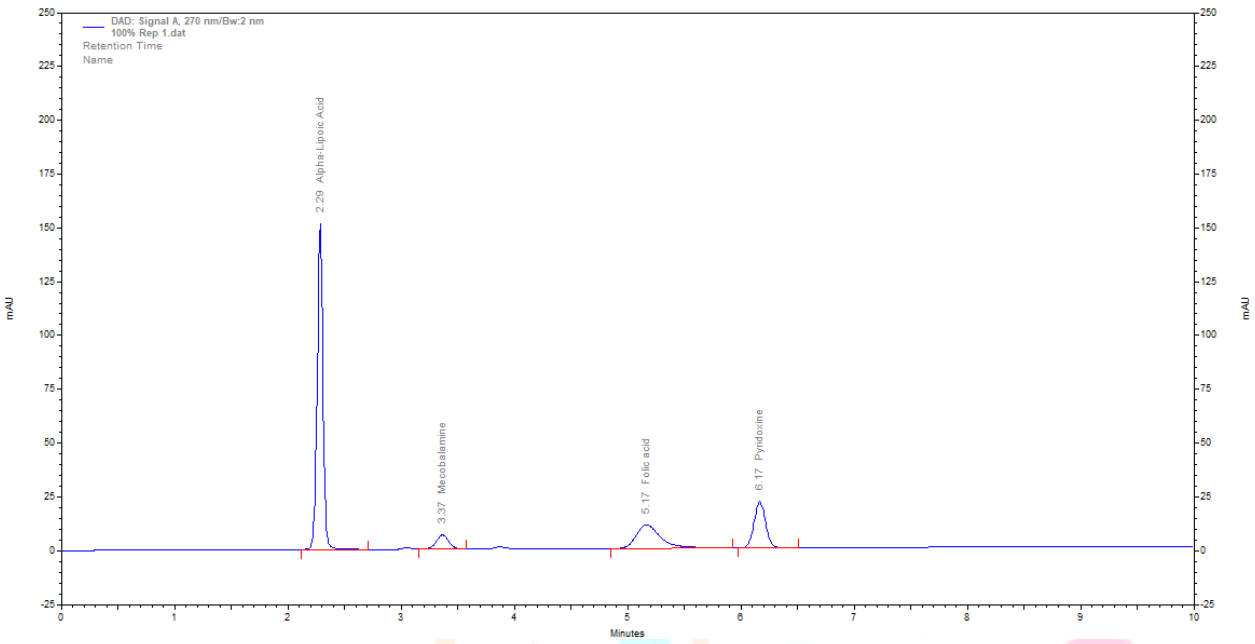


Figure No. Instrument Precision Rep 1

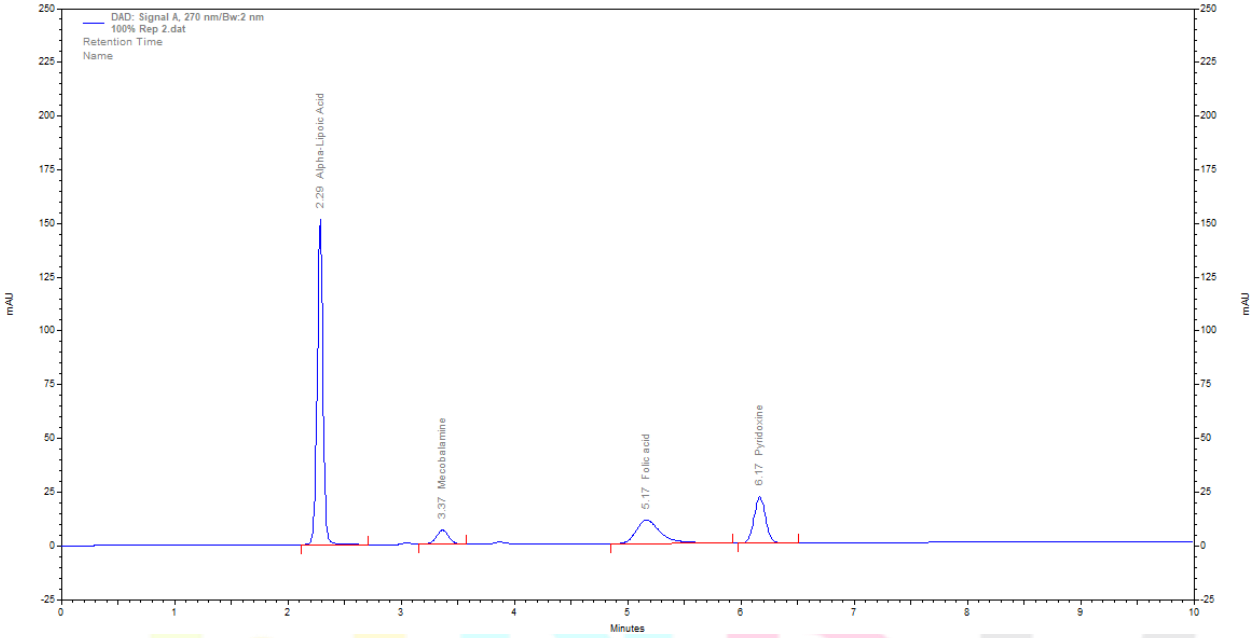


Figure No. Instrument Precision Rep 2

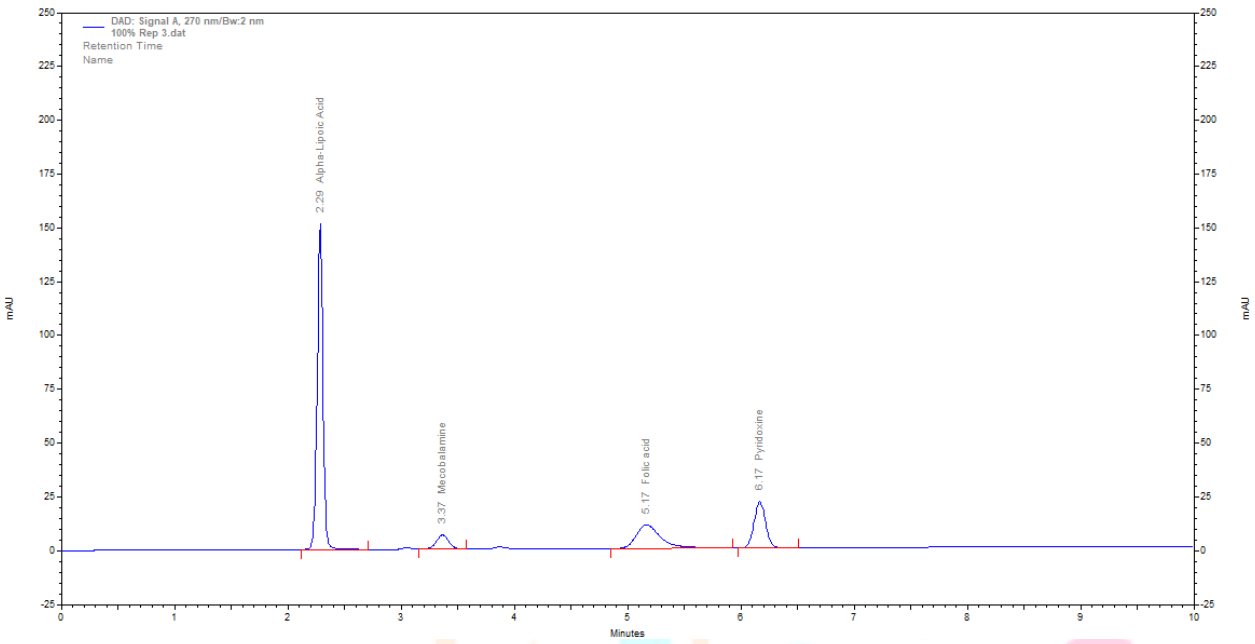


Figure No. Instrument Precision Rep 3

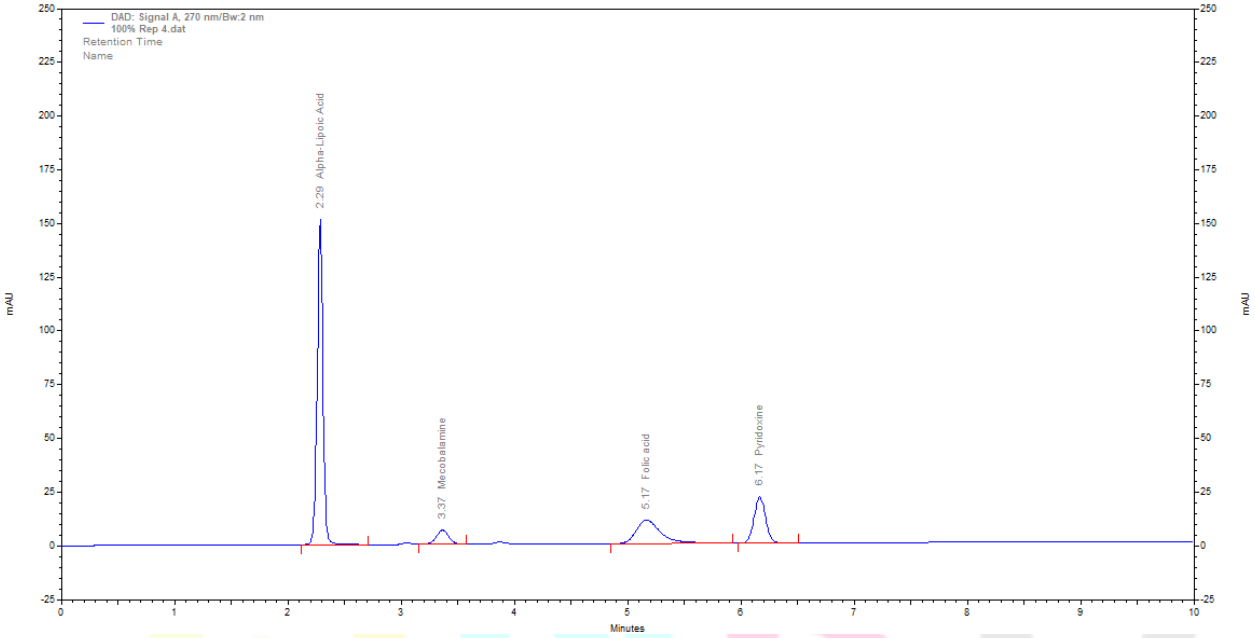


Figure No. Instrument Precision Rep 4

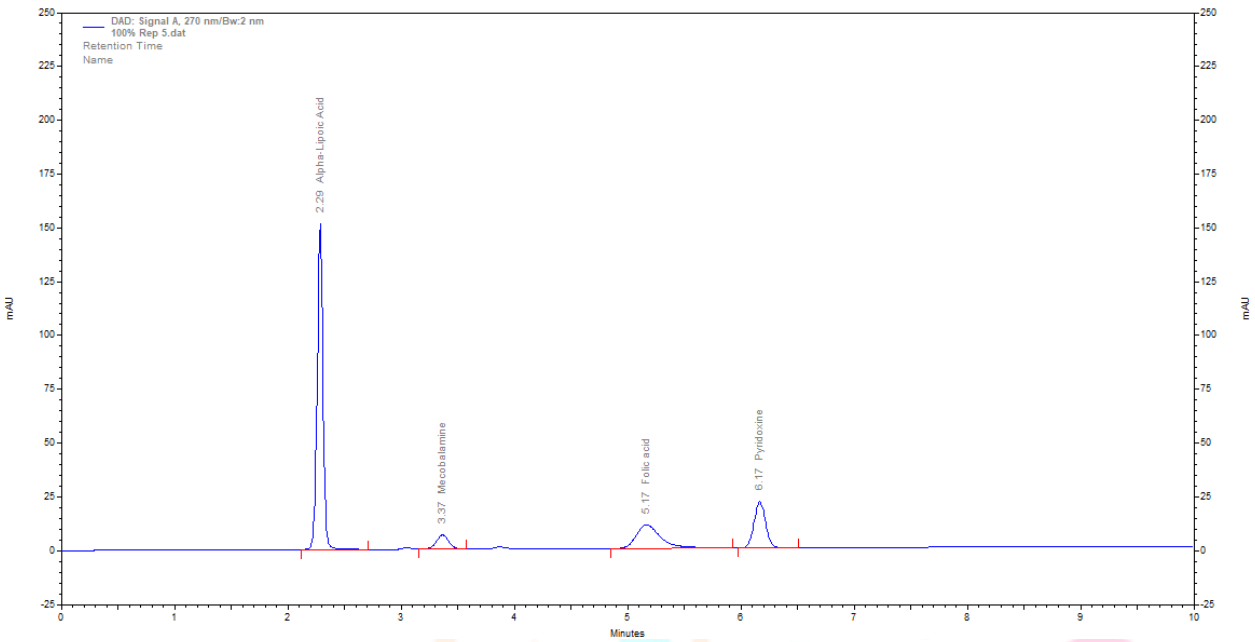


Figure No. Instrument Precision Rep 5

7.2.5. Linearity

The 5 points Linearity was performed for each Working Standard API. The results obtained is detailed below:

Table No. Linearity for Alpha Lipoic acid

A-LA		
% Level	**Conc (ug/ml)**	**Area**
80	80	909518
90	90	1001190
100	100	1134214
110	110	1275937
120	120	1403598

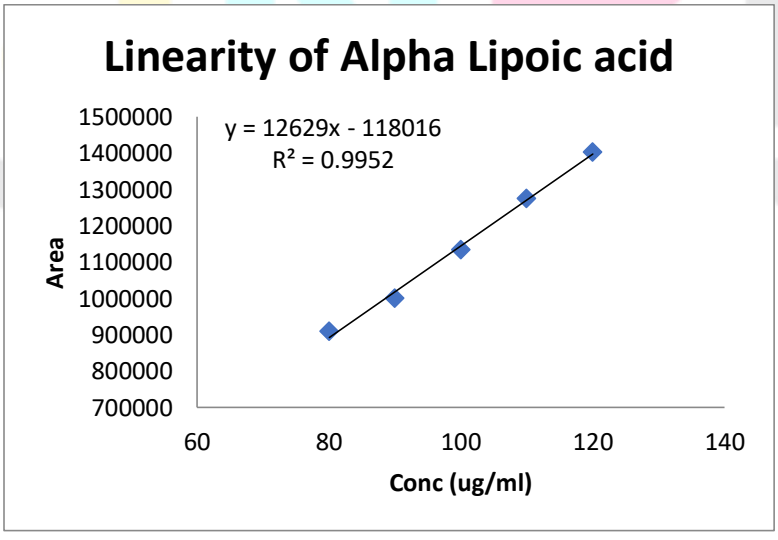


Figure: Linearity Graph for Alpha Lipoic acid

Table No. Linearity data for Mecobalamine

MeCbl		
% Level	Conc (ug/ml)	Area
80	12	84097
90	13.5	96618
100	15	105887
110	16.5	118408
120	18	126741

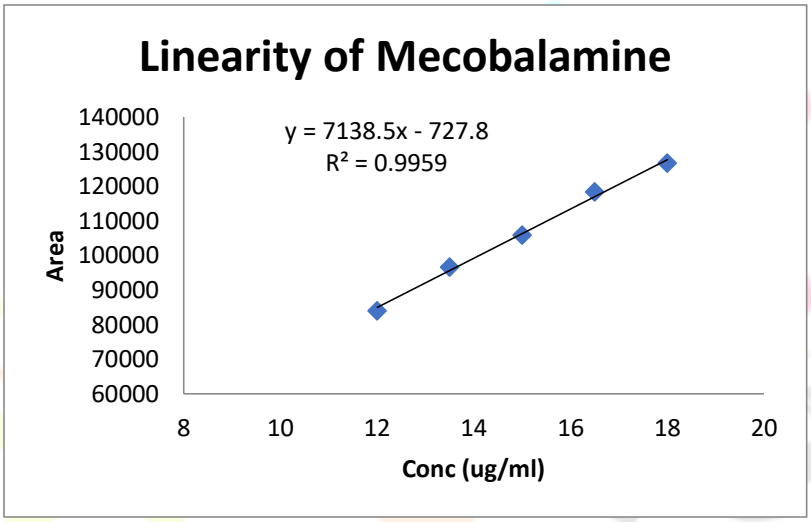


Figure No. Linearity Graph for Mecobalamine

Table No. Linearity data for Folic acid

FA		
% Level	Conc (ug/ml)	Area
80	12	278061
90	13.5	310823
100	15	345564
110	16.5	382556
120	18	412663

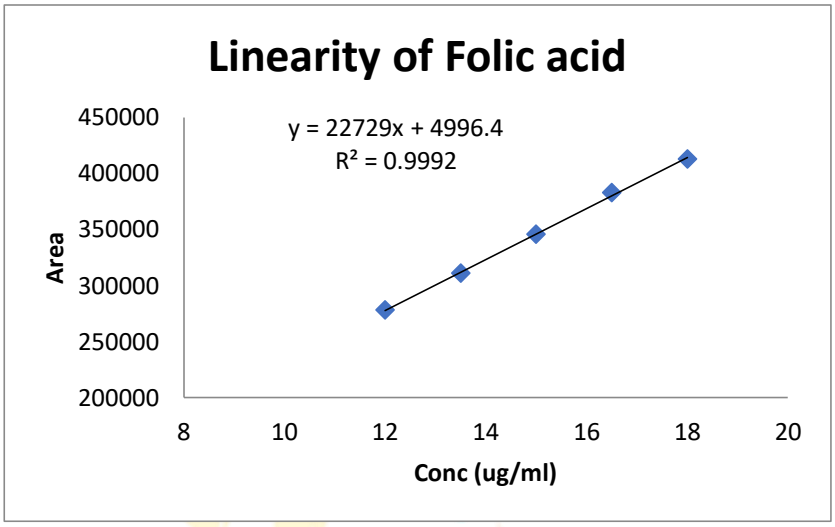


Figure No.: Linearity graph for Folic acid

Table No. Linearity data for Pyridoxine

PDX		
% Level	**Conc (ug/ml)**	**Area**
80	24	255130
90	27	286367
100	30	318638
110	33	348771
120	36	380350

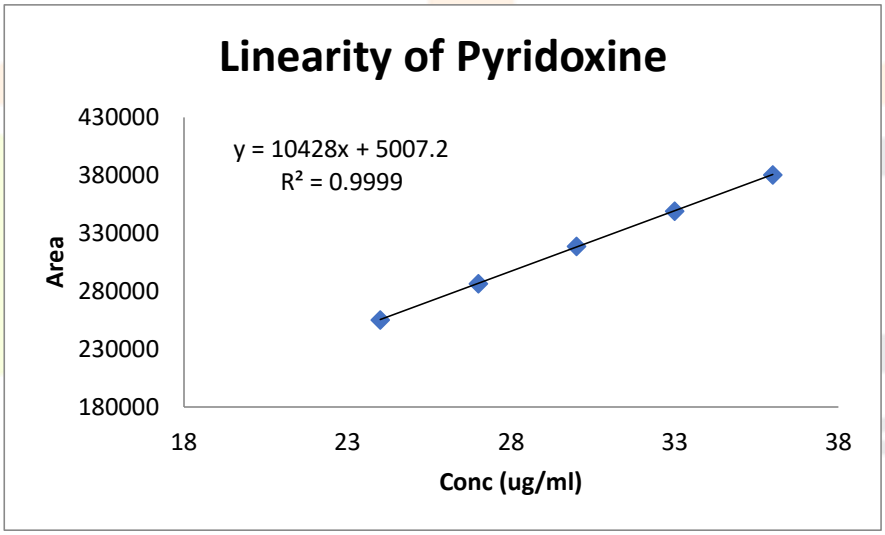


Figure No.: Linearity graph for Pyridoxine

Linearity Summary

Table No Linearity Summary

Parameter	Alpha Lipoic acid	Mecobalamine	Folic acid	Pyridoxine
y Intercept	118016	727.8	4996.4	5007.2
Slope (m)	12629	7138.5	22729	10428
r^2	0.9952	0.9959	0.9992	0.9999

Chromatogram of Linearity data

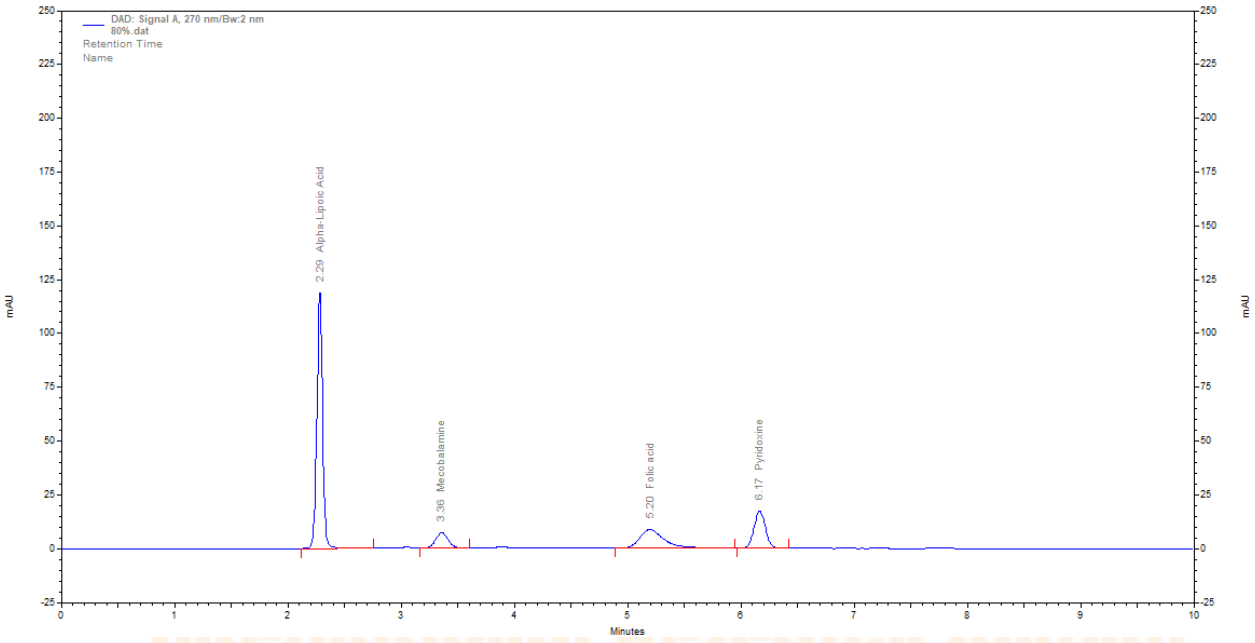


Figure No. : Linearity at 80% concentration

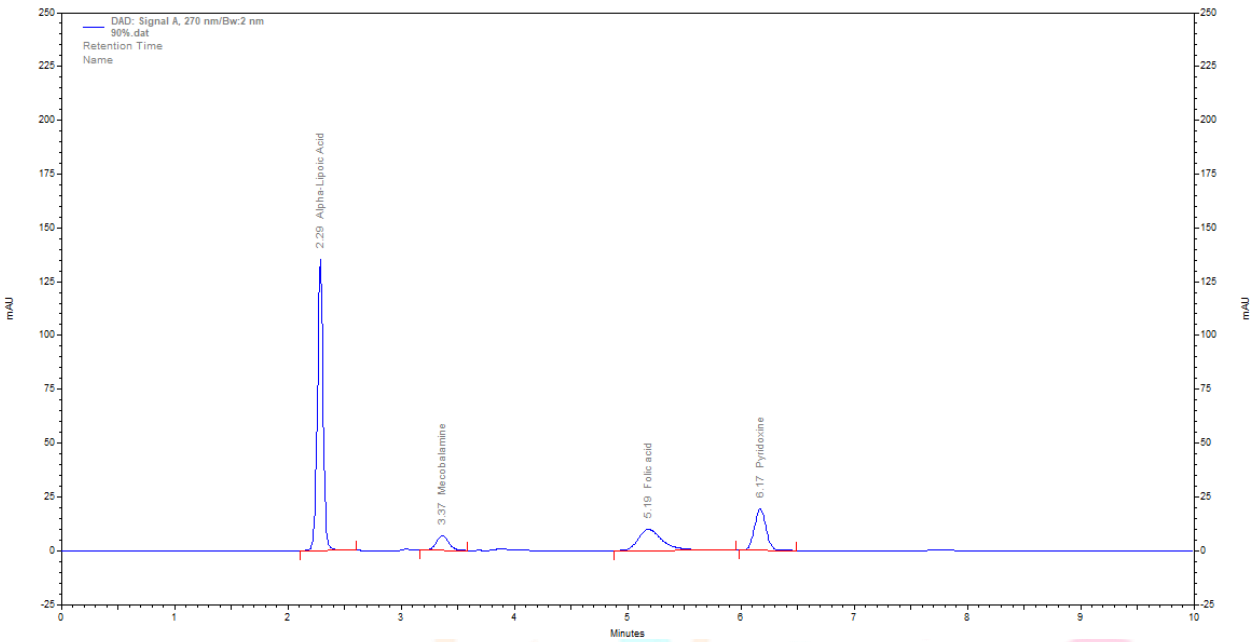


Figure No. Linearity at 90% concentration

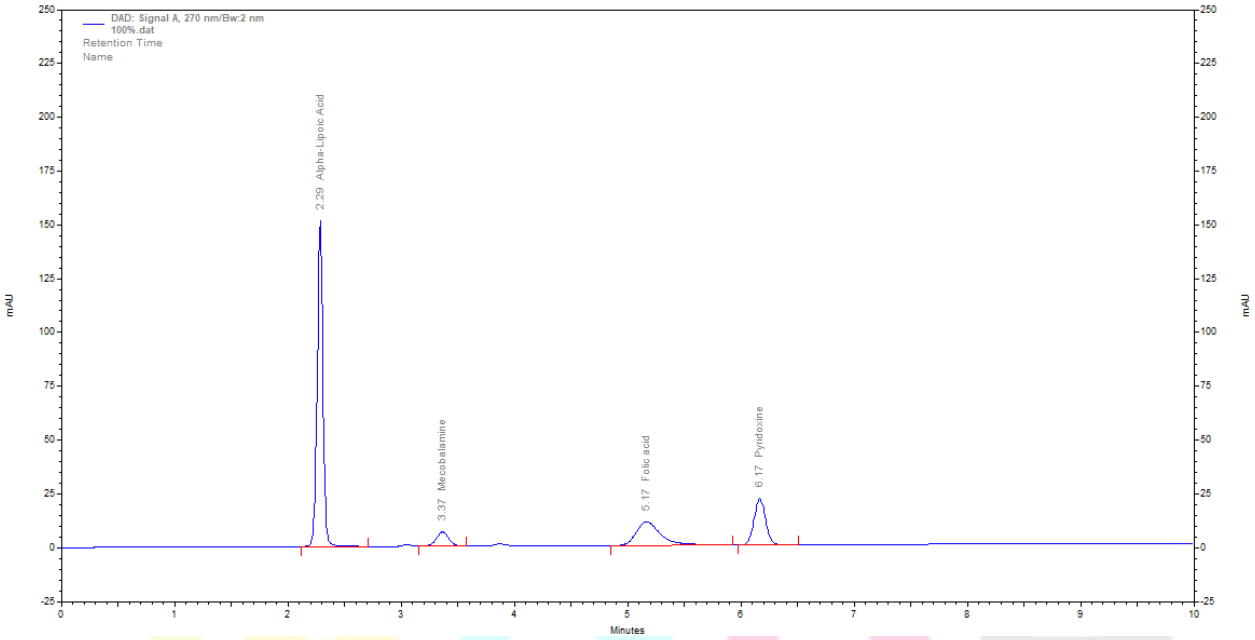


Figure No. Linearity at 100% concentration

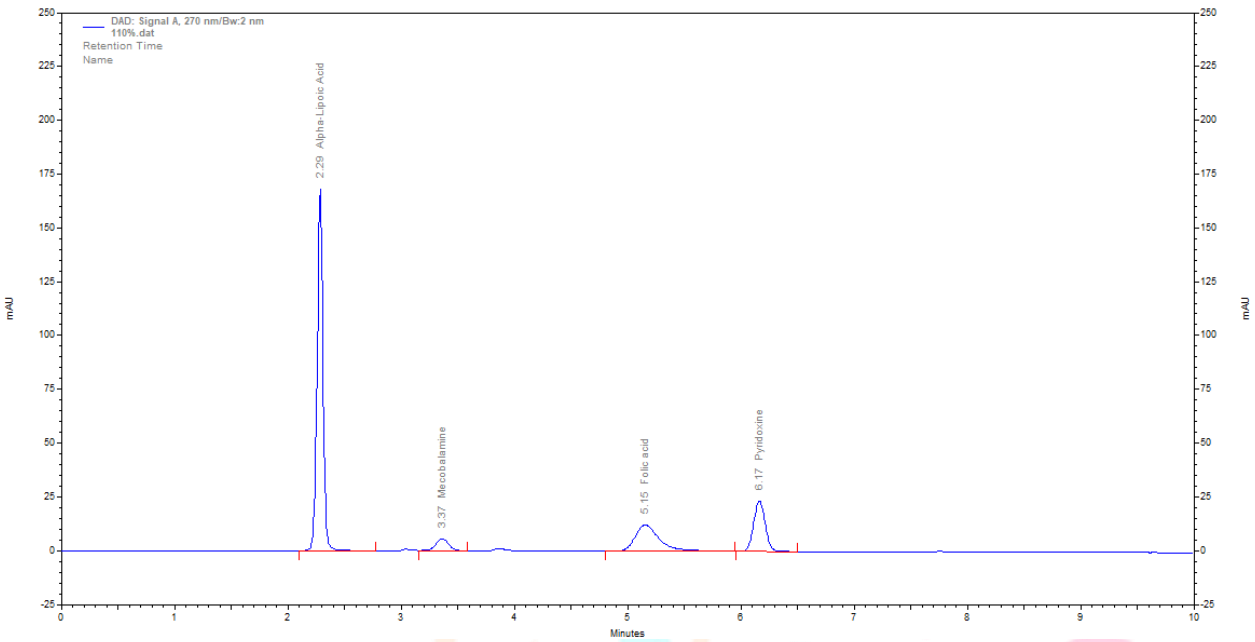


Figure No. Linearity at 110% concentration

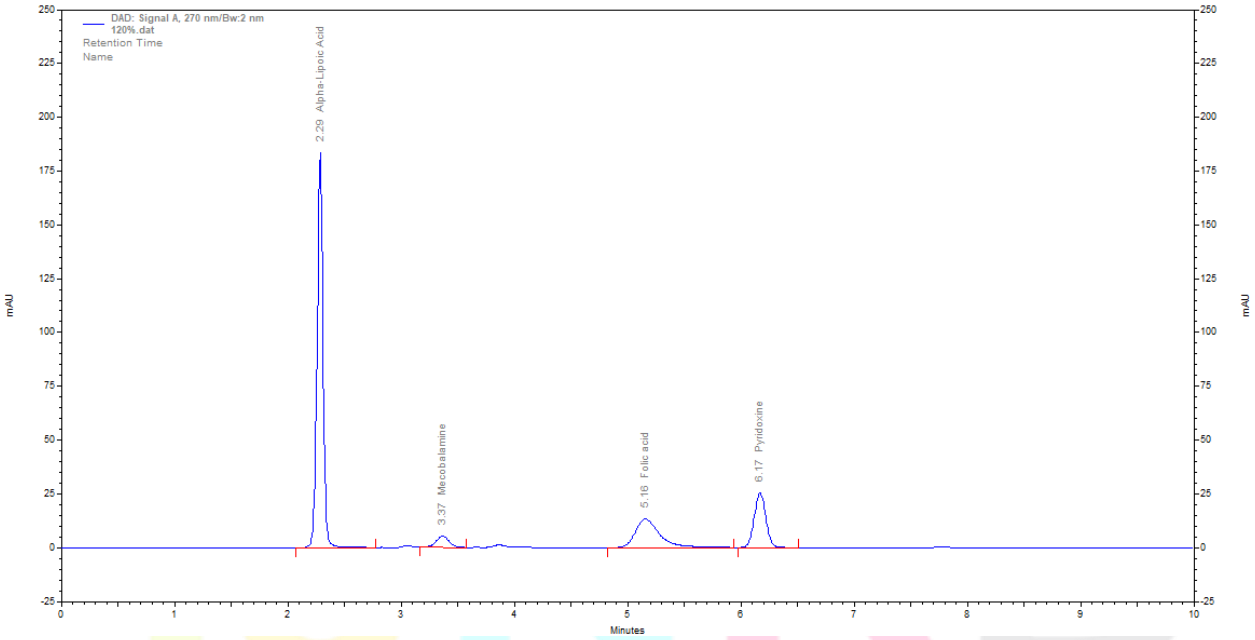


Figure No. Linearity at 120% concentration

7.2.6. Range

The range of linearity and analysis if summarized in the table below:

Drug	**Range ug/ml**
Alpha Lipoic acid	80– 120

Mecobalamine	12– 18
Folic acid	12 – 18
Pyridoxine	24 – 36

The analysis was found to be linear in this range of analysis.

7.2.7. LOD and LOQ

7.2.7.1. Alpha Lipoic acid LOD and LOQ

SUMMARY
OUTPUT

Regression Statistics	
Multiple R	0.997584287
R Square	0.99517441
Adjusted R Square	0.993565881
Standard Error	16055.96211
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1.59493E+11	1.59493E+11	618.6856906	0.000142477
Residual	3	773381758.3	257793919.4		
Total	4	1.60267E+11			

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	-118015.6	51278.63081	-2.301457705	0.104848143
X Variable 1	12629.07	507.7341031	24.87339323	0.000142477

LOD	13.40	ug/ml
LOQ	40.60	ug/ml

7.2.7.2. Mecobalamine LOD and LOQ

SUMMARY
OUTPUT

Regression Statistics	
Multiple R	0.997939075

R Square	0.995882398
Adjusted R Square	0.994509864
Standard Error	1257.065153
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1146569808	1146569808	725.5793703	0.000112278
Residual	3	4740638.4	1580212.8		
Total	4	1151310447			

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	-727.8	4014.744146	-0.181281789	0.867702514
X Variable 1	7138.533333	265.0126035	26.93658052	0.000112278

LOD	1.86	ug/ml
LOQ	5.62	ug/ml

7.2.7.3. Folic acid LOD and LOQ

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999579153
R Square	0.999158482
Adjusted R Square	0.998877976
Standard Error	1806.458441
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	11623803797	11623803797	3561.986926	1.03632E-05
Residual	3	9789876.3	3263292.1		
Total	4	11633593673			

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	4996.4	5769.365599	0.866022427	0.450186256
X Variable 1	22729.13333	380.8348782	59.68238372	1.03632E-05

LOD	0.84	ug/ml
LOQ	2.54	ug/ml

7.2.7.4. Pyridoxine LOD and LOQ

SUMMARY
OUTPUT

Regression Statistics	
Multiple R	0.999958109
R Square	0.99991622
Adjusted R Square	0.999888294
Standard Error	522.8234884
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	9787136834	9787136834	35805.1485	3.25468E-07
Residual	3	820033.2	273344.4		
Total	4	9787956867			

	Coefficients	*Standard Error*	*t Stat*	*P-value*
Intercept	5007.2	1669.764319	2.998746556	0.05772651
X Variable 1	10428.13333	55.11043458	189.2224841	3.25468E-07

LOD	0.53	ug/ml
LOQ	1.60	ug/ml

7.2.8. Accuracy

Accuracy was performed for range 80%, 100% and 120%. The data is reported as below:

Table No. : Accuracy data for Alpha Lipoic acid

A-LA		
Stdwt (mg)	**Purity (%)**	**Potency (ug/ml)**
10	99.7	997

Std Area	1138397

% Level	Reps	Spiked Conc (ug/ml)	Area	Amount Recovered (ug/ml)	% Recovery	AVG	STDEV	RSD
80%	Rep 1	79.76	909518	79.65	99.87	99.65	0.30832	0.31
	Rep 2	79.76	905547	79.31	99.43			
100%	Rep 1	99.70	1134214	99.33	99.63	99.55	0.122179	0.12
	Rep 2	99.70	1132247	99.16	99.46			
120%	Rep 1	119.64	1403598	122.93	102.75	102.19	0.780362	0.76
	Rep 2	119.64	1388522	121.61	101.64			

Table No. Accuracy data for Mecobalamine

MeCbl		
Stdwt (mg)	Purity (%)	Potency (ug/ml)
10	99.7	149.55

Std Area	105271

% Level	Reps	Spiked Conc (ug/ml)	Area	Amount Recovered (ug/ml)	% Recovery	AVG	STDEV	RSD
80%	Rep 1	11.964	84097	11.95	99.86	99.94	0.116709	0.12
	Rep 2	11.964	84236	11.97	100.02			
100%	Rep 1	14.955	105887	15.04	100.59	100.44	0.201511	0.20
	Rep 2	14.955	105587	15.00	100.30			
120%	Rep 1	17.946	126741	18.01	100.33	99.82	0.718723	0.72
	Rep 2	17.946	125457	17.82	99.31			

Table No. Accuracy data for Folic acid

FA		
Stdwt (mg)	Purity (%)	Potency (ug/ml)
10	99.7	149.55

Std Area	344365

% Level	Reps	Spiked Conc (ug/ml)	Area	Amount Recovered (ug/ml)	% Recovery	AVG	STDEV	RSD
80%	Rep 1	11.964	278061	12.08	100.93	100.46	0.669653	0.67
	Rep 2	11.964	275452	11.96	99.99			

100%	Rep 1	14.955	345564	15.01	100.35	99.71	0.907381	0.91
	Rep 2	14.955	341145	14.82	99.06			
120%	Rep 1	17.946	412663	17.92	99.86	99.97	0.151264	0.15
	Rep 2	17.946	413547	17.96	100.07			

Table No. Accuracy data for Pridoxine

PDX		
Stdwt (mg)	**Purity (%)**	**Potency (ug/ml)**
10	99.7	299.1

Std Area	318125

% Level	Reps	Spiked Conc (ug/ml)	Area	Amount Recovered (ug/ml)	% Recovery	AVG	STDEV	RSD
80%	Rep 1	23.928	255130	23.99	100.25	99.93	0.455939	0.46
	Rep 2	23.928	253489	23.83	99.60			
100%	Rep 1	29.91	318638	29.96	100.16	99.99	0.243834	0.24
	Rep 2	29.91	317541	29.86	99.82			
120%	Rep 1	35.892	380350	35.76	99.63	99.75	0.167446	0.17
	Rep 2	35.892	381254	35.85	99.87			

Accuracy analysis summary

% Level	Alpha Lipoic acid	Mecobalamine	Folic acid	Pyridoxine
80%	0.31	0.12	0.67	0.46
100%	0.12	0.20	0.91	0.24
120%	0.76	0.72	0.15	0.17

All the %RSD for 80%, 100% and 120% are within the specification of less than 2%. The method is accurate for Alpha Lipoic acid, Mecobalamine, Folic acidand Pyridoxine.

Chromatogram of Accuracy for all 3 levels.

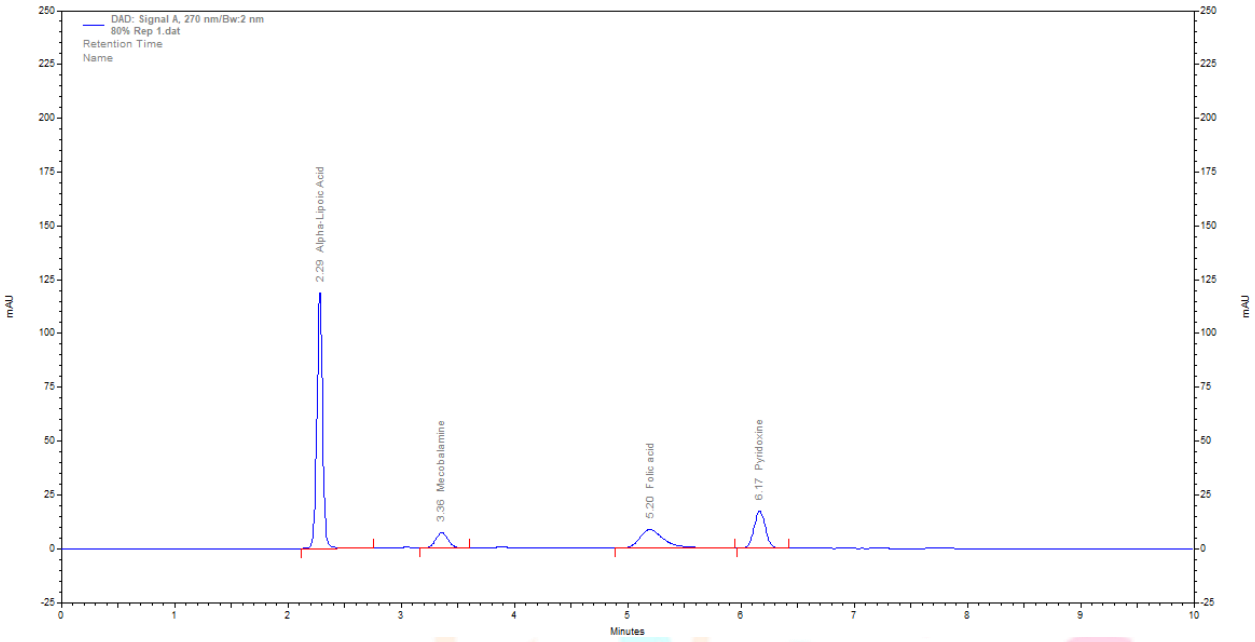


Figure No.: Accuracy 80% Rep 1

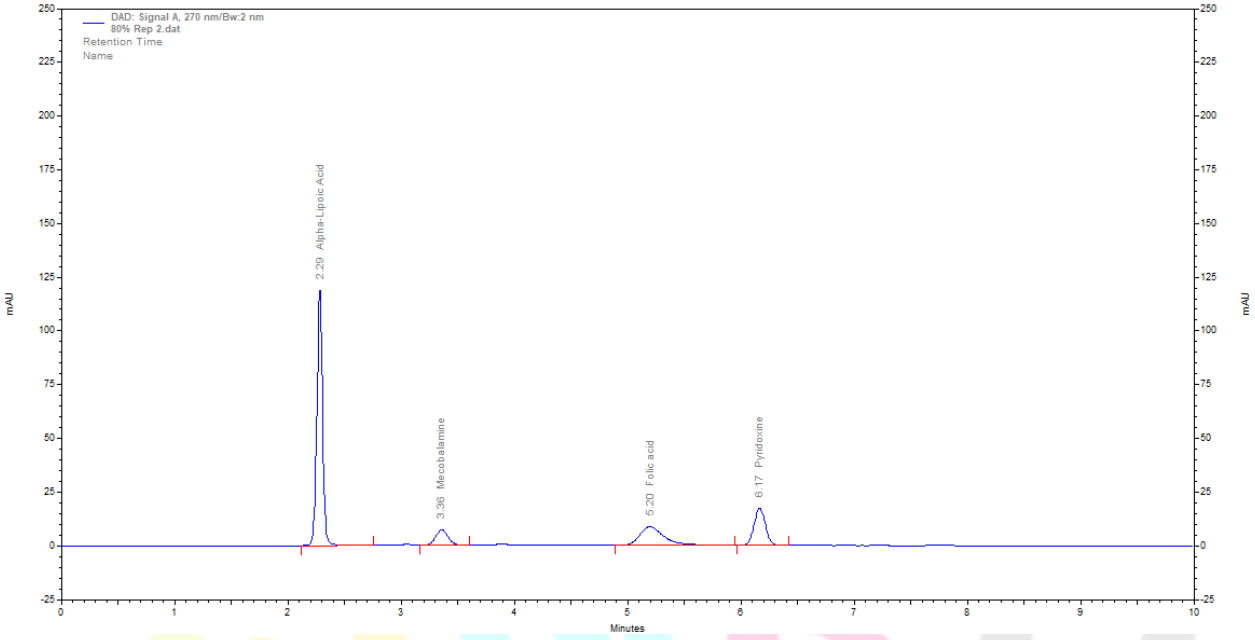


Figure No.: Accuracy 80% Rep 2

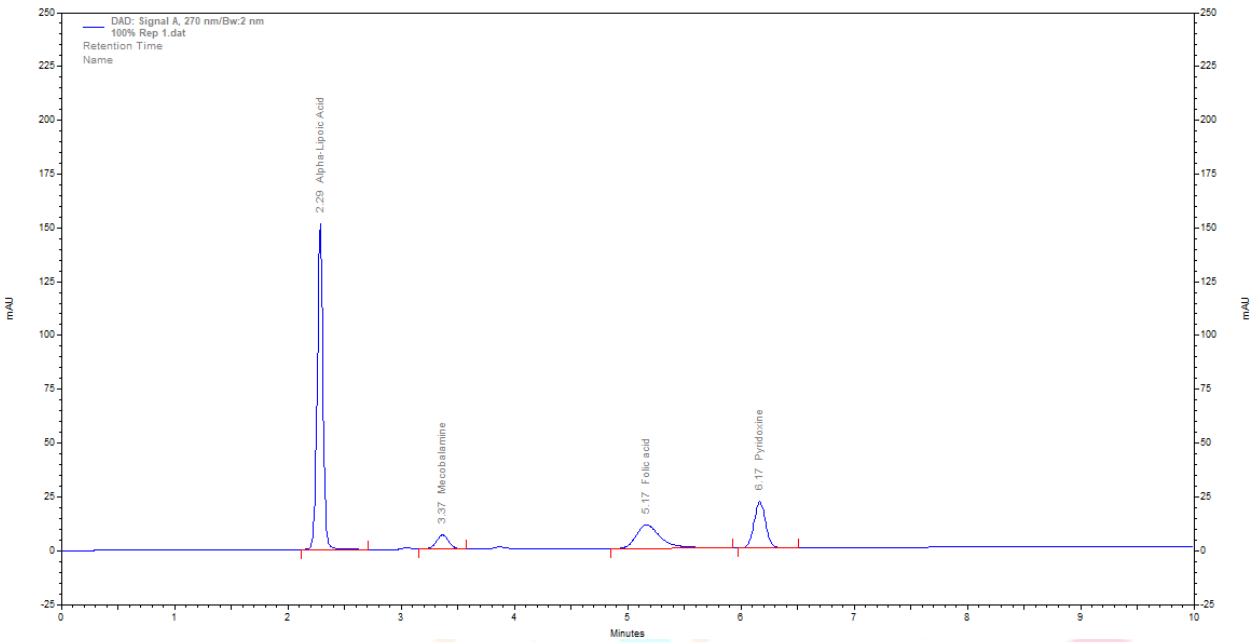


Figure No.: Accuracy 100% Rep 1

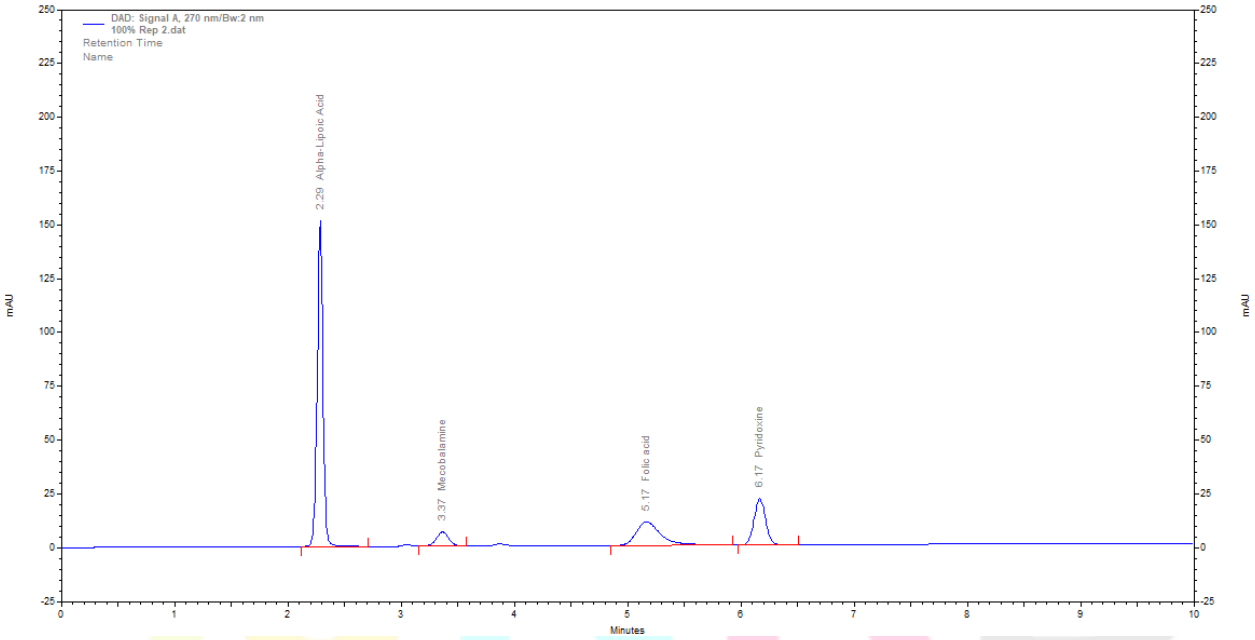


Figure No.: Accuracy 100% Rep 2

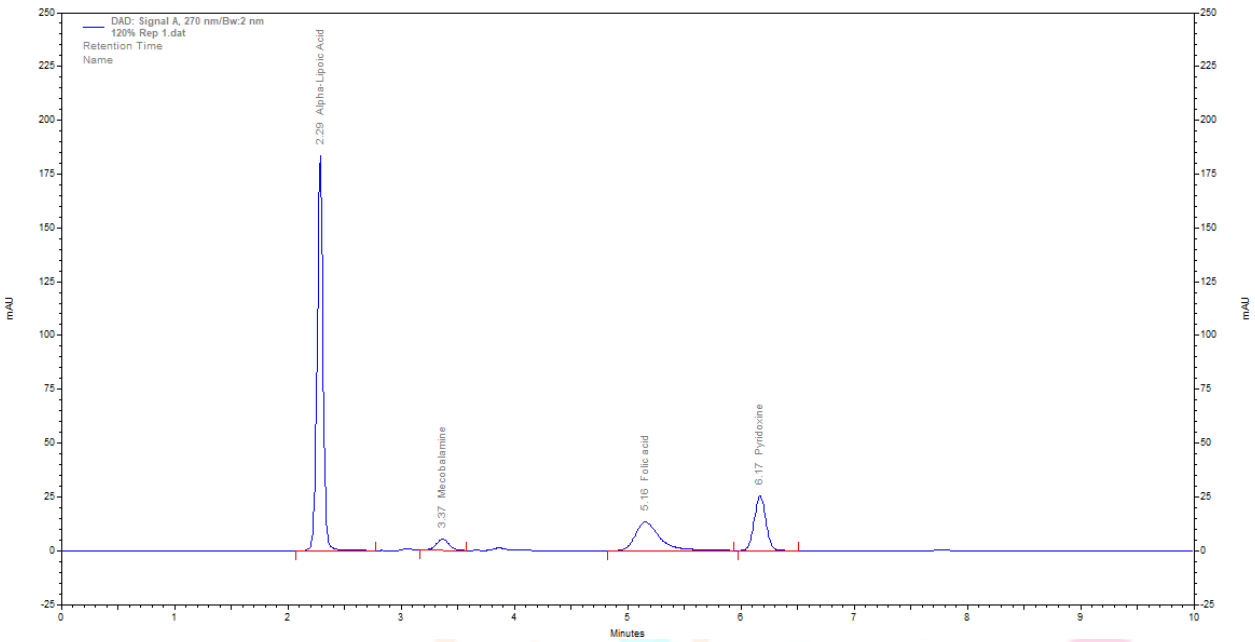


Figure No.: Accuracy 120% Rep 1

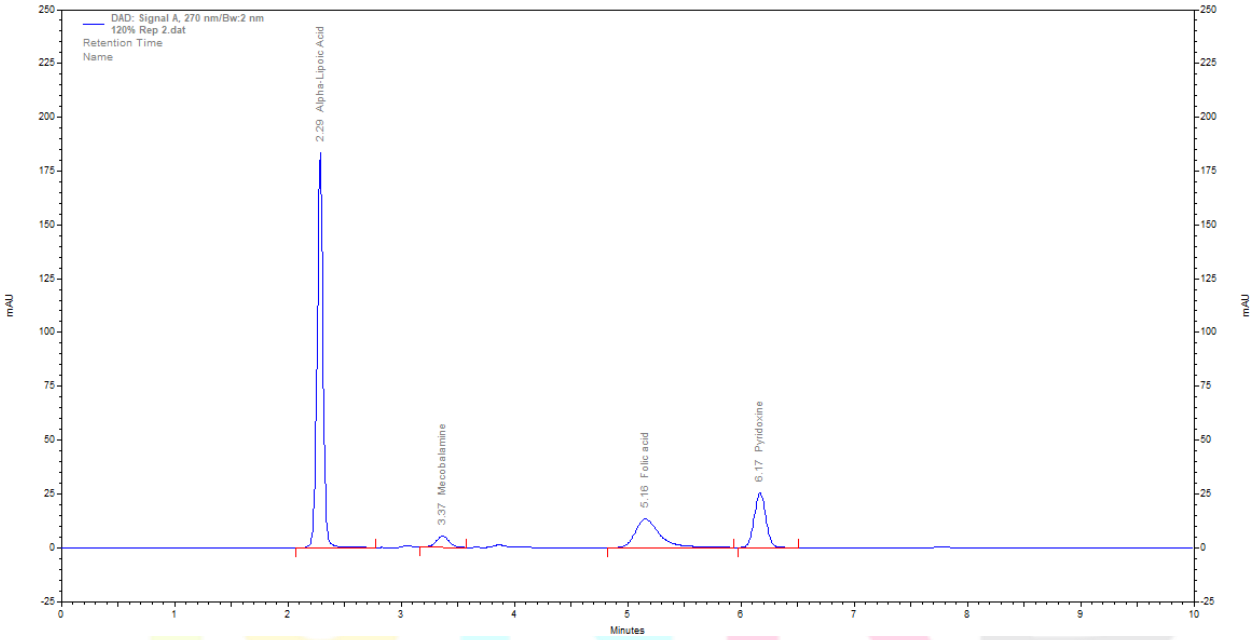


Figure No.: Accuracy 120% Rep 2

7.2.9. Robustness

Robustness was performed for change in column temperature and mobile phase ratio. The analysis is reported below:

7.2.9.1. Change in Column Temperature

Column Oven Temp Change																									
Condition	Sample	A-LA						MeCbl						FA						PDX					
		Area	Assay	RT	Asymmetry	TP	Resolution	Area	Assay	RT	Asymmetry	TP	Resolution	Area	Assay	RT	Asymmetry	TP	Resolution	Area	Assay	RT	Asymmetry	TP	Resolution
28℃	WS	1212453	-	2.35	1.06	104215	0.00	104225	-	3.39	1.03	431644	7.40	349521	-	5.29	1.49	2902	6.41	3175 21	-	6.21	1.06	37155	3.23
	DP	1211485	99.92	2.35	1.05	104879	0.00	104125	99.90	3.39	1.05	432220	7.40	348512	99.71	5.29	1.50	2905	6.41	3152 55	99.29	6.21	1.05	37541	3.23
30℃	WS	1134214	-	2.29	1.04	108293	0.00	105887	-	3.35	1.04	452744	7.43	345564	-	5.23	1.51	2903	6.38	3186 38	-	6.15	1.04	37573	3.23
	DP	1133785	99.96	2.29	1.11	105475	0.00	105714	99.84	3.35	1.06	452144	7.43	339872	98.35	5.23	1.49	3014	6.38	3125 56	98.09	6.15	1.02	35489	3.23
32℃	WS	1198711	-	2.21	1.05	105674	0.00	103485	-	3.27	1.05	442978	7.43	347216	-	5.15	1.53	2954	6.38	3125 87	-	6.11	1.03	37551	3.21
	DP	1195544	99.74	2.21	1.08	105324	0.00	103254	99.78	3.27	1.03	445822	7.43	346538	99.80	5.15	1.52	2916	6.38	3121 45	99.86	6.11	1.05	35214	3.21

There was no significant change in Retention time, Asymmetry, Resolution and Theoretical plate after change the column temperature to 28°C and 32°C. The method is robust from change in column temperature by 2°C.

7.2.9.2. Change in Mobile Phase Composition (MP-A: O-phosphoric acid; MP-B: Methanol)

MP Change																									
Condition	Sample	A-LA						MeCbl						FA						PDX					
		Area	Assay	RT	Asymmetry	TP	Resolution	Area	Assay	RT	Asymmetry	TP	Resolution	Area	Assay	RT	Asymmetry	TP	Resolution	Area	Assay	RT	Asymmetry	TP	Resolution
MPA: 52 MPB: 48	WS	1122486	-	2.33	1.01	1063621	0.00	1051023	-	3.39	1.08	45210	7.43	3421757	-	5.27	1.52	2905	6.38	3208064	-	6.19	1.04	371522	3.23
	DP	1123135	100.06	2.33	1.12	1063838	0.00	1052333	100.10	3.39	1.07	45982	7.43	3421220	99.99	5.27	1.52	2966	6.38	3192777	99.51	6.19	1.02	371299	3.23
MPA: 50 MPB: 40	WS	1134214	-	2.29	1.04	1082893	0.00	1058587	-	3.35	1.04	45274	7.43	3455564	-	5.23	1.51	2903	6.38	3186638	-	6.15	1.04	375733	3.23
	DP	1133785	99.96	2.29	1.11	1054775	0.00	1057714	99.84	3.35	1.06	45214	7.43	3398772	98.35	5.23	1.49	3014	6.38	3125556	98.09	6.15	1.02	354889	3.23
MPA: 48 MPB: 52	WS	1122357	-	2.24	1.05	1076778	0.00	1021223	-	3.30	1.05	45674	7.43	3536332	-	5.17	1.56	3015	6.38	3168657	-	6.11	1.03	375688	3.23
	DP	1121575	99.93	2.24	1.06	1076725	0.00	1012336	99.13	3.30	1.05	45660	7.43	3523556	99.64	5.17	1.56	3054	6.38	3164992	99.88	6.11	1.04	372599	3.23

There is no significant change in Retention time, Theoretical plats, Asymmetry and resolution of the peaks after change in Mobile phase by 2%. Implying the method is robust to change in Mobile phase by 2%.

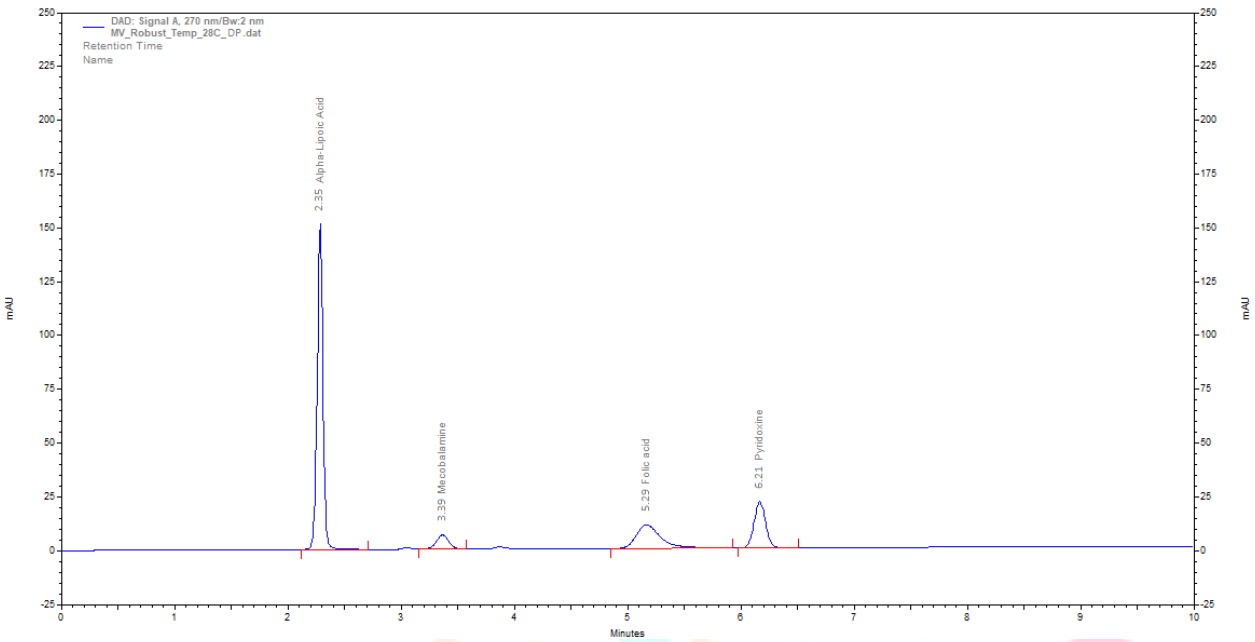


Figure No. Robustness Column Temperature 28C Drug Product

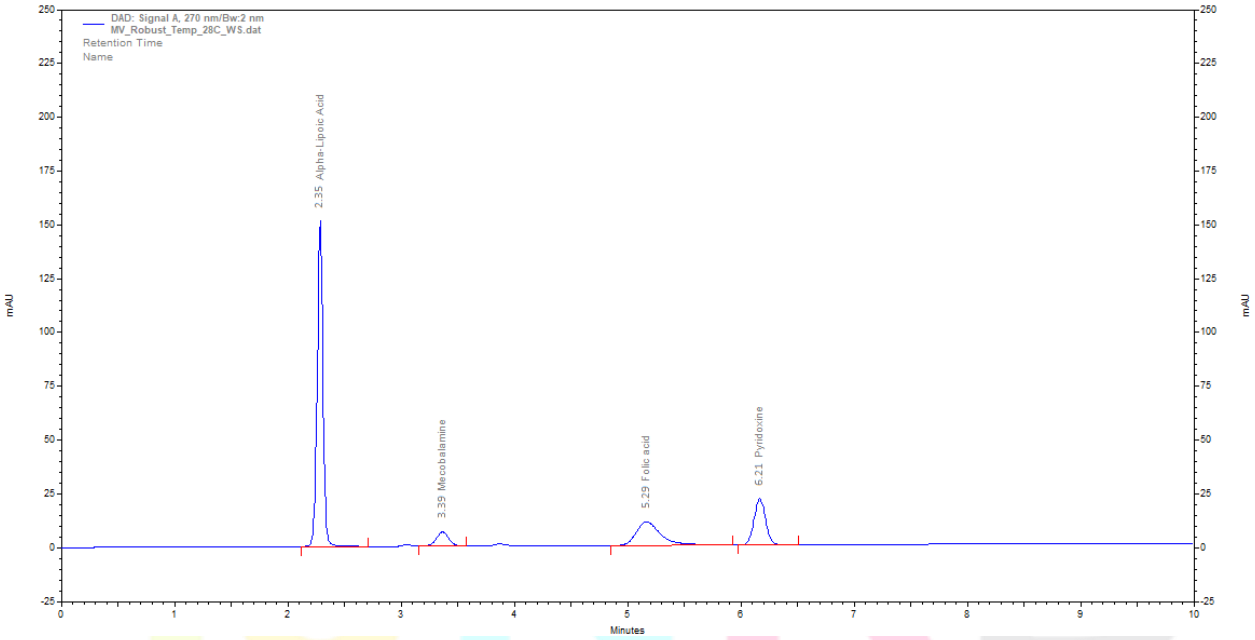


Figure No. Robustness Column Temperature 28C Working Standard

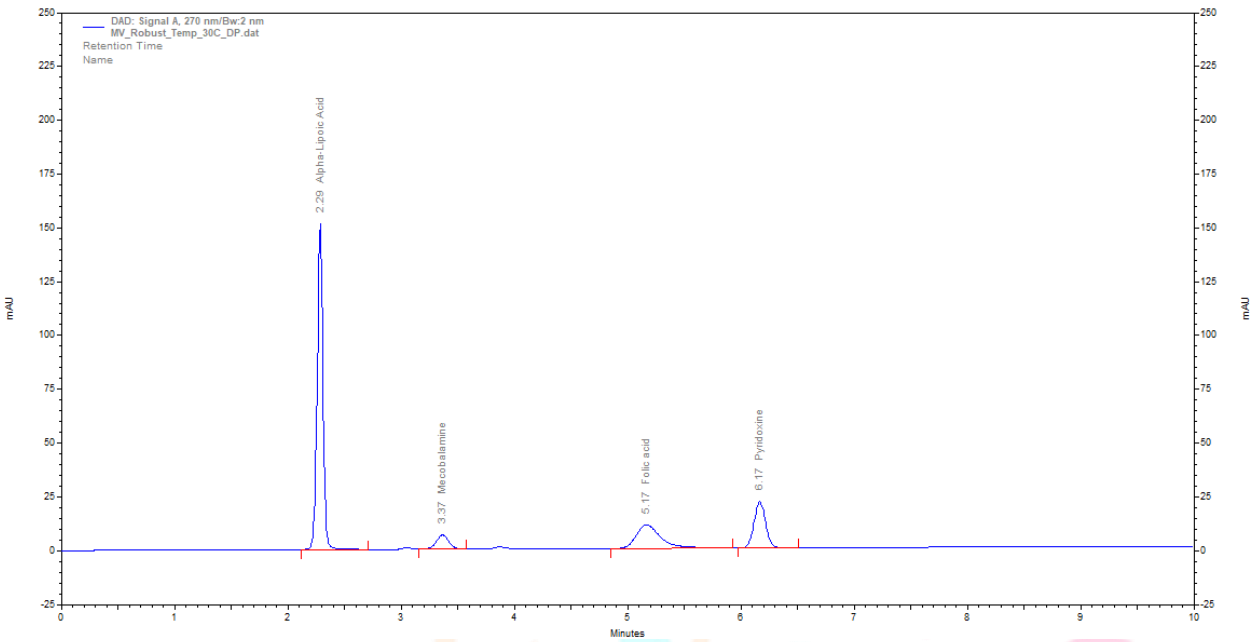


Figure No. Robustness Column Temperature 30C Drug Product

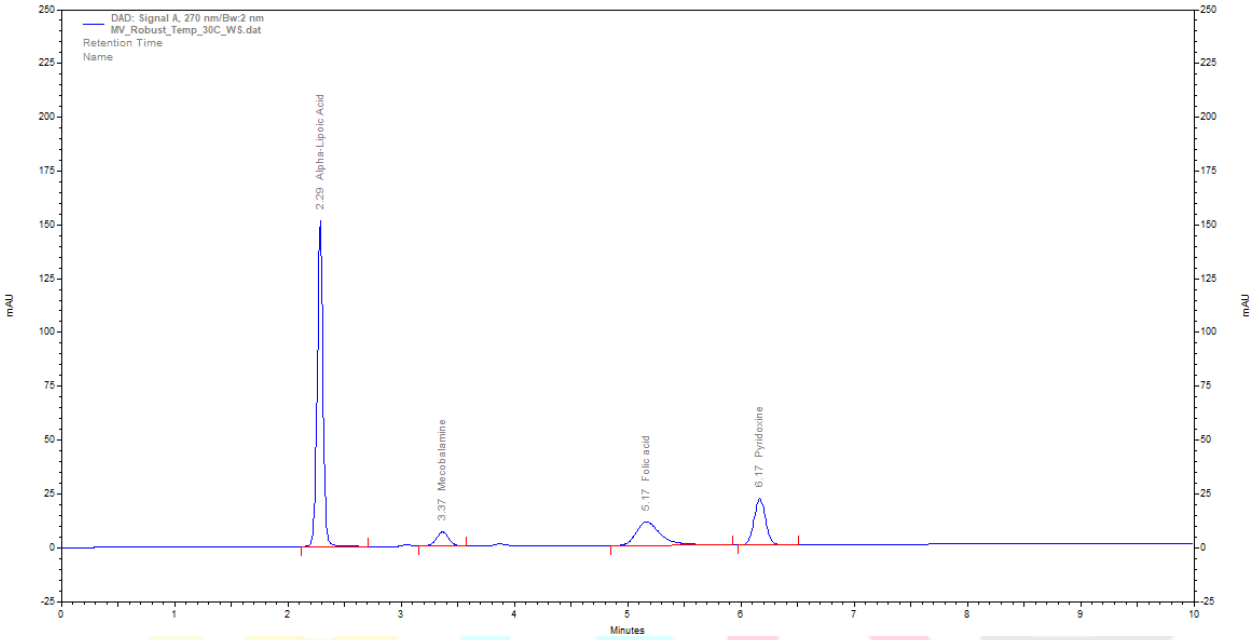


Figure No. Robustness Column Temperature 30C Working Standard

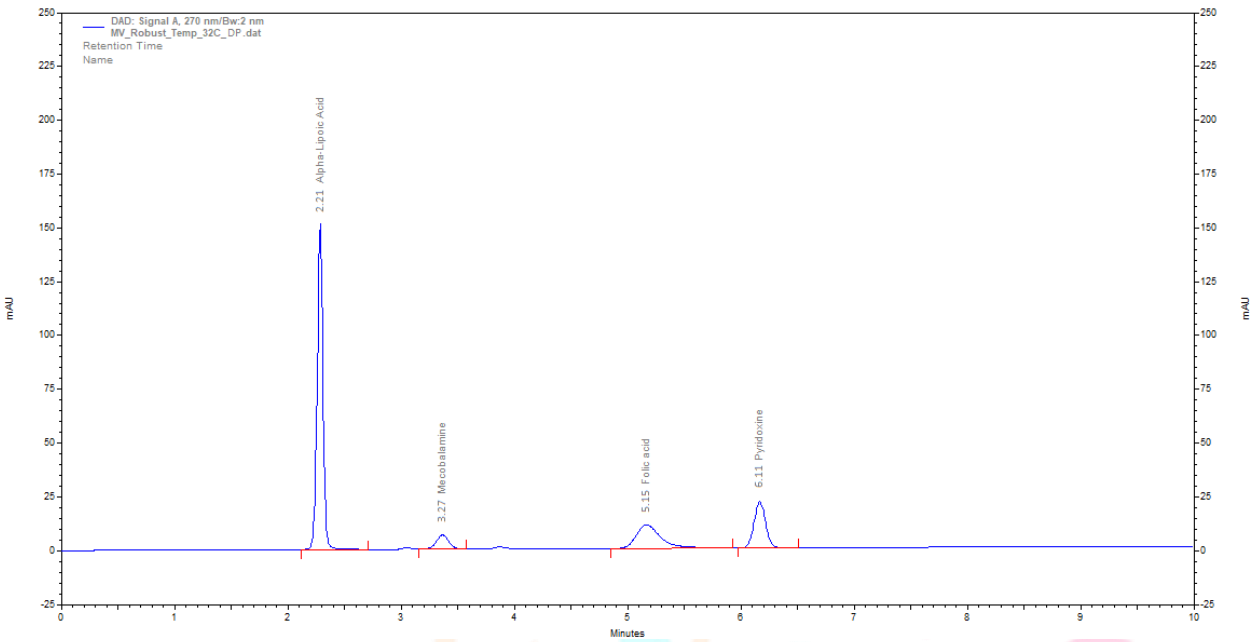


Figure No. Robustness Column Temperature 32C Drug Product

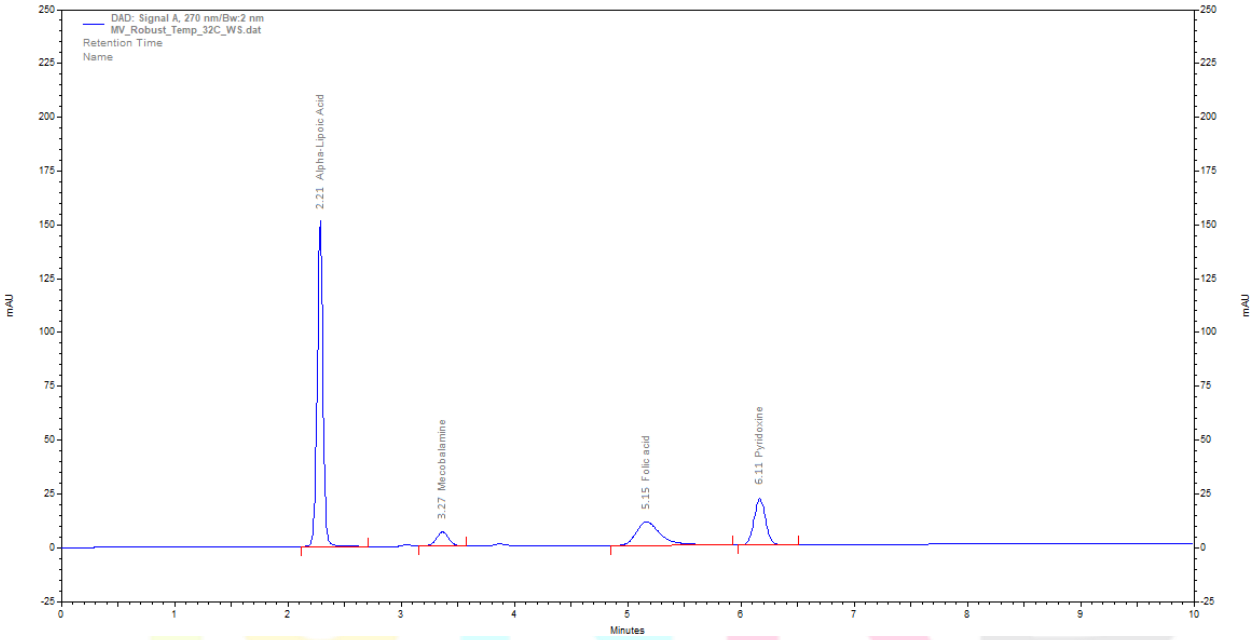


Figure No. Robustness Column Temperature 32C Working Standard

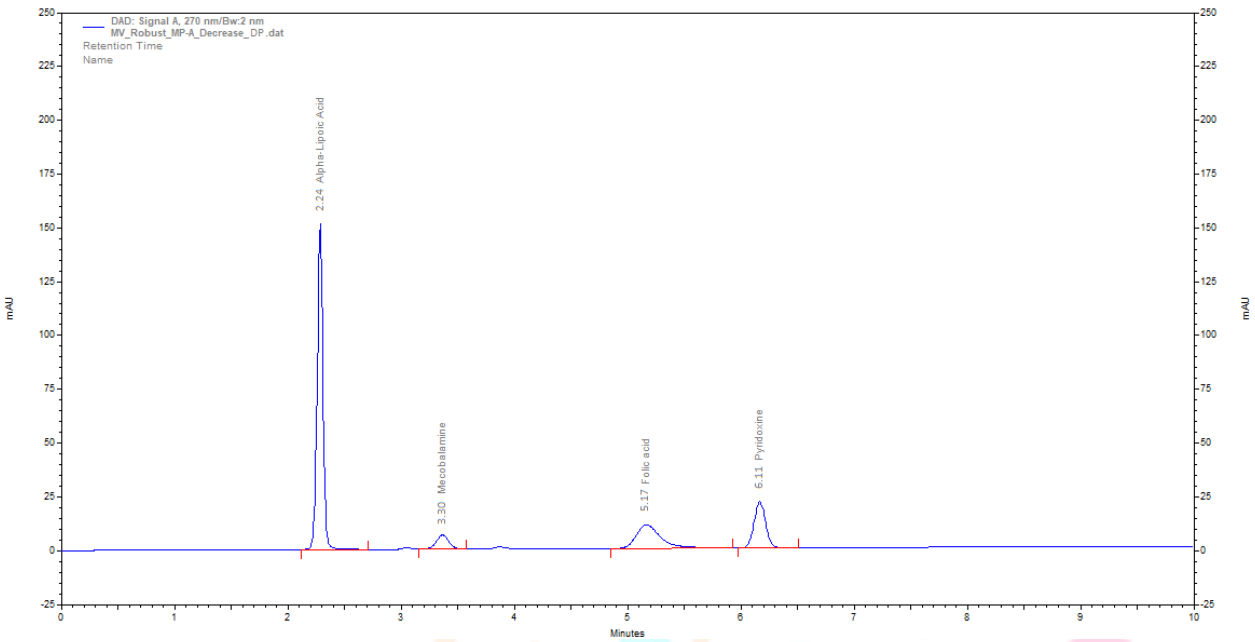


Figure No.Robustness Mobile Phase-A Decrease Drug product.

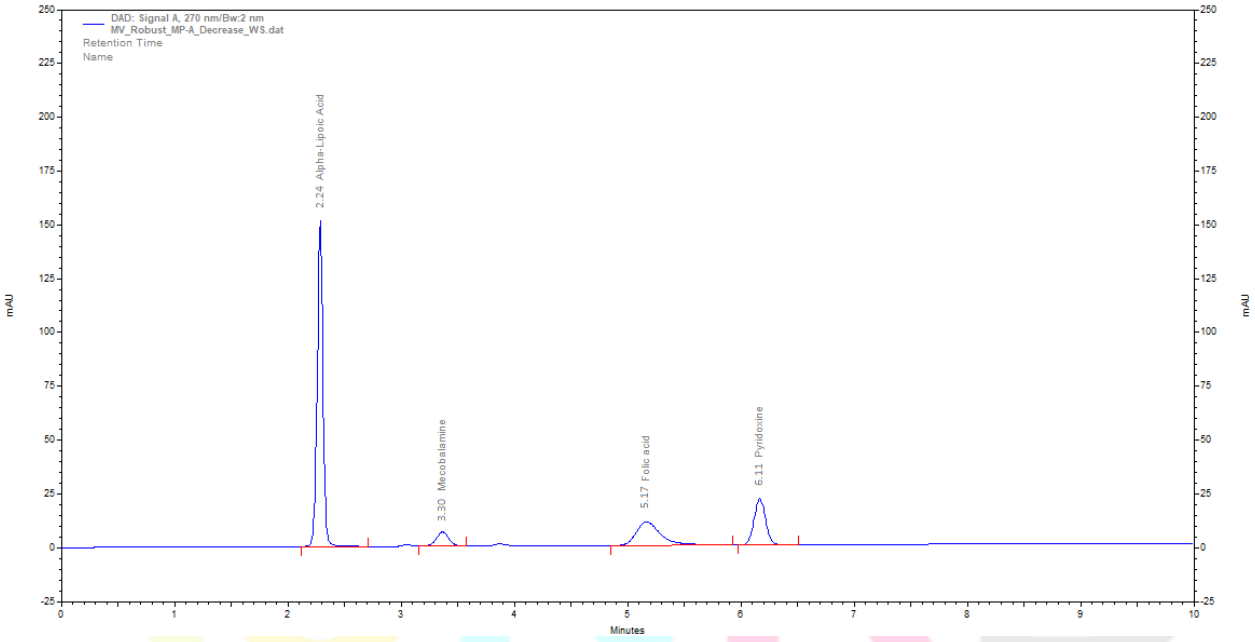


Figure No.Robustness Mobile Phase-A Decrease Working standard

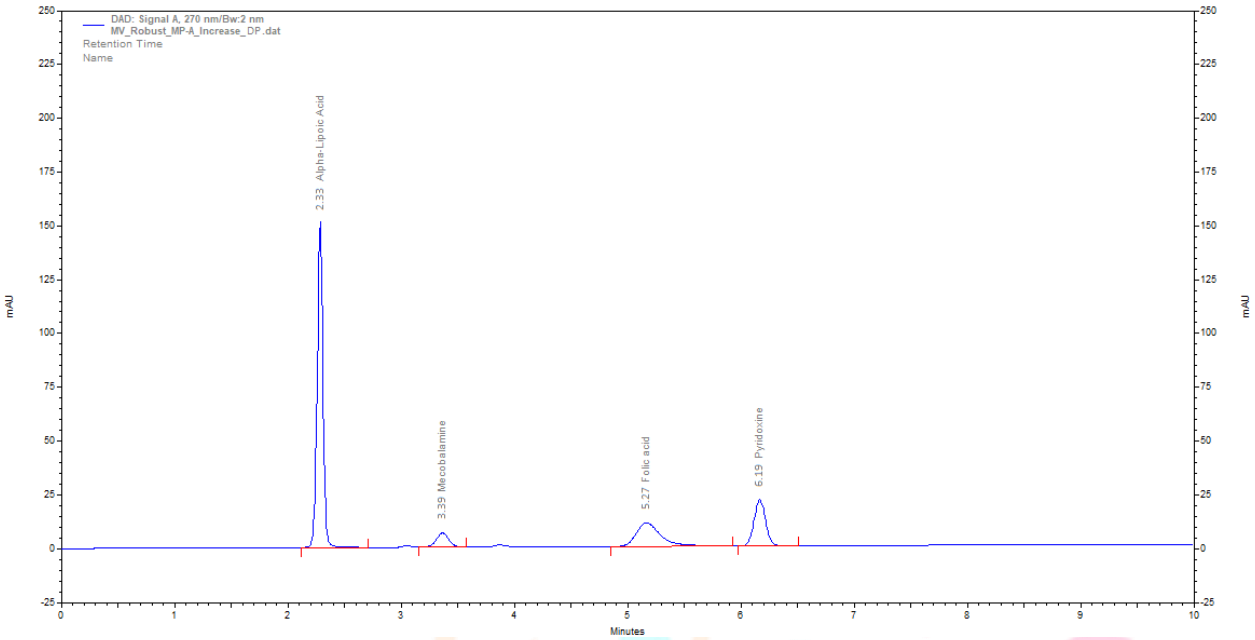


Figure No.Robustness Mobile Phase-A Increase Drug product.

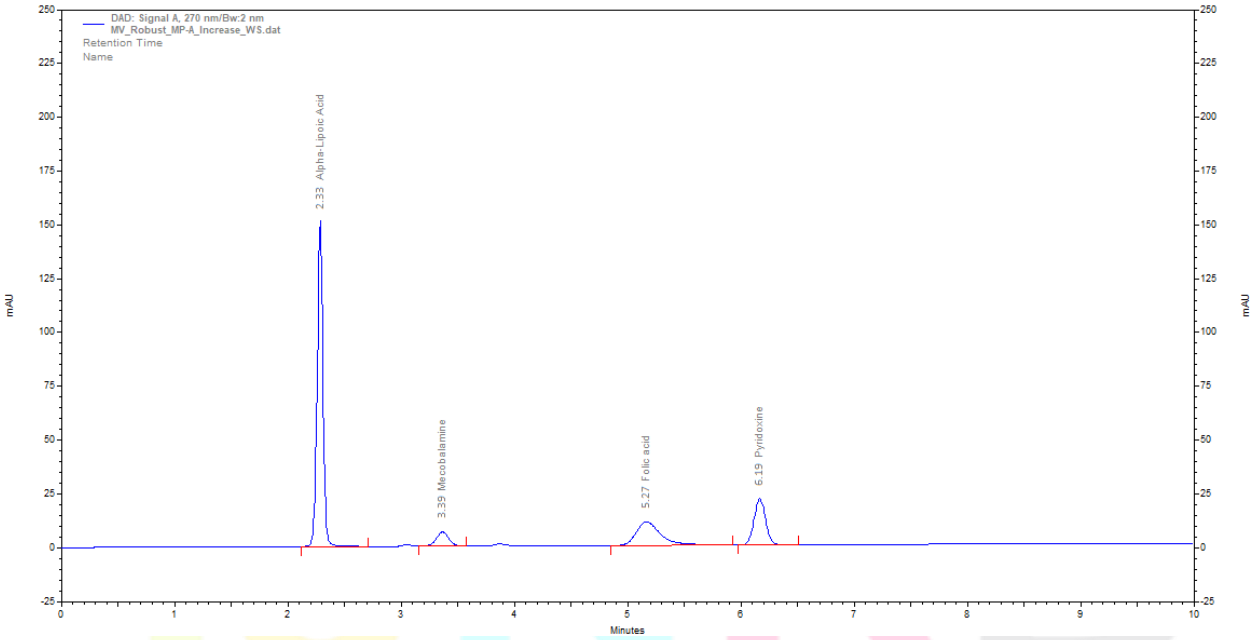


Figure No.Robustness Mobile Phase-AIncrease Working Standard.

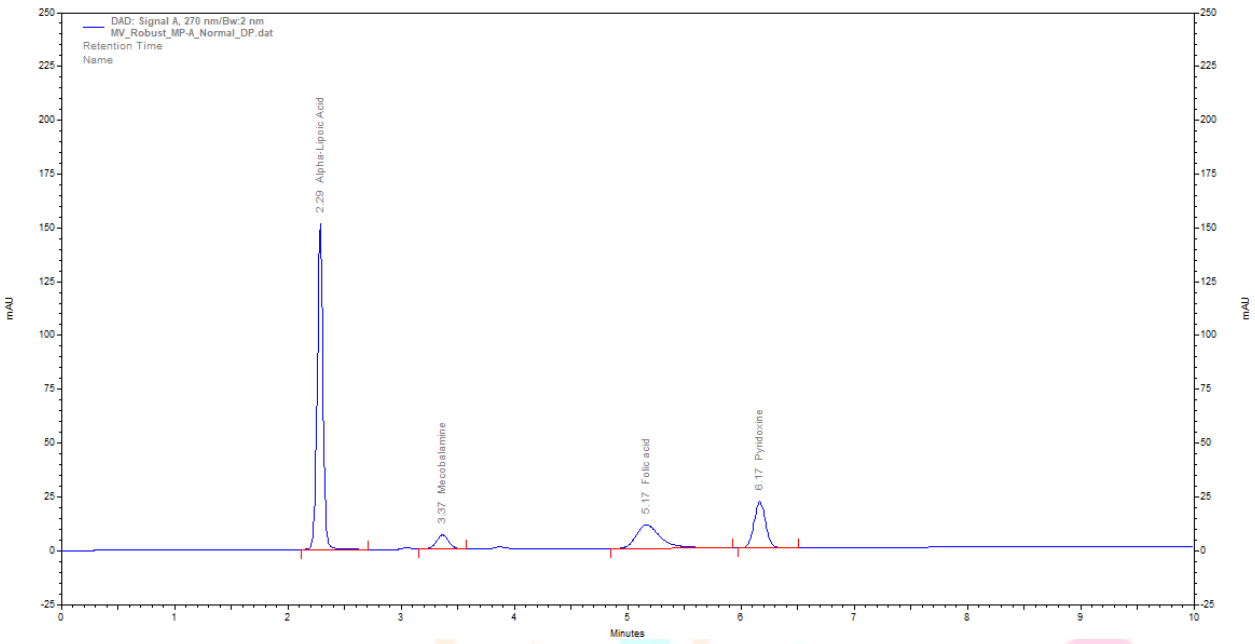


Figure No.Robustness Mobile Phase-A NormalDrug product.

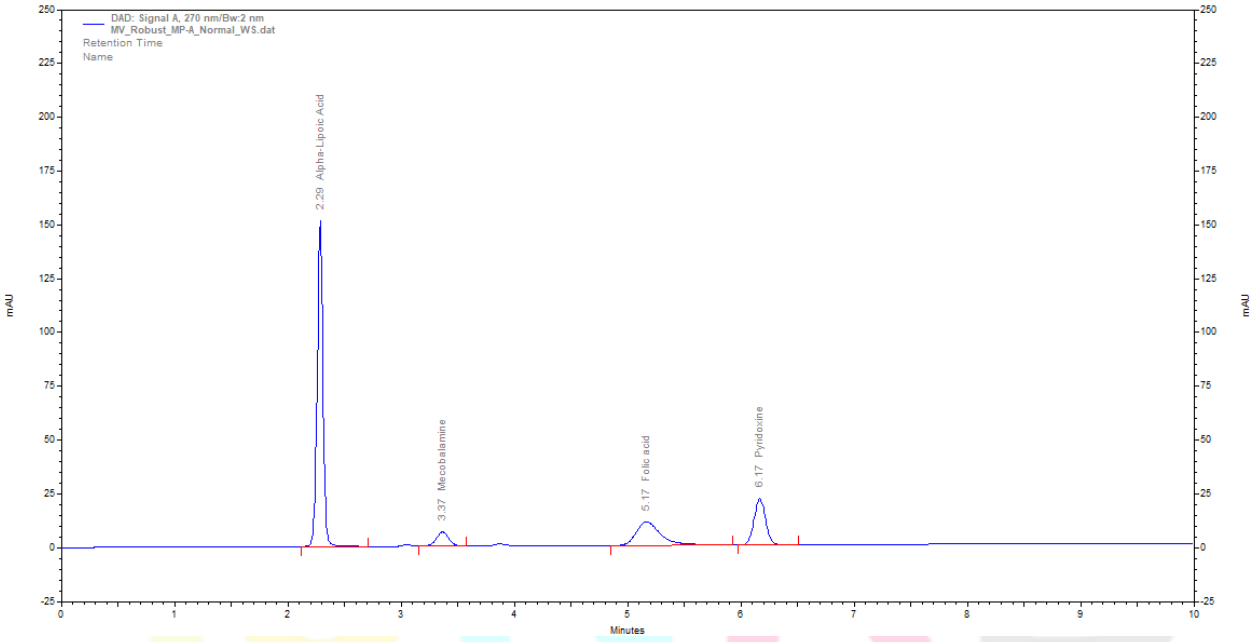


Figure No.Robustness Mobile Phase-A Normal Working Standard.

7.2.10. Intra Day precision

Intra-day precision was performed for working standard solution. Analysis observed is reported below:

Table No. Intraday Precision

Intra Day precision									
Day 1	Sample ID	A-LA		MeCbl		FA		PXD	
		Area	Assay	Area	Assay	Area	Assay	Area	Assay
Morning	WS	1134214	-	105887	-	345564	-	318638	-
	DP	1133785	99.96	105714	99.84	339872	98.35	312556	98.09
Evening	WS	1132556	-	104367	-	342168	-	316475	-
	DP	1123548	99.20	104221	99.86	346552	101.28	315476	99.68

There no significant change in the peak area of the working standard and drug product. Therefore the solution is stable for 24 hours. And the method is precise if performed at 2 different times on a same day.

7.2.11. Inter day precision

Inter day precision was performed for working standard solution. Analysis observed is reported below:

Inter Day precision									
Day	Sample ID	A-LA		MeCbl		FA		PXD	
		Area	Assay	Area	Assay	Area	Assay	Area	Assay
Day 2	WS	1123256	-	104662	-	349821	-	318242	-
	DP	1121485	99.84	104281	99.64	349732	99.97	318120	99.96

The Method is precise for inter day analysis. Therefore, the solution is stable for 48 hours. And the method is precise if performed at 2 different times on 2 different days.

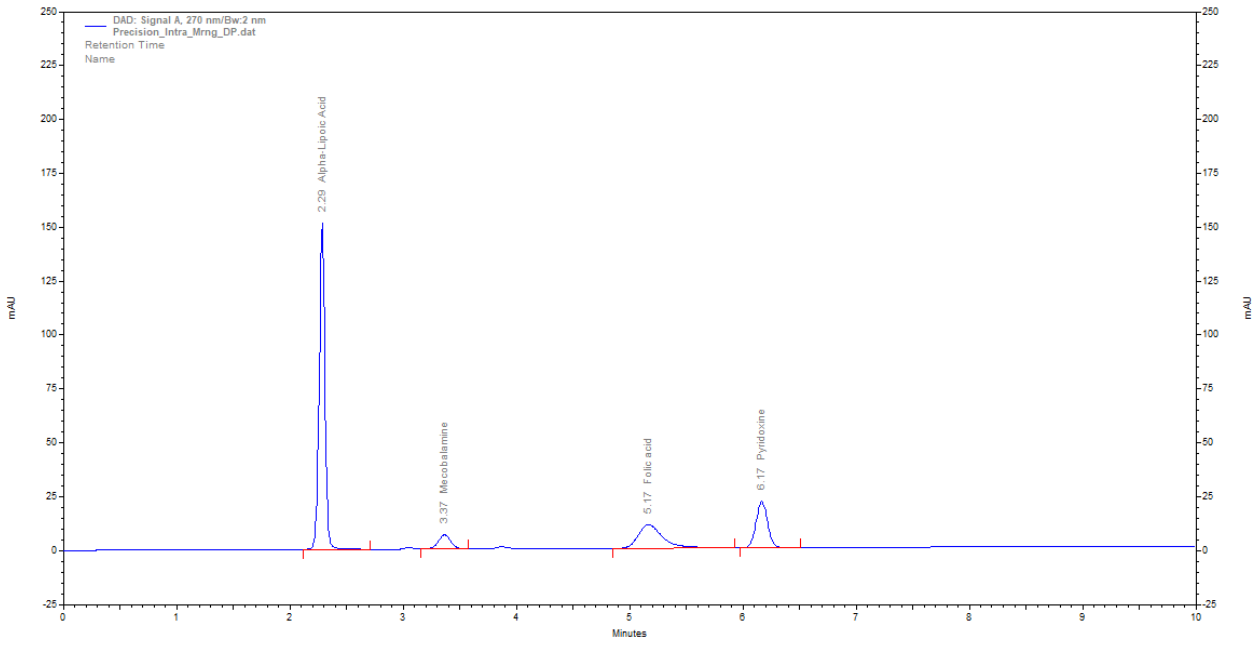


Figure No. : Intraday Morning analysis Drug Product

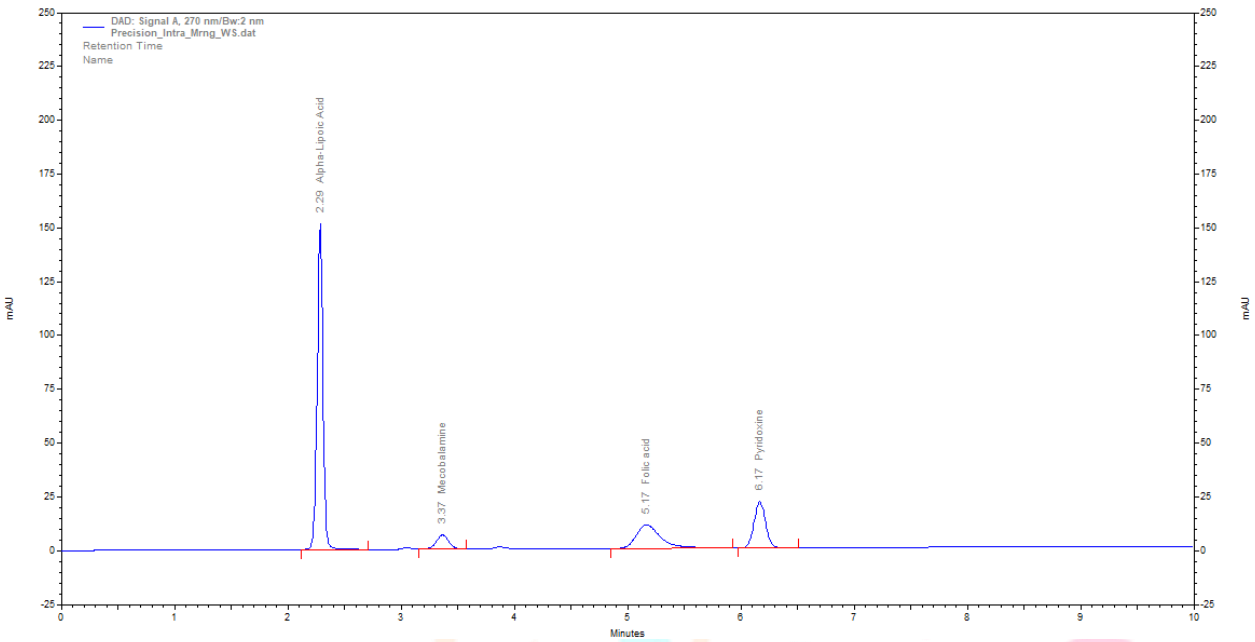


Figure No. : Intraday Morning analysis Working Standard

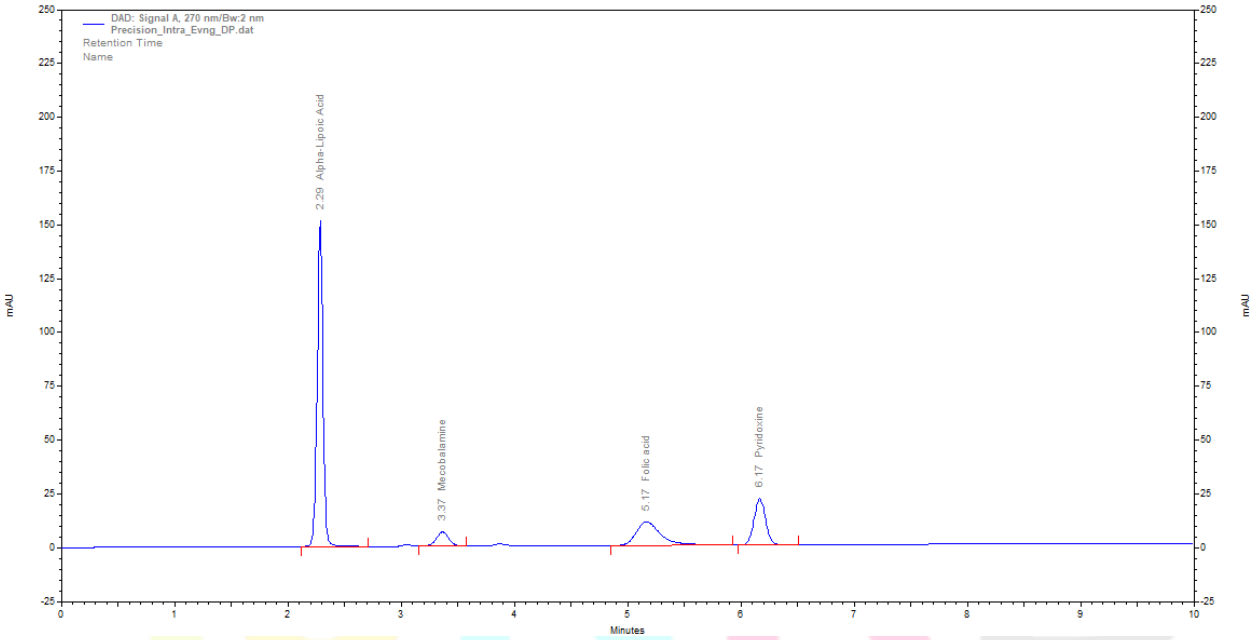


Figure No. : Intraday Evening analysis Drug Product

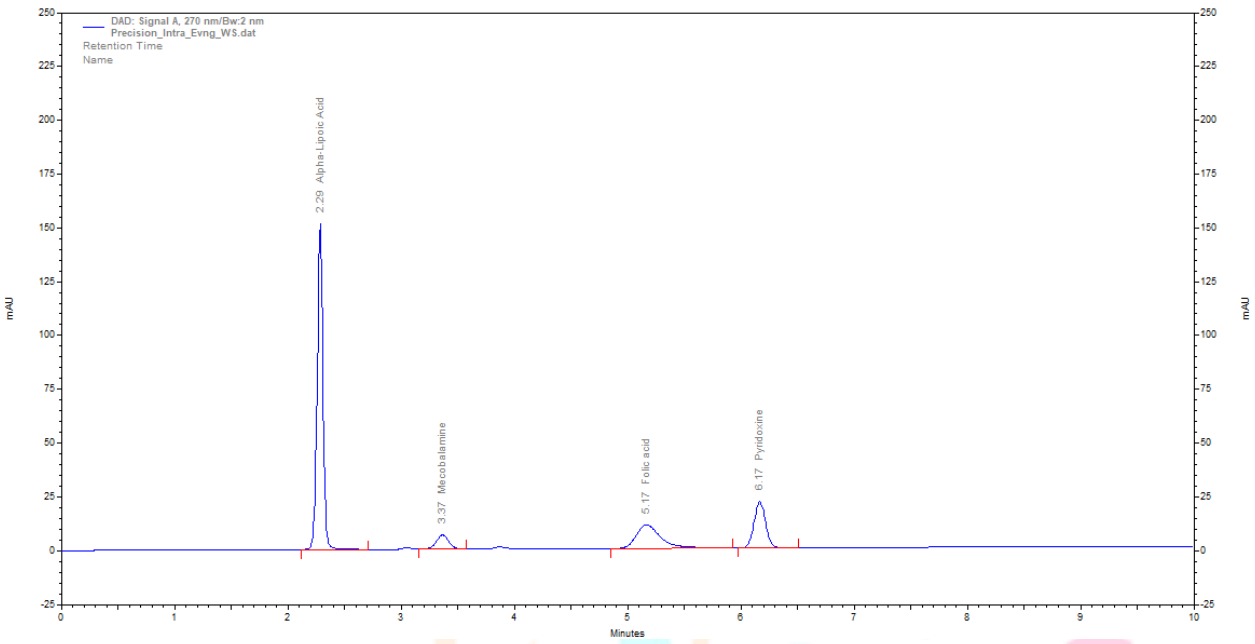


Figure No. : Intraday Evening analysis Working Standard

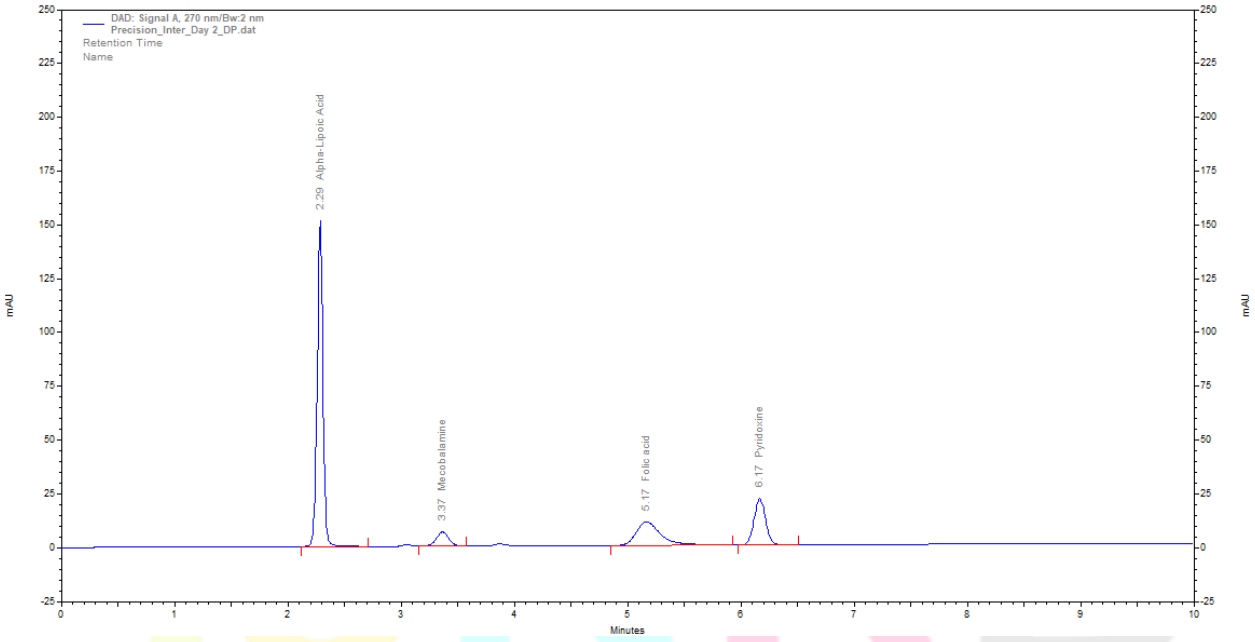


Figure No. : Inter day 2 analysis Drug Product

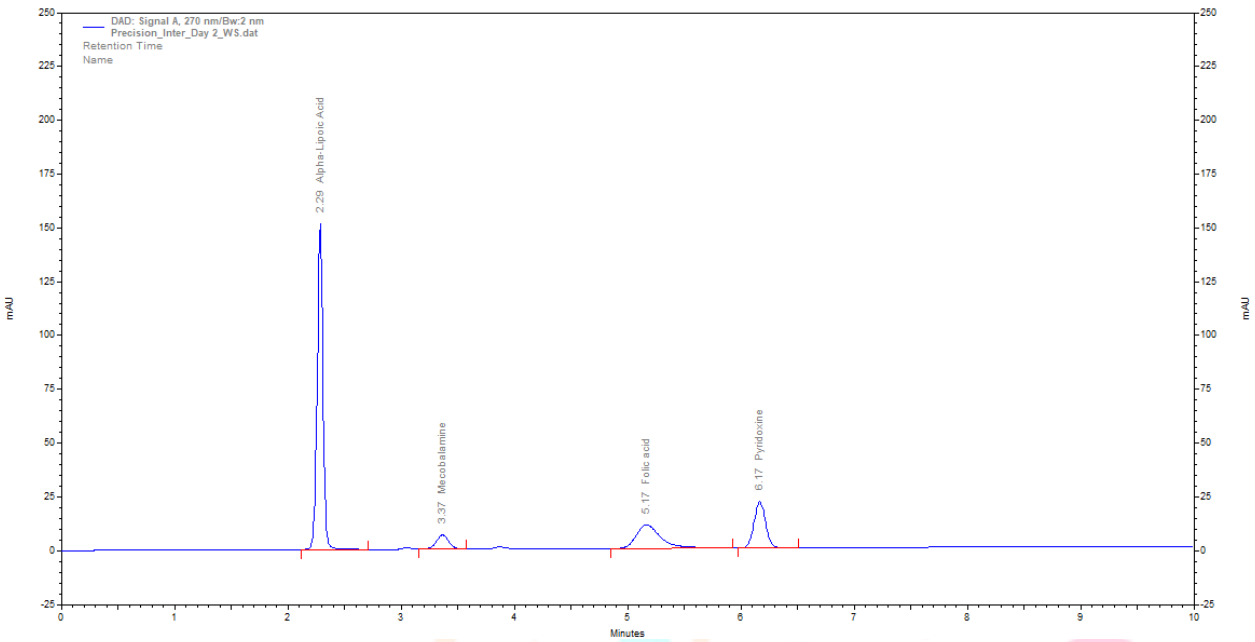


Figure No. : Inter day 2 analysis Working Standard

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