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Method Development and Validation of Gabapentin, Methyl cobalamin and their degradative products by RP-HPLC

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Abstract

A new RP-HPLC method was developed for the determination of Gabapentin and mecobalamin. The HPLC method was then validated to indicate that the analytical procedure used is suitable for intended use. The HPLC separation and quantification was achieved on waters C18(250x4.6mm x 5□). The mobile phase was prepared by mixing orthophosphoric acid and methanol in the ratio of (500:500) v/v that run isocratically at the flow rate of 0.8ml/min. The temperature maintained in sample compartment is 25oC. The injection volume is 10□l. the wavelength at which detector was set is 275nm.

The wide linearity range, Accuracy, Short retention times, & Simple Mobile phase imply that the proposed method can be successfully employed for routine quantification of Gabapentin &Mecobalamin in combined dosage form. The method is economic too as the cost of mobile phase used is less compared to costly solvents that has to be used like acetonitrile for the quantification of GBP & MCB.

Also the forced degradation studies imply that this method is Stability Indicating Method development & validated according to ICH guidelines, one can adopt in an industry confidently for routine analysis.

Keywords: Gabapentin, Mecobalamin ,RP-HPLC

1. INTRODUCTION

Analytical chemistry is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and the quantitative measurement of the substances present in bulk and pharmaceutical preparation.

Measurements of physical properties of an analytes such as conductivity, electrode potential, light absorption or emission, mass to charge ratio, and fluorescence, began to be used for quantitative analysis of variety of inorganic and biochemical analytes. Highly efficient chromatographic and electrophoretic techniques began to replace distillation, extraction and precipitation for the separation of components of complex mixtures prior

to their qualitative or quantitative determination. These newer methods for separating and determining chemical species are known collectively as instrumental methods of analysis. Most of the instrumental methods fit into one of the three following categories vise spectroscopy, electrochemistry and chromatography.

Advantages of instrumental methods

- Small samples can be used
- High sensitivity is obtained
- Measurements obtained are reliable
- Determination is very fast
- Even complex samples can be handled easily

Limitations of instrumental methods

- An initial or continuous calibration is required
- Sensitivity and accuracy depends on instrument
- Cost of equipment is large
- Concentration range is limited
- Specialized training is needed
- Sizable space is required

Principle types of chemical instrumentation:

Spectrometric techniques

1. Ultraviolet and visible spectrophotometry
2. Fluorescence and phosphorescence spectrophotometry.
3. Atomic Spectrometry (emission and absorption)
4. Infrared Spectrophotometry
5. Raman Spectroscopy
6. X-Ray Spectroscopy
7. Radiochemical Techniques including activation analysis
8. Nuclear Magnetic Resonance Spectroscopy
9. Electron Spin Resonance Spectroscopy

Electrochemical techniques

1. Potentiometric
2. Voltammetry
3. Voltammetry Techniques
4. Amperometry Techniques
5. Colorimetric
6. Electro gravimetric
7. Conductance Techniques

Chromatographic techniques

1. Gas Chromatography
2. High Performance Liquid Chromatography
3. High Performance Thin Layer Chromatography

Miscellaneous techniques

1. Thermal Analysis
2. Mass Spectrometry
3. Kinetic Techniques
4. Hyphenated techniques
5. GC-MS (Gas Chromatography – Mass Spectrometry)
6. GC-IR (Gas Chromatography – Infrared Spectroscopy)
7. MS-MS (Mass Spectrometry – Mass Spectrometry)

1.1 INTRODUCTION TO HPLC:

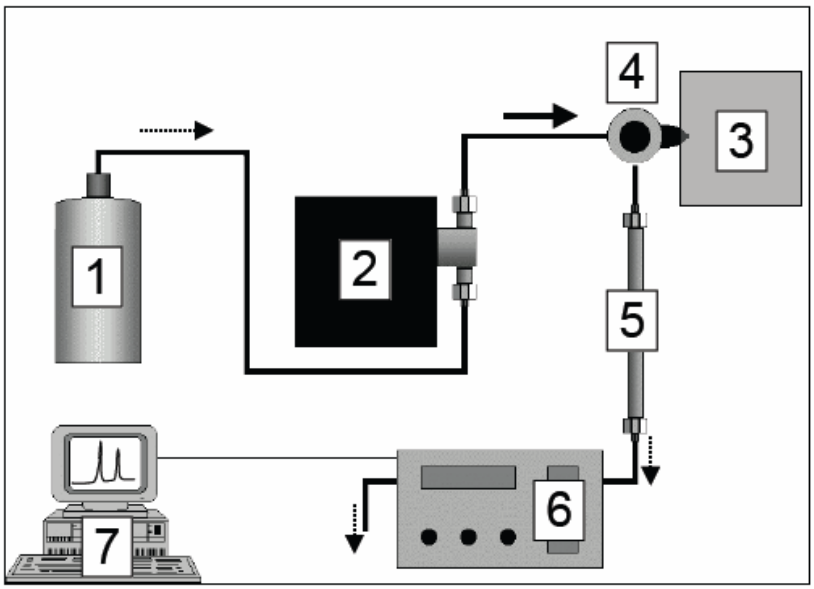
HPLC is a type of liquid chromatography that employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rate liquid must be pressurized to a few thousands of pounds per square inch.

The rate of distribution of drugs between Stationary and mobile phase is controlled by diffusion process. If diffusion is minimized faster and effective separation can be achieved .The techniques of high performance liquid chromatography are so called because of its improved performance when compared to classical column chromatography advances in column chromatography into high speed, efficient ,accurate and highly resolved method of separation.

For the recent study metformin and Sitagliptin was selected for estimation of amount of analyte present in formulation and bulk drug. The HPLC method is selected in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages

- Speed many analysis can be accomplished in 20min (or) less.
- Greater sensitivity(various detectors can be employed).
- Improved resolution(wide variety of stationary phases).
- Reusable columns(expensive columns but can be used for many analysis).
- Ideal for the substances of low viscosity.
- Easy sample recovery, handling and maintenance.
- Instrumentation leads itself to automation and quantification (less time and less labour).
- Precise and reproducible.
- Integrator itself does calculations.
- Suitable for preparative liquid chromatography on a much larger scale.

HPLC Basic Instrumentation:



1 – Reservoir

2 – Pump

3 – Auto Sampler

4 – Injection Valve

5 – Column

6 – Detector

7 – Data System

Fig 1.1: Schematic diagram of a basic HPLC system.

HPLC components

The essential components of a complete HPLC system are solvent delivery system(Pump), detector, fixed volume injector loop or auto sampler, solvent reservoirs, packed column, data system and recorder. A schematic of a simplified HPLC system is shown in Figure 1.

Column

The column is probably the heart of HPLC system. The development of this column technology leads to the evolution of the HPLC instrumentation systems used today. The conventionally used HPLC columns are particle packed columns. The key of column selection when previous separation is not available resides in knowing the chemistry of the sample. Columns should never be dry. A dry column will eventually have voids because the packing will shrink away from the wall, which would result in band broadening. Before running a sample in HPLC the column should be equilibrated. Usually column equilibrium is achieved after passage of 10 – 20 column volumes of the new mobile phase through the column. Insufficient column equilibrium usually leads to retention difference.

Pump

The solvent delivery system or as it is commonly called the pump includes two major types, constant volume or flow and constant pressure. Constant volume pumps are mechanically driven systems, most commonly using screw driven syringes or reciprocating pistons. On the other hand, constant pressure pumps are driven or controlled by gas pressure.

Injector or Auto sampler

Samples are usually introduced by syringe injection via a manual injector into the mobile phase stream or by the use of an auto sampler. The important aspects in sample introduction are precise and reproducible injections. This is especially important with quantitative analysis where the reproducibility of the peak response is dependents on the precision of the sample introduction. Direct syringe injection through a manual injector was the first popular method of sample introduction. As

HPLC instrumentation evolved, many auto sampler techniques were applied so that sample introduction has become more precise and rapid.

Detector

HPLC detectors include ultraviolet-visible, fluorescence, electrochemical, refractometer, mass spectrometer and others. The UV visible absorption detector is the most widely used detector in liquid chromatography, since most organic compounds show some useful absorption in the UV region. This detector is fairly universal in application, although sensitivity depends on how strongly the sample absorbs light at particular wavelength.

Solvent reservoir

Different containers are used as a solvent delivery system reservoir. The best material from which the containers are made is glass. Plastic containers are not recommended as it leads to plasticizer leaching. The container should be covered to prevent solvent evaporation. The tubing from the reservoir can be made of stainless steel or Teflon, and both are satisfactory.

Data handling and analysis

Data handling in HPLC is as important to the success of any experiment or analysis as any other components in the system. It is part of good HPLC techniques to properly label and document the analytical results. The advanced computer software used now in data handling and analysis allow easy recording and storage of all chromatographic data.

Types of HPLC techniques

Based on modes of chromatography:

- Normal phase chromatography
- Reverse phase chromatography

Based on principle of separation:

- Adsorption chromatography
- Ion exchange chromatography
- Size exclusion chromatography
- Affinity chromatography
- Chiral phase chromatography

Based on elution technique

- Isocratic separation
- Gradient separation

Based on the scale of operation

- Analytical HPLC
- Preparative HPLC

Normal phase chromatography

In normal phase mode the stationary base (eg; silica gel) is polar in nature and the mobile phase is non polar. In this technique, non polar compound travel faster and are eluted first. This is because less affinity between solute and stationary phase and take more time to elute.

Reverse phase chromatography

The popularity of reversed phase liquid chromatography is easily explained by its unmatched simplicity, versatility and scope. Neutral and ionic analytes can be separated simultaneously. Retention in RPLC is believed to occur through nonspecific hydrophobic interaction of the solute with the stationary phase. The near universal application of RPLC stems from the fact that almost all organic compound shave hydrophobic regions in their structure and are capable of interacting with the stationary phase.

A decrease in the polarity of the mobile phase leads to a decrease in retention. It is also generally observed in RPLC that branched chain compounds are retained to lesser extent than their straight chain analogues and that unsaturated compounds are eluted before their fully saturated analogs. A wide variety of RP-HPLC columns are available. Most columns are silica based. Silica offers good mechanical stability. A typical stationary phase is formed by chemically bonding a long-chain hydrocarbon group to porous silica. Typical ligands are n-octadecyl (C18), n-octayl (C8), n-butyl (C4), diphenyl (C2),and cyano propyl.

Parameters affecting separation:

Separation in reversed phase chromatography is affected by stationary phase type and column length. It is also affected by organic solvent type and percentage in the mobile phase and by mobile phase pH. Flow rate could also affect separation in reversed phase chromatography; however it is usually limited by the developed backpressure. Moreover temperature of the column also has an effect on separation.

Parameters affecting separation in reversed phase chromatography are shown

Schematically:

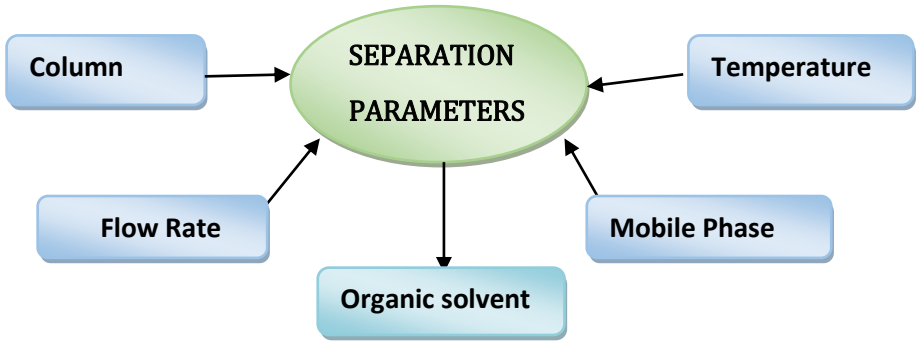


Fig 1.2. Parameters affecting separation in reversed phase chromatography

Quantitative Methods in chromatography:

Internal standard method : In this technique a known quantity internal standard is chromatographed and area is ascertained then a quantity of the internal standard is added to the raw sample prior to any sample pretreatment or separation operation .The peak area of the standard in sample run is compared with the peak are when the standard is run separately This ratio serves as correction factor for variation in sample size ,for losses in any preliminary operations, or for incomplete resolved adjacent sample component, must not interfere with the sample component and must never be present in sample.

$$\text{Area ratio} = \frac{\text{Area of sample}}{\text{Area of internal standard}}$$

$$\text{Sample concentration} = \frac{\text{Area of sample}}{\text{Area of internal standard}} \times \text{concentration of standard}$$

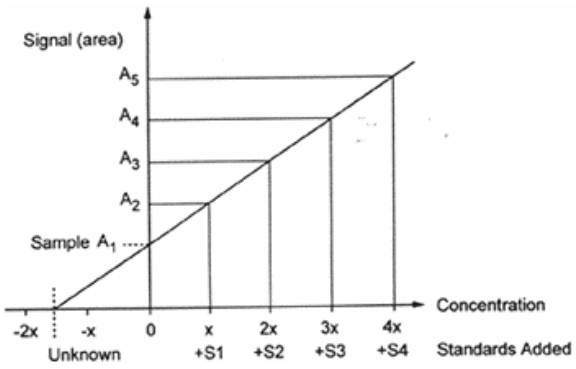
This technique is often used for the samples having components. It is used to evaluate the absolute purity of sample .the procedure is to total up the areas under all peaks and then calculate the percentage of total area that is contributed by compound of interest. For this method the entire sample must be eluted all components must be separated and peak must be completely resolved.

Standard addition method

Standard addition method is used in many techniques in analytical chemistry. It is of limited use in chromatography because of the difficulty of injecting accurately known amounts of sample. A sample mixture is analyzed for the analyte of interest by adding a specified amount of this analyte to the sample, thus increasing its concentration. The analysis is then repeated and the resulting increase in peak area due to addition of the standard amount is noted. Hence, the concentration of the analyte in the original sample may be calculated. If the peak area for the first analysis is A1 and with the standard addition of x mg is A2, then the peak area corresponding to x mg (or x mg/litre) is (A2 - A1). Thus, the original amount of the analyte x in the sample corresponding to A1, is given by

Amount x = (x A1) / (A2 - A1) mg/litre.

An allowance for dilution due to addition of the standard amount has to be made. The main difficulty with this method concerns the reproducibility of the sample injection. A precision of better than 1 % should be achieved if valid quantitative results are to be obtained. An alternative approach is first to analyse the sample, noting the area, A1,for the analyte. Successive standard amounts of the analyte are then added, each sample standard mixture being analysed and the areas recorded. A graph of peak area versus concentration is drawn and the amount of analyte in the sample obtained by extending the calibration line to intersect the abscissa as shown in graph.



Graph 1.1 Calibration graph for the API

External standard method

Automated sample injection systems and multiport injection valves (HPLC)have good reproducibility so that a series of injections can be made with a variation in sample volume of < 1 %. A set of standard mixtures containing known concentrations of the analytes is analysed and their peak areas recorded. A calibration graph of area versus concentration can be drawn for each analyte to confirm a linear detector response and from

which the amount of the analyte in a mixture can be determined. Alternatively for unestablished method a replicate series of one standard mixture is injected and the area/unit amount of analyte calculated.

$A_{STANDARD}$ = xmg/litre.

The mixture is then analysed and the amount of the components in the sample calculated using the peak area data for the standard mixture. Therefore, if the recorded peak area for the component in a sample mixture is AMIX then the amount of component x is

Amount x = (x AMIX) / (ASTANDARD) mg/litre.

PARAMETERS USED IN HPLC

Retention time (Rt);

Retention time is the difference in time between the point of injection and appearance of peak maxima .Retention time is the time required for 50% of a component to be eluted from a column Retention time is measured in minutes or seconds retention time is also proportional to the distance moved on a chart paper, which can be measured in cm.

Retention volume (Vr):

Retention volume is the volume of mobile phase required to elute 50% of the component from the column .It is the product of retention time and flow rate.

Retention volume = Retention time * flow rate

Tailing factor (T):

The accuracy of quantification decreases with increase in peak tailing as the integrator encounters difficulty in determining where and when the peak ends. Hence, the calculation of the area under the peak differs with tailing. If the integrator is unable to determine exactly when an up slope or down slope occurs, accuracy drops.

$$\mathbf{T = W_x / 2f}$$

Recommendations: T of< 1.5 is preferred.

Resolution (Rs):

Resolution is the measure of extent of separation of two component and the baseline separation achieved. Resolution is generally defined as the distance between the centers of two eluting peaks as measured by retention time or volume divided by average width of respective peaks. for example an Rs value of 1.0 indicates 98% purity has been achieved base line resolution between two well formed peaks indicates 100% purity and requires an Rs value greater than 1.5 resolution can be determined by using the following formula

Rs=2(Rt1—Rt2)

W1+W2

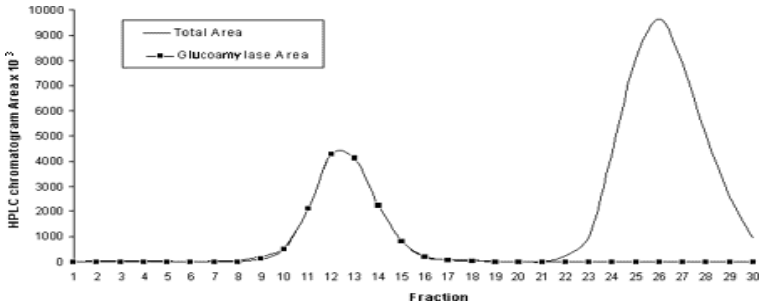
Calculating Rs is the simplified method for quantitating the actual separation achieved between two solute molecules the parameters that contribute to peak resolution column selectivity, column efficiency and the column retention time

Rs = 1/4* (*α-1)* (√N)* *_ k′ _

1 + k'

α=Separation Factor,k'=capacity factor,N=Column Plate Number.

Resolution Rs is a function of selectivity, efficiency (number of theoretical plates N and average retention factor K for peaks 1and2.



Graph 1.2 Resolution for a samples

CHROMATOGRAM

Capacity factor

The capacity factor is related to the retention time and is a reflection of the proportion of time a particular solute resides in the stationary phase as opposed to the mobile phase . long retention times result in large values of K^1.TheCapacity Factor is not same as the available binding capacity ,which refers to the mass of the solute that a specified amount of medium is capable of binding under defined conditions the capacity factor K^1can be calculated.

Capacity Factor= Moles of solute in stationary phase Moles of solute in mobile phase

K^1= Tr—To *Vr—Vo

To Vo

Tr&To are retention times Vr& Vo are retention volumes respectively.

Recommendations:

The peak should be well resolved from other peaks and the void volume. Generally the value of k' is >2.

Efficiency (N): The efficiency of packed column is expressed by number of theoretical plates N. It is a measure of the band spreading of a peak smaller the band spread higher the theoretical plates indicates good column and system performance. Theoretical plates is an imaginary or hypothetical unit of a column where equilibrium has been established between stationary based on mobile phase.

The greater the number of theoretical plates a column has greater its efficiency and corresponding the higher the resolution which can be achieved.

The column plate's number increases with several factors;

1. Well packed columns
2. Longer columns
3. Lower flow rates
4. Smaller column packing particles
5. Lower mobile phase viscosity and higher temperature
6. Smaller samples molecules

The column plates number N is defined by

$$N = 16(Rt/W)^2$$

N = Number of theoretical plates

Rt =Retention time, W=Peak width at base

Manual measurement of baseline bandwidth may be subject to error .Therefore a more practical equation for N is

$$N = 5.54(Rt/W_{1/2})^2 = L/H$$

$W_{1/2}$=Band width at half height,

Selectivity:

Selectivity is equivalent to relative retention time of the solute peaks and unlike efficiency depends strongly on the chemical properties of the chromatography medium.

1.2 ANALYTICAL METHOD DEVELOPMENT

Method development is done

a. For new products
b. For existing products

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available.

Steps of method development

Documentation starts at the very beginning of the development process, a system for full documentation of the development studies must be established. All data relating to these studies must be recorded in laboratory notebook or an electronic database. Analyte standard characterization

1. All known information about the analyte and its structure is collected i.e., physical and chemical properties, toxicity, purity, hygroscopic nature, solubility and stability.
2. The standard analyte (100% purity) is obtained. Necessary arrangement is made for the proper storage (refrigerator, desiccators, freezer).
3. When multiple components are to be analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.
4. Only those methods (MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

Method Requirements

The goals or requirements of the analytical method that need to be developed are considered and the analytical figures of merit are defined. The required detection limits, selectivity, linearity, range, accuracy and precision are defined. Literature search and prior methodology The literature for all types of information related to the analyte is surveyed. For synthesis, physical and chemical properties, solubility and relevant analytical methods. Books, periodicals, chemical manufacturers and regulatory agency compendia such as USP / NF, Association of Official Analytical Chemists (AOAC) and American Society for Testing and Materials (ASTM) publications are reviewed. Chemical Abstracts Service (CAS) automated computerized literature searches are convenient. Choosing a method

a) Using the information in the literatures and prints, methodology is adapted. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples.
b) If there is no prior method for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out. There is usually one compound for which analytical method already exist that is similar to the analyte of interest.

Instrumental setup and initial studies

a) The required instrumentation is setup. Installation, operational and performance qualification of instrumentation using laboratory standard operating procedures (SOP's) are verified.
b) Always new consumables (e.g. solvents, filters and gases) are used, for example, method development is never started, on a HPLC column that has been used earlier.
c) The analyte standard in a suitable injection / introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix. If the sample is extremely close to the standard (e.g., bulk drug), then it is possible to start work with the actual sample.
d) Analysis is done using analytical conditions described in the existing literature.

Optimization

During optimization one parameter is changed at a time, and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan and every step is documented (in a lab notebook) in case of dead ends.

Documentation of analytical figures of merit

The originally determined analytical figures of merit Limit of quantitation (LOQ),Limit of detection (LOD), linearity, time per analysis, cost, sample preparation etc. Are documented.

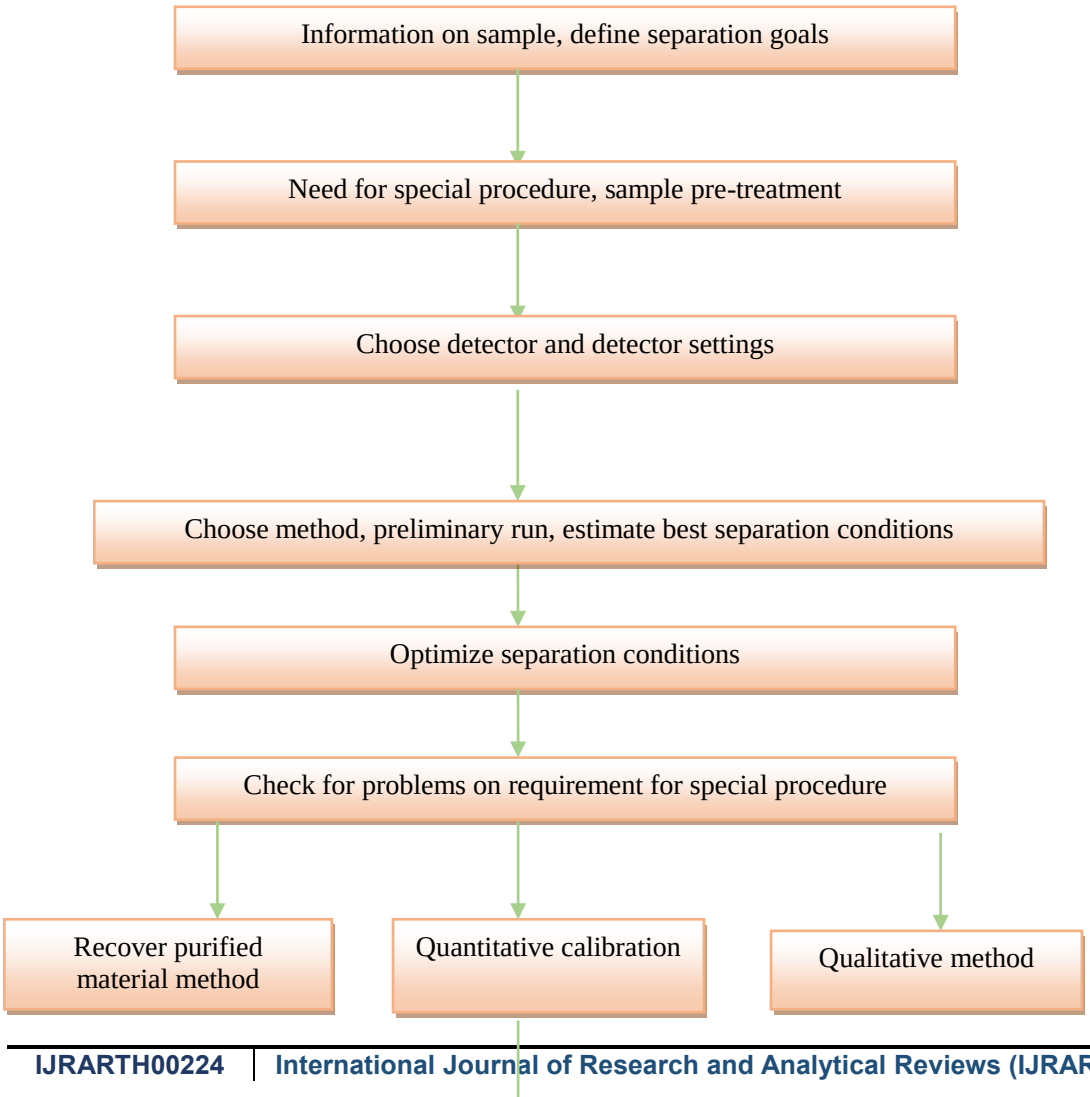
Evaluation of method development with actual samples

The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

Determination of percent recovery of actual sample and demonstration of quantitative sample analysis

Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average +/-standard deviation) from sample to sample and whether recovery has been optimized has been shown. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with a high degree of certainty. The validity of analytical method can be verified only by laboratory studies. Therefore documentation of the successful completion of such studies is a basic requirement for determining whether a method is suitable for its intended applications.

Strategy for Method Development:



Validate the method for release to routine laboratory

Fig No 1.3 Strategy for Method Development

1.3 ANALYTICAL METHOD VALIDATION

Validation is defined as follows by different agencies

Food and Drug administration (FDA):

Establishing documentation evidence, which provides a high degree of assurance that specific process, will consistently produce a product meeting its predetermined specification and quality attributes.

World Health Organization (WHO):

Action of providing that any procedure, process, equipment, material, activity, or system actually leads to the expected results

European Committee (EC)

Action of providing in accordance with the principles of good manufacturing practice, that any procedure, process, equipment material, activity or system actually lead to the expected results. In brief validation is a key process for effective Quality Assurance.

Analytical method validation

Analytical monitoring of a pharmaceutical product or of specific ingredients within the product is necessary to ensure its safety efficacy throughout all phases of its shelf life. Such monitoring is in accordance with the specifications elaborated during product development.

Analytical method validation is the corner stone of process validation without a proven measurement system it is impossible to confirm whether the manufacturing process has done what it purports to do. All new analytical methods developed are validated

Steps followed for validation procedures

1. Proposed protocols or parameters for validations are established
2. Experimental studies are conducted
3. Analytical results are evaluated
4. Statistical evaluation is carried out
5. Report is prepared documenting all the results

Types of validation

The following are frequently required to be validated on a pharmaceutical process: Equipment, environment, materials, methods, controls, process, personnel's, facilities and operating procedure. Based on these, the validation programmed comprises.

- Equipment validation
- Facility validation including utilities
- Process validation
- Cleaning validation

- Analytical method validation

Objective of the validation: The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, quality and purity they purpose are represent posses.

- Assurance of quality
- Government regulation

Importance of validation: As the quantity of product cannot always be assured by routine quality control because of testing of statistically insignificant number of sample, the validation thus should provide adequacy and reliability of a system or product to meet the pre-determined criteria or attributes to providing high degree of confidence that the same level of quality if consistently built into each of finished product from batch to batch. For taking appropriate action in case of non-compliance.

Method validation: Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Analytical method needs to be validated or revalidated: Before their introduction into routine use. Whenever the condition changes for which the method has been validated. Example: sample with a different matrix, an instrument with different characteristics. Whenever the method is changed and changes are outside the original scope of the method.

Strategy for the validation of method: (Ludwig Huber, 1999): The preparation and execution should follow a validation protocol, preferably written in a step-by-step instruction format. The proposed procedure assumes that the instrument has been selected and method developed.

It meets criteria such as:

1. Ease of use.
2. Ability to be automated and to be controlled by computer system.
3. Cost per analysis.
4. Sample through put.
5. Turnaround time.
6. Environmental, health and safety requirement.

Steps in Method validation: (Pharmacopeial forum 2001).

- Develop a validation protocol, an operating procedure or a validation master plan.
- Define the application, purposes and scope of the method.
- Define the performance parameters and acceptance criteria.
- Define validation experiments.
- Verify relevant performance characteristics of equipment.
- Qualify materials. E.g. Standards and reagents for purity, accurate amount and sufficient stability.
- Perform revalidation experiments.
- Adjust method parameter and for acceptance criteria if any.
- Perform full internal or external validation experiments.
- Develop a SOP’s for executing the method in routine analysis.
- Define the criteria for revalidation Define type and frequency of system suitability test and/or

analytical QC checks for routine analysis.

- Document validation experiment and results in validation report.
- The scope of the method and its validation criteria should be define dearly in the process. What analyte should be detected?
- What are the expected concentration levels?
- What are the sample matrixes?
- Are their interfering substances expected and if so, they should be separated from the mixture, detected and quantified.
- Are there any legislative or regulatory requirements?
- Should information be qualitative of quantitative?
- What are the detection and quantitation limit.
- What precision and accuracy is expected.
- How robust the method should be.
- Which type equipment should be used?
- What skills do the anticipated users of the method have?

The scope of the method also includes the different type of the equipment and the location where the method will be run. For example, if the method is to be urn on a specific instrument in specific laboratory, there is no need to use instrument in specific laboratory, there is no need to use instrument from other vendors or to include other laboratories in the validation experiments. An experienced analyst who should be very well versed in the technique and operation of the instrument should carry out the validation experiments. Before an instrument is used to validate a method it's performance specification (USP 25) should be verified using chemical standards and such methods are not deduced as analytically rugged. The care should be taken during the development of method itself that method can be applied on different column of same size and packing. The use of specific column shall be mentioned by setting interest of method like related substances. Any chemicals used to determine critical validation parameters, such as reagents and reference standard should be:

- Available in sufficient quantities.
- Sufficiently stable
- Checked for exact composition and purity.

Any other materials and consumables, for example, chromatographic columns should be new and easily available. This ensures that one set of consumables can be used for most experiments and avoids unpleasant surprises during method validations. If there is little or no information on the method's performance characteristics, it is recommended to prove the suitability of the method for its intended use in initial experiments.

These studies should include the specificity including degradation studies accuracy at 100% precision, working range and detection limits. If the preliminary data appear to be inappropriate, the method itself, the equipment, the analysis technique or the acceptance limit should be changed method development and validation are, therefore, an interactive process. For example, in LC selectivity is achieved through the selection of mobile phase composition. For quantitative measurements, the resolution factor between two

peaks should be 2.5 or higher. If this value is not achieved, the mobile phase composition needs further optimization. The influence of operating parameters on the performance of the method should be assessed at this stage if this was not done during development and optimization of the method. For a liquid chromatographic method, the following sequence has proven to be useful

- Selectivity if standards (optimizing separation and detection of standard mixtures if selectivity is insufficient).
- Linearity, limit of quantitation limit of detection, range.
- Repeatability (short-term precision) of retention times and peak areas.
- Intermediate precision.
- Selectivity with real samples.
- Trueness/accuracy at different concentrations.

Once the method has been developed and validated, a validation report should be prepared that includes the following:

- Objective and scope of the method
- Summary of the methodology.
- Type of compounds and matrix.
- All chemicals, reagents, reference standards, required working standard, finished
- Product and placebo samples, their source or detailed instructions on their preparation
- Procedure for quality checks of standards and chemicals used.
- A plan and procedure for method implementation from the method development lab to routine analysis.
- Listing of equipment and its functional and performance requirements.
- Detailed conditions on how experiments were conducted, including sample preparation, the report must be detailed enough to ensure that it can be reproduced by competent technician with comparable equipment.
- Statistical procedures and representative calculations.
- Procedure of QC routine analysis, e.g. System suitability tests calibration curves.
- Method acceptances limit performance data.
- Criteria for revalidation.
- The person who developed and validated the method.
- Summary and conclusions.
- Approval with names, titles, date, signature of those responsible for the validation, review and approval of the analytical procedure.

1.4. Validation Parameters

1. Specificity

2. Precision

- System precision
- Method precision

3. Accuracy

4. Linearity

5. Robustness

- Flow rate variation
- Temperature variation
- Mobile phase variation
- pH variation

6. Ruggedness

- System to system variation
- Analyst to analyst variation
- Column to column variation

7. Filter validation

The parameters as defined by the ICH and by other organization and authors, are as follows

1.4.1. Specificity: In practice this can be done by spiking the drug substance or product (placebo formulation, excipients degradation product, process impurity) with appropriate level and demonstrating the assay result is unaffected by the presence of these extraneous materials.

1.4.2. Precision: The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation. In the case of system precision, ten replicates of the standard solution are made for the test performance of the chromatographic instrument. In the case of method precision, six replicates from the same batch are analyzed for the assay observing the amount of scatter in the results.

1.4.3. Accuracy: In the case of a drug in the formulated product. Accuracy may be determined by application of to analytical synthetic mixtures of the drug components to which the known amount of analyte have been added within range of the method. If it is not possible to obtain all the components, it may be acceptable either known quantities of the analyte to the product i.e. “to spike” or to compare the results the results with those of a second, well characterized method, the accuracy of which has been stated or defined. The ICH recommends that accuracy should be assessed during the minimum of nine determinations over a minimum of three concentrations levels, covering a specified range (i.e. three concentrations and three replicates of each concentration). Accuracy is calculated the percentage recovery by the assay of the known amount of analyte in the sample or as the difference between the mean and the accepted true value together with confidence intervals. For assay method, spiked samples are prepared in triplicate at three intervals over a range of 50,100,150% of the target concentration. Potential impurities should be added to the matrix to mimic impure samples. The analyte levels in the spiked samples should be determined using the same quantization procedure as will be used in the final method procedure (i.e. Same levels of standards and same number of samples and standard injections).

1.4.4. Linearity: ICH recommended that, for the establishment of linearity, a minimum of 5 concentrations. It is also recommended that the following minimum specified range should be considered. For assay of a drug substance or a finished product 50-150% of the concentration should be taken. Acceptability of the linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for

the response versus concentration plot. The correlation coefficient of > 0.999 is generally considered as evidence of acceptable fit of the data to the regression line the analyte at to target level.

1.4.5. Robustness (ICH 1994): The robustness of the methods was determined by performing the assay of the triplicate by deliberately alternating parameters and that the results are not influenced by different changes in the below parameters.

- Change in column temperature: + 2^0C
- Change in flow rate: + 0.2ml/min.
- Change in organic phase : + 10%
- Change in pH: + 0.2

1.4.6. Ruggedness: The ruggedness of an analytical method is determined by the analysis of aliquots form homogeneous loss in different laboratories by different analyst using operational and environmental condition that may differ but or still within the specified parameters of the assay and dissolution. The degree of reproducibility of the result is then determined as a function of assay and dissolution variables this reproducibility was compared to precision of assay to obtain a measure of the ruggedness of the analytical method.

a. When reproducibility is performed, intermediate precision is not needed

b. May be needed in some cases

Table-1.3Validation Parameters Recommended by (ICH)

ASSAY TYPE VALIDATIONS	
Identification tests are intended to ensure the identity of an analyte in a sample. This is normally achieved by comparison of a Specificity property of the sample to that of a reference standard.	Specificity
Impurities Quantitation are intended to accurately reflect the purity characteristic of the sample. Different validation characteristics are required for a quantitative test than for a limit test.	Accuracy Precision Specificity Detection limit Quantitation limit Linearity Range
Impurities limits are intended to reflect the purity characteristics of the sample.	Specificity Detection limit
Content / Potency, Dissolution are intended to measure the analyte present in a given sample. A quantitative measurement of the major	Accuracy

component (s) in the drug substance.

Precision

Specificity

Linearity

Range

Table-1.2 Validation characteristics versus type of analytical procedures

Type of Procedure	Identification	Quantitation	Limit	Dissolution Measurement (Content / Potency)
Accuracy	NO	YES	NO	YES
Precision	NO	YES	NO	YES
Intermediate Precision	NO	YES[a]	NO	YES[a]
Specificity	YES	YES	YES	YES
Detection Limit	NO	NO[b]	YES	NO
Quantitation Limit	NO	YES	NO	NO
Linearity	NO	YES	NO	YES
Range	NO	YES	NO	YES

The comparison of different official guidelines in case of parameters required to be validated for different assays is shown in Table-1.3.

Table-1.3 Comparative Table Representing FDA, USP and ICH Requirements

Criteria	GMP	FDA	USP	ICH
Accuracy	X	X	X	X
Reproducibility	X	-	-	X
Sensitivity	X	-	-	-
Specificity	X	X	X	X
Linearity	-	X	X	X

Precision	-	X	X	X
Detection limit	-	-	X	X
Quantitation Limit	-	-	X	X
Range	-	-	X	X
Recovery	-	X	-	-
Ruggedness	-	X	X	-

Analytical methods are required for the identification, batch analysis and storage stability data for active constituents of Pharmaceutical products, and for post-registration compliance purposes. Analytical method development as a first step is carried out to ensure that the API used and the dosage forms that are developed and manufactured for human consumption are meeting the regulated quality norms. Every newly developed method must be validated prior to sample analysis. Validation must also be repeated if a parameter has been modified or ifthe validation was strongly performed in another laboratory, to ensure that the methods are transferable. A verification is necessary if the analyst or instrument have been changed, or if the sample type has been modified.

The objective of validation of an analytical method is to demonstrate that the procedure, when correctly applied, produces results that are fit for purpose. Method validation is practical process designed and experimentally carried out to ensure that an analytical methodology is accurate, specific, reproducible and rugged over the specified range of analysis. Validation provides both assurance and reliability during normal use and documented evidence that the method is 'fit for purpose'.

Method validation ensures the validity of a measurement before it is carried out and is essential part of quality assurance procedures. The process of method validation provides information on the critical factors affecting the method output, thus enabling suitable quality control procedures to be implemented to ensure data quality. The extent of method validation will vary with applications, sector and regulatory compliance.

1.5. STABITY INDICATING FORCED DEGRADATION METHOD

"Stability is defined as the capacity of a drug substance or drug product to remain within established specifications to maintain its identity, strength, quality, and purity throughout the retest or expiration dating periods".

STABILITY ZONES:

Table 1.4.Acceptance Limits for System Suitability Test

S.No	ZONE	MAJOR COUNTRIES	TEMPERATURE	RH
1.	Temperature	US, UK, Russia, Northern Europe	21^{o}C	45%
2.	Subtropical/	Southern Europe, Japan	25^{o}C	60%

	Mediterranean			
3.	Hot /dry	India, Iraq	30°C	35%
4.	Hot /humid	Iran, Egypt	30°C	70%
5.	Hot /very	Brazil, Singapore	30°C	75%

1.5.1 Forced degradation:

Forced degradation studies are also known as stress testing, stress studies, stress decomposition studies, forced decomposition studies, etc. Forced degradation is a process that involves degradation of drug products and drug substances at conditions more severe than accelerated conditions and thus generates degradation products that can be studied to determine the stability of the molecule. The ICH guideline states that stress testing is intended to identify the likely degradation products which further helps in determination of the intrinsic stability of the molecule and establishing degradation pathways, and to validate the stability indicating procedures used.

Table1.5. Conditions generally used for forced degradation studies

Degradation type	**Experimental conditions**	**Storage conditions**	**Sampling time (days)**
Hydrolysis	Control API (no acid or base)	40°C, 60°C	1,3,5
	0.1M HCl	40°C, 60°C	1,3,5
	0.1 M NaOH	40°C, 60°C	1,3,5
	Acid control (no API)	40°C, 60°C	1,3,5
	Base control (no API)	40°C, 60°C	1,3,5
	pH: 2,4,6,8		
Oxidation	3%H2O2	25°C, 60°C	1,3,5
	Peroxide control	25°C, 60°C	1,3,5
	Azobisisobutyronitrile (AIBN)	40°C, 60°C	1,3,5
	AIBN control	40°C, 60°C	1,3,5
Photolytic	Light 1 × ICH	NA	1,3,5
	Light 3 × ICH	NA	1,3,5
	Light control	NA	1,3,5
Thermal	Heat chamber	60°C	1,3,5
	Heat chamber	60°C /75% RH	1,3,5
	Heat chamber	80°C	1,3,5
	Heat chamber	80°C /75% RH	
	Heat control	Room temp.	

Table :1.6. Conditions of Stress testing

Degradation factor	Conditions
Thermal	>/=60°C
Humidity	>/=75% RH
Acid	0.1N HCL
Base	0.1N NaOH
Oxidative	Oxygen gas, or 3% H_2O_2
Photolytic	Metal halide, Hg, Xe lamp, or UV-B fluorescent
Metal ions	0.05M Fe^{2+} or Cu^{2+}

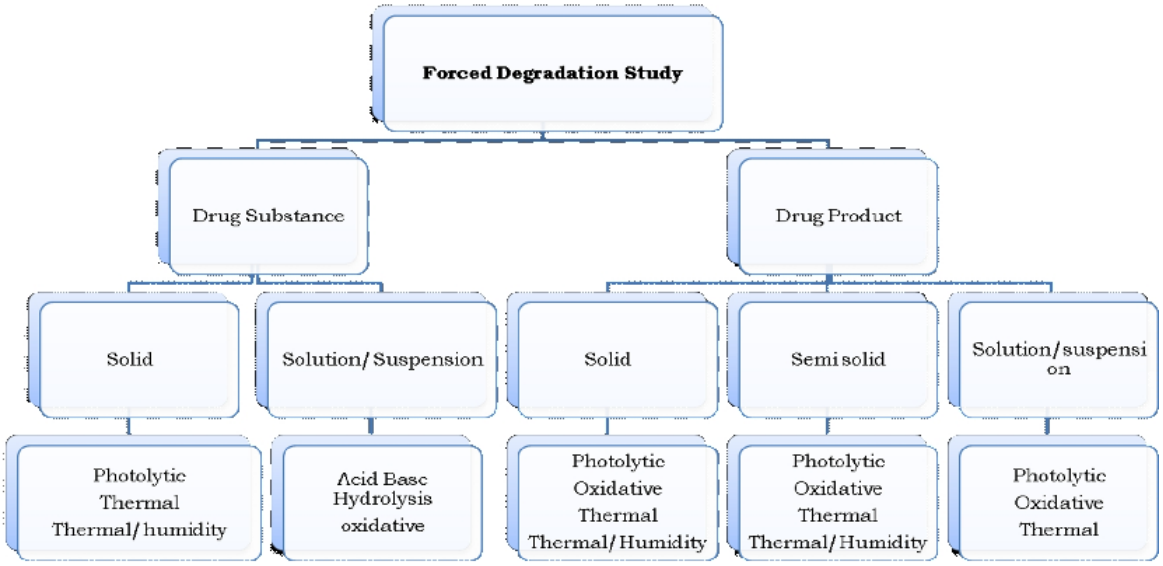


Fig.1.4. Flow chart of forced degradation study

1.5.2 Hydrolytic degradation

Hydrolysis is one of the most common degradation chemical reactions over wide range of pH. Hydrolysis is a solvolytic process in which drug reacts with water to yield breakdown products of different chemical compositions.

Water either as a solvent or as moisture in the air comes in contact with pharmaceutical dosage forms is responsible for degradation most of the drugs. For example, aspirin combines with water and hydrolyzed to salicylic acid and acetic acid Hydrolytic study under acidic andb asic condition involves catalyzation of ionisable functional groups present in the molecule. HCl and NaOHare employed for generating acidic and basic stress samples, respectively[11]. The hydrolytic degradation of a new drug in acidic and alkaline condition can be studied by refluxing the drug in 0.1 N HCl / 0.1 N NaOH. If reasonable degradation is seen, testing can be stopped at this point. However in case no degradation is seen under these conditions the drug should be refluxed in acid/alkali of higher strength and for longer duration of time. Alternatively if total degradation is seen after subjecting the drugs to initial condition, acid/alkali strength can be decreased with decrease in reaction temperature. In general temperature and pH are the major determinant in stability of the drug prone to hydrolytic decomposition. Hydrolysis of most of the drugs is dependent upon the relative concentration of hydronium and hydroxyl ions. Hence pH at which each drug is optimally stable can be determined.

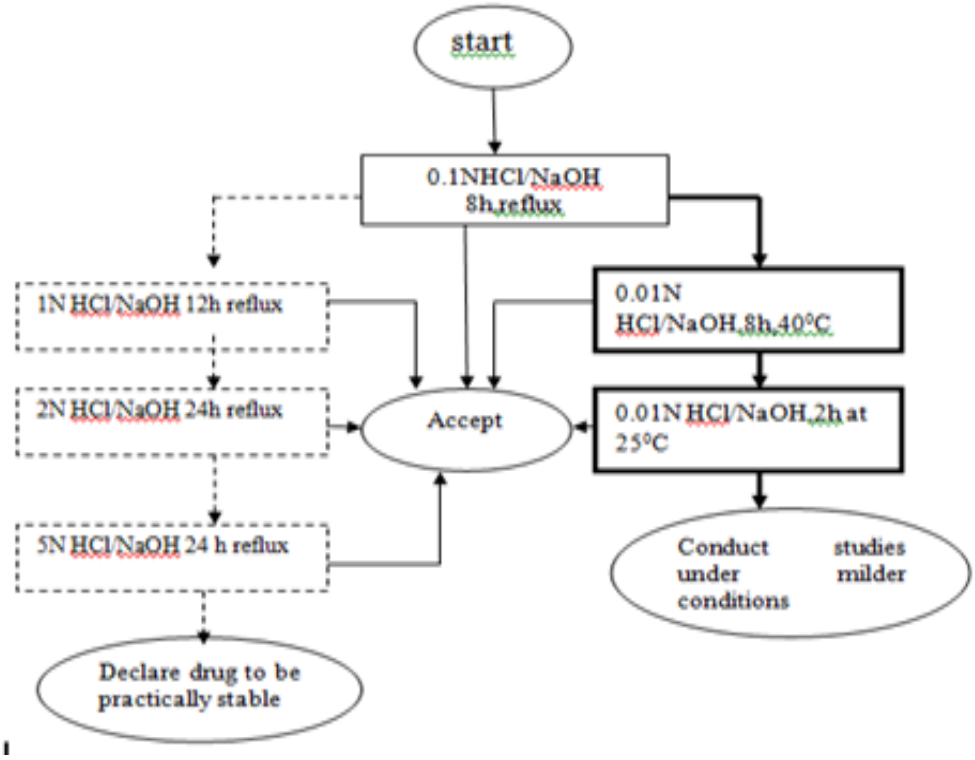


Fig: 1.5 – Flow chart for hydrolytic degradation under acid or alkali Conditions

1.5.3.Oxidative degradation

Hydrogen peroxide is widely used for oxidation of drug substances in forced degradation studies but other oxidizing agents such as metal ions, oxygen, and radical initiators (e.g.,azobisisobutyronitrile, AIBN) can also be used. Selection of an oxidizing agent, its concentration, and conditions depends on the drug substance. It is reported that subjecting the solutions to 0.1–3% hydrogen per oxide at neutral pH and room temperature for seven days or upto a maximum 20% degradation could potentially generate relevant degradation products. The mechanism of oxidative degradation of drug substance involves an electron transfer mechanism to form reactive anions and cations. Amines, sulphides and phenols are susceptible to electron transfer oxidation to give N-oxides, hydroxylamine, sulphones and sulphoxide .The functional group with labile hydrogen like benzylic carbon, allylic carbon, and tertiary carbon or α – positions with respect to hetro atom is susceptible to oxidation to form hydroperoxides, hydroxide or ketone.

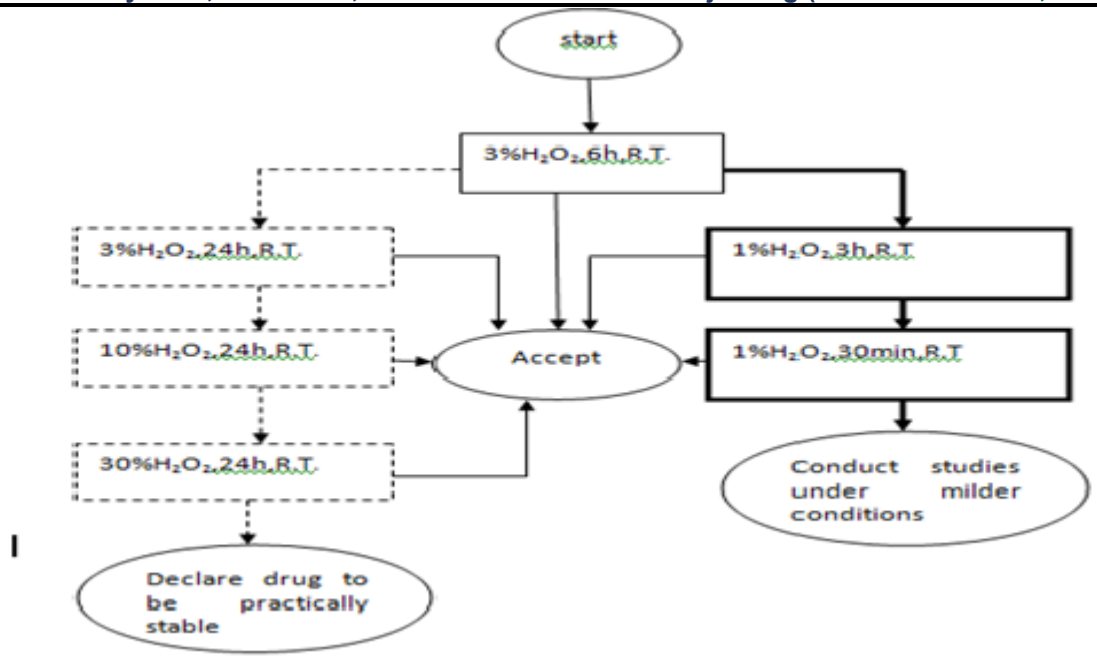


Fig: 1.6- Flow chart for oxidative degradation

1.5.4. Photo degradation

According to ICH Q1B guideline for photo degradation, samples should be exposed to light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 watt hours/square meter with spectral distribution of 320-400nm to allow direct comparisons to be made between the drug substance and drug product. Samples may be exposed side-by-side with a validated chemical actinometric system to ensure the specified light exposure is obtained or for the appropriate duration of time when conditions have been monitored using calibrated radiometers/lux meters]. The photolytic degradation can occur through non-oxidative or oxidative photolytic reaction. The non-oxidative photolytic reaction include isomerization, dimerization, cyclization, rearrangements, decarboxylation and haemolyticcleavage of X-C hetero bonds, N-alkyl bond (dealkylationand deamination), SO2- C bonds etc and while oxidative photolytic reaction occur through either singlet oxygen (1O2) or triplet oxygen (3O2) mechanism. The singlet oxygen reacts with the unsaturated bonds, such as alkenes, dienes, polynuclear aromatic hydrocarbon to form photoxidative degradation products whereas triplet oxygen react with free radical of the drug molecule, which than react with a triplet oxygen molecule to form peroxide. Hence, light can also act as a. catalyst to oxidation reactions.

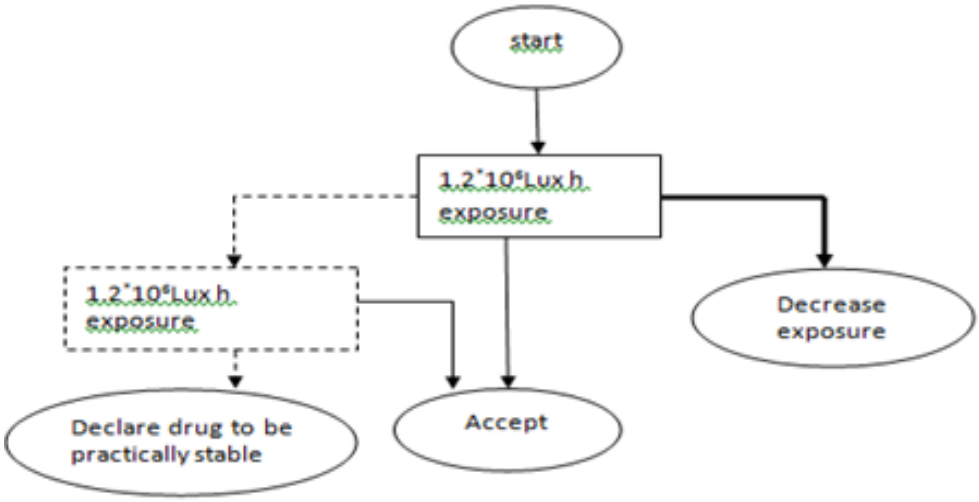


Fig:1.7-Flow chart for photolytic degradation

1.5.5. Thermal degradation

In general, rate of a reaction increase with increase in temperature. Hence, the drugs are susceptible to degradation at higher temperature. Many APIs are sensitive to heat or tropical temperatures. For example, vitamins,

peptides, etc. Thermal degradation involves different reactions like pyrolysis, hydrolysis, decarboxylation, isomerization, rearrangement and polymerization. Effect of temperature on thermal degradation of a substance is studied through Arrhenius equation:

$$K = Ae^{-Ea/RT}$$

Where k is specific reaction rate, A is frequency factor, Eais energy of activation, R is gas constant (1.987 cal/ degmole) and T is absolute temperature. Thermal degradation study is carried out at 40 ˚C to 80 ˚C. The most widely accepted temperature is 70 ˚C at low and high humidity for 1-2 months. The use of high-temperatures in predictive degradation studies assumes that the drug molecule will follow the same pathway of decomposition at all temperatures. This assumption may not hold true for all drug molecules, and therefore great care must be taken in using the extreme temperatures easily accessible in a sealed-vessel microwave experiment for predictive degradation studies.

1.6. Formulas used

1.6.1 Correlation coefficient(r):

When the changes in one variable are associated or followed by changes in the other, it is called correlation. The numerical measure of correlation is called the coefficient of correlation and is defined by the relation.

$$r = \frac{\sum(x - x')(y - y')}{\sqrt{\sum(x - x')^2 \sum(y - y')^2}}$$

1.6.2 Regression equation:

Regression equation= I + aC

$$a = \text{slope} = \frac{Y2 - Y1}{X2 - X1}$$

I = Intercept = regression – a C

As a percentage of mean absorbance.

1.6.3 Standard Deviation:

$$S = \sqrt{\Sigma (X - X^{!})^{2}/N - 1}$$

Where, X = observed values

$X^{!}$ = Arithmetic mean = ΣX/N

N = Number of deviations

For practical interpretation it is more convenient to express 'S' in terms of percent of the approximate average of the range of analysis is used in the calculation of 'S'. This is called co-efficient of variation (C.V) or percent relative standard deviation (%RSD).

C.V OR %RSD = 100* S/ $X^{!}$

1.6.4 Average = $\frac{\text{Sum of No. of Observations}}{\text{Total No. of Observations}}$

1.6.5 %Relative Standard Deviation = $\frac{\text{Standard Deviation}}{\text{Average}}$ * 100

1.6.6 Recovery or Practical conc. = $\frac{\text{Sample area}}{\text{Standard Area}}$ *Standard conc.

Standard concentration*100

1.6.7 %Recovery = ——————————————————————

Sample concentration amount (Label Claim)

Calculated or Sample Weight

1.6.8 %Assay of Drug = s* ——————————————

Label claim

Standard Deviation of Area of Linearity

1.6.9LOD = 3.3 * ——————————————————————

Slope

Standard Deviation of Area of Linearity

1.6.10 LOQ =10 * ——————————————————————

slope

1.7. AIM AND PLAN OF WORK

1.7.1.AIM

The present work is aimed to develop a simple, novel, rapid, precise, accurate and cost effective stability indicating analytical method development and validation of GABAPENTIN and METHYL COBALAMIN in bulk and its dosage form by using RP-HPLC.

1.7.2 PLAN OF WORK

- Solubility determination of GABAPENTIN and METHYL COBALAMIN various solvents and buffers.
- Determine the absorption maxima of both the drugs in UV–Visible region in different solvents/buffers and selecting the solvents for HPLC method development.
- Optimize the mobile phase and flow rates for proper resolution and retention times.
- Validate the developed method as per ICH guidelines.

1.8. NEED FOR THE STUDY

Analytical Method Development for Pharmaceutical Formulations

Quality investigation plays a very important role in quality specification establishment of chemical drugs. The number of drugs introduced into the market every year .very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. Hence, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

Basic criteria for new method development of drug analysis:

- The drug or drug combination may not be official in any pharmacopoeias.
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations.
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients.
- Analytical methods for a drug in combination with other drugs may not be available.
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

Analytical method development provides the support to track the quality of the product from batch to batch.

Method development involves considerable trial and error procedures. The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase.

Single dosage forms with combination of drugs are widely used today due to their advantages and their simultaneous estimation of individual component is a challenging task.

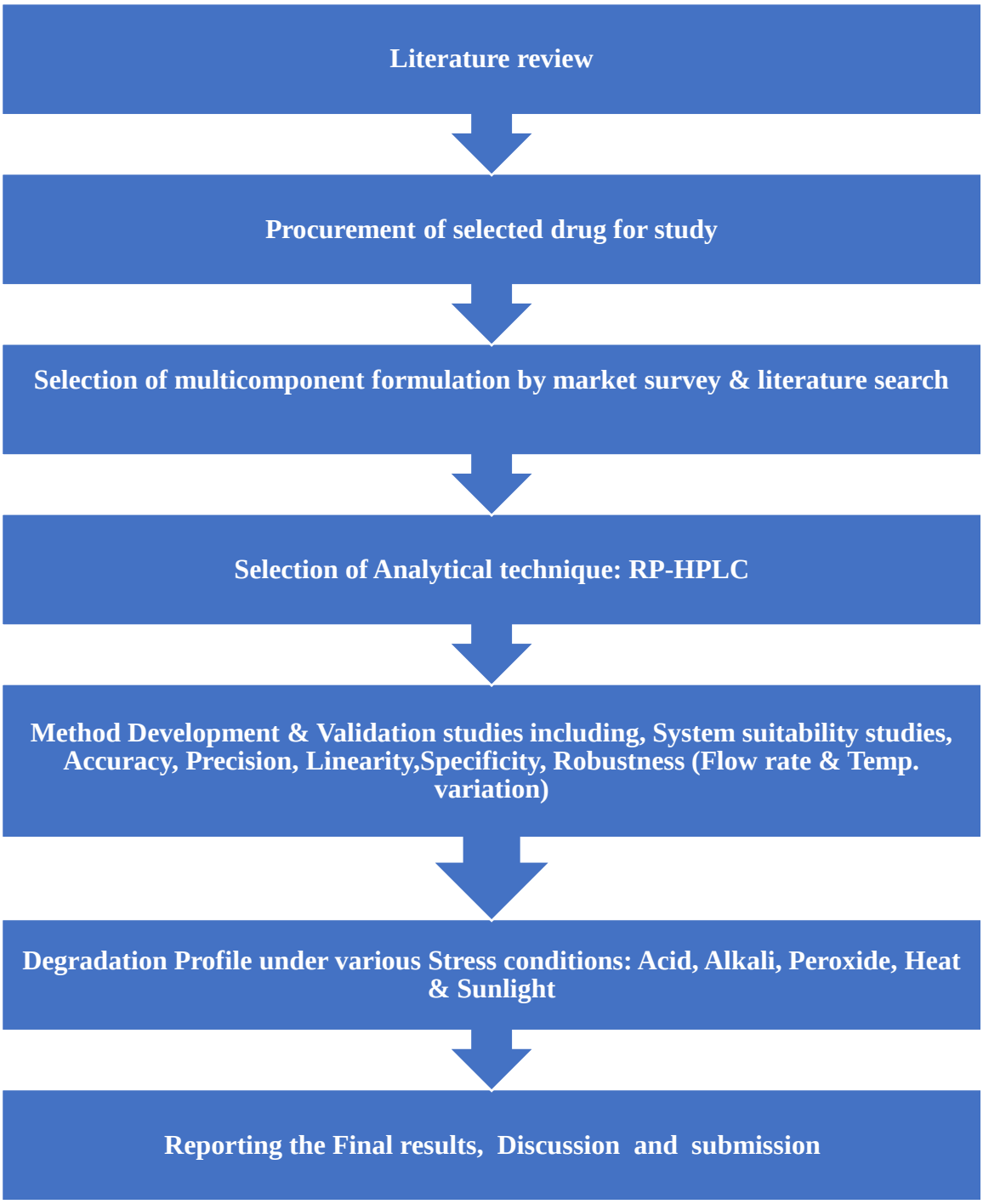
Gabapentin being the first line drug for pain due to diabetic neuropathy &postherpetic neuralgia, and mostly used in conjunction with vitamin B_{12}/ Mecobalamin, to treat the same, magnifies their importance as one of the most frequently used preliminary drugs.

Assaying these drugs plays an important role in industry and in welfare of mankind that is essential that it should be reliable, accurate, speedy and uncomplicated and the results readily understood by those who required to act on them.

The principal aim of this study was to provide accurate, precise, economic & reliable method for the simultaneous determination of gabapentin &mecobalamin in bulk drug & combined dosage form. The present work also describes an analytical procedure for stability indicating chromatographic assay method for Gabapentin and mecobalamin quantification in their combined dosage formulation.

Validate the proposed method in accordance with ICH guidelines for the intended analytical application i.e assay of these drugs in their combined form & also to study the effect of acid, base, peroxide, heat & sunlight.

PLAN OF WORK:



2.LITERATURE REVIEW

LITERATURE REVIEW OF GBP&MCB INDIVIDUALLY & COMBINED DOSAGE FORM:

The following methods have been reported for the estimation of Gabapentin & Mecobalamin individually & in combination with other drugs.

1.**B Lakshmi *et al*** developed a simple, precise, accurate RP-HPLC Method for the determination of Gabapentin in tablet dosage form using zodiac C18(250mmx4.6mm, ID,5µm PS) at ambient temperature isocratically at the flow rate of 1ml/min, using mobile phase methanol, acetonitrile, orthophosphoric acid 65:33:2% (v/v/v) at a UV detection at a wavelength of 216nm. Rt for gabapentin was 3.72min. The % RSD for precision & accuracy of method were found to be <2%.

2. **Varsha R Galande, K G Baheti, M H Dehghan** developed a UV-VIS Spectrophotometric Method for estimation of Gabapentin and Methylcobalamin in bulk & tablets based upon measurement of absorbance of tablet in distilled water at λmax 351nm for methylcobalamin and 405nm for gabapentin after reacting with 0.2% ninhydrin. Beer lamberts law obeyed in the concentration range of 50-300 µg/mlfor gabapentin and 1-7 µg/ml for mecobalamin.

3. **KG. Baheti and VR. Galande** developed a simple, precise and accurate HPTLC method for the estimation of gabapentin in presence of mecobalamin in tablet based upon measurement of peak area of gabapentin after separation from mecobalamin in potassium dihydrogen orthophosphate (0.05M,pH6.3) acetonitrile, methanol (7.5:1.5:1v/v/v) mobile phase.Separated gabapentin was reacted with 0.25%alcoholic ninhydrin and estimated at 500nm. Beer lamberts law obeyed over concentration range of 50-350 ng/ml for gabapentin.

4.**Sharma M.C, Sharma S, Sharma A.D** developed an UV spectrophotometric method and validated the same for quantitative determination of gabapentin & mecobalamin in combined dosage form using 50M sodium benzoate; as a hydrotropic agent.This method based upon the simultaneous equation method. The absorbance maximum of gabapentin and mecobalamin were 313nm and 334nm respectively. Calibration curves were prepared.The correlation coefficient were found to be 0.9993 & 0.9989 respectively. % recovery was found in the range of 99.8% to 100.33%. Analyzing the combination in tablet dosage form , recovery studies showed a good agreement in the assay of results. The method is simple, precise, accurate and can be employed for routine analysis of gabapentin and mecobalamin in tablet dosage form.

5. **Sameer A. M., Abdulrahman, prof. KanakapuraBasavaiah** reported the Highly Sensitive Spectrophotometric method for the determination of Gabapentin in capsules using sodium hypochloride, based on reaction of gabapentin & sodium 1,2 napthoquinone 4-sulphonate(NQS) in presence of clark and lub buffer of pH11 to form orange coloured product, measured at 495 nm. The parameters that affect were carefully optimized & under the optimized conditions, linear relationship was obtained in concentration range of 7.5 -75µgml^{-1}G. The molar absorptivity , LOD, LOQ and sandell sensitivity were also reported.

6. **Pratik PravubhaiGoti&Parula B Patel** reported a simple, economic, specific,rapid,reliable and reproducible method for the simultaneous estimation of gabapentin, mecobalamin and alpha lipoicacid.The derivative ratio absorbance of these drugs were measured at 731.10nm, 768.53nm & 242.21nm for their qualification % label claim for gabapentin, mecobalamin & alpha lipoic acid were found to be 98.71, 98.94 & 98.44 respectively, which shown linearity in the concentration range of 100-500µg/ml, 0.5-2.5µg/ml & 100-500µg/ml respectively.

7. **Maryam Kazemipoura, ImanFakhari& Mehdi Ansari** reported a sensitive spectrophotometric method for determination of gabapentin in human plasma & capsule by coupling of solid phase extraction, derivatization reaction & UV Visible spectrophotometry.The quantitation limit of gabapentin in human plasma was 0.8mg/L.The method was linear over the concentration range of 10.0-90.0mg/L and 0.8-10.0mg/L for pharmaceutical dosage form and plasma respectively.

8.**Ganesan.M, Solairaj.P, Rajesh S.C. Senthilkumar.T, Thangathirupathi**.A reported a Simple Spectrophotometric Method for the estimation of Mecobalamin in injections. The standard (10µg/ml) was scanned between 200-400nm and maximum absorbance recorded at 353nm. Assay results were found to be

98.94%. % recovery 99.05-100.50%. Linearity range of 10-50µg/ml proved that it obeyed Beer's law.

9. **Nandini R. Pai and Seema S. Sawant** reported a simple and validated RP-HPLC method for the estimation of methylcobalamin and Alphalipoic acid in soft gelatine capsule dosage form.This method was validated for assay determination of mecobalamin 1500mcg & alpha lipoic acid 300mg in soft gelatin capsule formulation.Aqueous mobile phase containing 0.02 M phosphate buffer adjusted to pH 3.5. Separation and quantification was achieved by changing the proportion of the system linearity with a time scheduled programme. Detection carried out in the range of 200-600 nm for mecobalamin and further analysis was carried out using UV detector.The precision was exemplified by rsd of 0.78% for mecobalamin & 0.53% for alpha lipoic acid. Good linearity was observed between concentration of analytes with correlation coefficient of 0.99995 and 0.99941 respectively.

10.**Rajinder Singh Gujral and S. K ManirulHaque** reported development and validation of a new HPLC Method for the determination of Gabapentin in pure form. HPLC separation was achieved on C18, 5µm waters column (150mm x 4.6mm) using mobile phase of methanol: potassium dihydrogen orthophosphate solution (20:80 v/v)containing 10% NaOH to adjust pH6.2 at a flow rate of 1.0ml/min.UV detection was operated at 275nm. The method was found to be simple, specific, precise, accurate and reproducible.

11.**Syed Sultan Qasim; Mohammed Mustafa Ali Siddiqui ; Ehab Youssef Abueida;Mohammed AbulKhair, Abudhab** reported validation of an isocratic HPLC assay of gabapentin in pharmaceuticalformulations and stress test for stability of drug substances. This paper describes the validation of isocratic HPLC method for assay of gabapentin capsule and evaluation of the stability of drug substance after stress test by photo diode array detection. HPLC separation was carried out on Beckman ultrasphere C18, 4.6mmx 25cm is suitable using mobile phase acetoitrile : methanol: phosphate buffer solution (55:35:10:0.1) at flow rate 1ml/min and detection at 210nm. Resolution between gabapentin and gabapentin related compound A is NLT 1.5.

12. **Binaya Das** reported the estimation of gabapentin in human plasma using LC-MS/MS method. A simple, sensitive & specific liquid chromatography- tandem mass spectrometry was developed for the quantification of gabapentin in human plasma . The analytical method consists of liquid-liquid extraction of plasma sample followed by the determination of gabapentin by LC-MS/MS.

13.**Narmada P, Vijaya Lakshmi G, Nalini G, Gowtham Y, Suhasini B, Jogi K.V** developed a simple, precise & accurate RP-HPLC method & validated the same for the estimation of Pregablin and Mecobalamin in capsule formulation using HPLC instrument shimadzu prominence with UV detection employing inertsil ODS (octadecylsilane) C18 column (250 x 4.0x3) , mobile phase 60 volumes of buffer potassium dihydrogen phosphate and dipotassium hydrogen phosphate and 40 volumes of methanol at flow rate of 0.6ml/min at 210 nm.

3. Materials & Methods

3.1. MATERIALS & TOOLS:

Chemicals Used for the studies

Gabapentin and Mecobalamin were obtained from Lara drugs pvt ltd.with the percentage purity of 99.1% and 99.7%, respectively. A commercial tablet formulation NUROKIND G from Mankind Pharma, (New Delhi, India) containing 300mg of Gabapentin and 500 mcg of Mecobalamin was purchased from local market and

used within their shelf life period. Analytical grade methanol, orthophosphoric acid, was obtained from Rankem. Distilled water obtained from milli-Q. HPLC grade water was obtained from Rankem. All other chemicals used were of pharmaceutical or analytical grade.

Instruments / Apparatus Used

Chromatographic conditions:

The HPLC system (LC Waters, Empower II) line degasser (Waters, model AF), photodiode array detector (Water, 2695 model) and auto sampler (Waters, model 717 plus). Data was processed using Empower Pro software (Waters).

Isocratic elution of the mobile phase Methanol: orthophosphoric acid buffer (pH adjusted to 2.2), in the ratio of 500:500 v/v, with the flow rate of 0.8 ml/min. Separation was performed on a waters C18 (250 x 4.6 mm x 5 µ particle size) analytical column and a pre column to protect the analytical column from strongly bonded material.

Integration of the detector output was performed using the Waters Empower software to determine the peak area. The contents of the mobile phase were filtered through 0.45 µ membrane filter and degassed by sonication prior to its use. Mobile phase was used as diluent. The flow rate of the mobile phase was optimized to 0.8 ml/min. The run time was set at 6 min and the column temperature was set at 25^0C. The volume of injection was 10 µl, prior to injection of the analyte, the column was equilibrated for 30-40 min with the mobile phase. The eluents was detected at 275 nm. The developed method was validated in terms of specificity, linearity, accuracy, limit of detection (LOD), limit of quantification(LOQ), precision, robustness for the assay of Gabapentin and Mecobalamin as per ICH guidelines[Q_2].Also forced degradation studies were carried out using 0.1M Hcl, 0.1M NaOH,1% H_2O_2, heat and sunlight exposure.

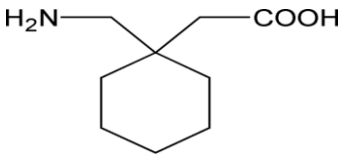
3.1. Drug Profile

3.1.1.Drug Profile of Gabapentin:

Description: Gabapentin (brand name Neurontin) is a medication originally developed for the treatment of epilepsy. Presently, gabapentin is widely used to relieve pain, especially neuropathic pain.

Appearance: white to off white crystalline solid.

Structure:



Synonyms:

- Neurontin
- Gabapentin
- Gabapetine
- Gabapentinum

Brand names:

- Neurostil

- Neurontin
- Fanatrex
- Gabarone
- Gralise
- Gabrion
- Nupentin
- Penral
- Gabapin

Category Anti epileptic, Diabetic & Peripheral neuropathy,

Trigeminal neuralgia

Weight: 171.237 g/mol

Melting point: 162-1660C

Solubility: water soluble

Chemical formula: C9H17NO2

IUPAC name: 2-[1-(aminomethyl) cyclohexyl]acetic acid

Mechanism of Action: Gabapentin interacts with voltage-sensitive calcium channels in cortical neurons. Gabapentin increases the synaptic concentration of GABA, enhances GABA responses at non-synaptic sites in neuronal tissues, and reduces the release of mono-amine neurotransmitters. One of the mechanisms implicated in this effect of gabapentin is the reduction of the axon excitability measured as an amplitude change of the presynaptic fibre volley (FV) in the CA1 area of the hippocampus. This is mediated through its binding to presynapticNMDA receptors. Other studies have shown that the antihyperalgesic and antiallodynic effects of gabapentin are mediated by the descending noradrenergic system, resulting in the activation of spinal alpha-2 adrenergic receptors. Gabapentin has also been shown to bind and activate the adenosine A1 receptor.

Adverse Effects: The most common side effects of gabapentin in adults include dizziness, fatigue, drowsiness, weight gain & peripheral edema. Gabapentin may also produce sexual dysfunction in some patients, symptoms of which may include loss of libido, inability to reach orgasm, and erectile dysfunction. Gabapentin should be used carefully in patients with renal impairment due to possible accumulation and toxicity.

Overdose:

Persons who accidentally or intentionally ingested overdoses have manifested drowsiness, sedation, blurred vision, slurred speech and somnolence or coma. Serum gabapentin concentrations may be measured to confirm diagnosis.

Other uses:

There is some evidence of benefit in acquired pendularnystagmus and infantile nystagmus but not in periodic alternating nystagmus.

Gabapentin may help with certain menopausal symptoms, such as hot flashes. It may be effective in reducing pain and spasticity in multiple sclerosis. Gabapentin may reduce symptoms of alcohol withdrawal but it does not prevent the seizures often associated with this condition. Use for smoking cessation has had mixed results. Gabapentin helps with itching (pruritus) associated with renal failure (uremic pruritus) and other conditions.

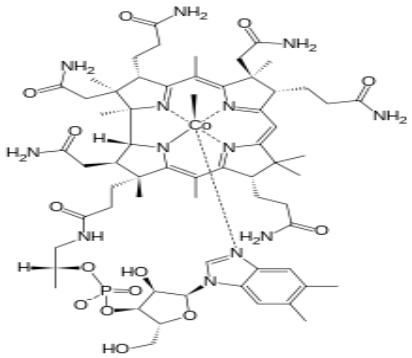
3.2. Drug Profile of Mecobalamin

Description: Methylcobalamin (mecobalamin, MeCbl, or MeB12) is a

cobalamin,aform ofvitamin B12., usedin the treatmentofmegaloplasticaneamia, diabetic neuropathy and peripheral neuropathy.

Appearance: Bright red crystals

Structure:



Synonyms: Mecobalamin, Mecobalamine, Cobaltmecobalamine

Category: Peripheral & Diabetic neuropathy

Weight: 1344.40g/mol

Melting point: >1900C

Solubility: Moderately soluble in water

Chemical formula: C63H91CoN13O14P

Pharmacokinetics of Mecobalamin:

Absorption: Absorb dafter oral, sublingual, injection

Excretion: Excretion via urine

Side Effects of Mecobalamin:

Oral: Anorexia, nausea, Vomiting and diarrhea

Parenteral: Rash, headache, hot sensation, diaphoresis and pain/induration at IM injection site

IUPAC name: cobalt (3+); mecobalamin

Mechanism of Action: Mecobalamin is the neurologically active form of

vitamin B12 and occurs as a water-soluble vitamin in the body. It is a cofactor in the enzyme methionine synthase, which functions to transfer methyl groups for the regeneration of methionine from homocysteine. In anaemia, it increases erythrocyte production by promoting nucleic acid synthesis in the bone marrow and by promoting maturation and division of erythrocytes.

Storage Requirements for Mecobalamin:

Oral: Store at room temperature. Protect from moisture and light.

Parenteral: Store at room temperature. Do not expose to direct light.

Brand names of GBP & MCB formulations:

- Alcobal
- Doloneuron
- Gabamin
- Gabatop M
- Gabana M

Brand Mixtures of GBP & MCB Tablet:

NEUROKIND G; each film coated tablet contains Gabapentin USP- 300mg & Mecobalamin-500 mcg.

3.3.METHODOLOGY:

A Stability indicating assay method was developed for the determination of Gabapentin & Mecobalamin in bulk & combined dosage formulation. The RP-HPLC method was then validated according to ICH Guidelines for various parameters like Accuracy, Precision, Linearity, Robustness, Specificity, LOD, LOQ, System & Forced degradation studies were performed as well.

3.4.PREPARATION OF STANDARD SOLUTIONS:

Wt taken: 600mg of gabapentin and 1mg of methylcobalamin

Standard stock solution I:

An accurately weighed quantity of the above-mentioned weights were dissolved in mobile phase and sonicated for 10 min room temperature, later the volume made upto 50 ml in volumetric flasks. For simultaneous quantitative studies of both drugs, a series of standard working solutions containing both the drugs were prepared by an appropriate dilution of a mixture of stock solutions.

The potency of working standard/ reference standard used for GBP & MCB was 99.1 and 99.7 respectively.

Preparation of test Solution I: (Analysis of gabapentin and mecobalamin in Tablets)Ten tablets of Nurokind G were weighed and finely powdered. A powder equivalent to 809 mg was accurately weighed, transferred into a 50 ml volumetric flask containing mobile phase. The above mixture was sonicated for about 10 min for complete mixing. This solution was filtered through Whatman No.1 filter paper. From the filtrate different aliquots were taken in separate 10 ml volumetric flasks and diluted with mobile phase up to the mark so as to get a concentration ranging from 50-150 µg ml^{-1} each of gabapentin and mecobalamin. Each of these solutions (10 µl) was then injected three times into the column. The mean peak areas of each drug were calculated and the drug contents in the tablets were quantified using the respective regression equations.

Preparation of buffer OPA: Weighed accurately 6.8gms of potassium dihydrogen Ortho-phosphate in a 1000 ml standard flask and dissolved in minimum quantity milli-Q water and made upto the mark with milli-Q

water.

Preparation of mobile phase: About 600ml of above buffer solution was transferred into a 1000 ml volumetric flask, to this 400ml of methanol was added, shaken well, filtered and degassed.

Method Guidelines:

- Solubility profile
- Analytical profile
- Stability profile
- Selection of optimization of mobile phase
- Buffer and its Strength
- pH of the Buffer or pH of the mobile phase
- Selection of mobile phase and its composition
- Selection of suitable detection wavelength
- Selection of column
- Selection of solvent delivery system
- Selection of flow rate
- Selection of column temperature
- Selection of diluents for test preparation and extraction procedure
- Methods of extraction
- Selection of test concentration, injection volume
- Establishment of stability of test preparation
- Establishment of system suitability

SELECTION OF CHROMATOGRAPHIC METHOD:

- Proper selection of the method depends upon the nature of the sample, molecular weight, and solubility.
- The drug selected for the present study was polar. So reversed phase chromatography can be used, this reverse phase HPLC was selected for the initial separation from the knowledge of properties, C_{18} column(250X4.6mm,5µ) was chosen as stationary phase with mobile phase orthophosphoric acid & methanol, composition 500:500(v/v).

A variety of mobile phases were investigated in the development of an RP-HPLC method suitable for analysis of Gabapentin & Mecobalamin in the bulk drug and in combined pharmaceutical dosage form. These included dipotassium hydrogen phosphate: methanol, 600:400 (%v/v), dipotassium hydrogenphosphate: methanol, 400:600, potassium dihydrogenphosphate:methanol, 600:40, potassium dihydrogen phosphate:methanol,400:600, orthophosphoric acid: methanol (pH), 400:600, and ortho–phosphoric acid: methanol buffer (pH2.2), 500:500; finally orthophosphoric acid : methanol, 500:500. The suitability of the mobile phase was decided on the basis of the sensitivity of the assay, suitability for stability studies, time required for the analysis, ease of preparation, and use of readily available cost-effective solvents.

Solubility test:

Gabapentin &Mecobalamin were checked for their solubility by adding a pinch of drug sample in solvents like water, methanol, ethanol and dimethylsulfoxide. It was found that both the drugs were comparitively more soluble in methanol. Thus methanol is used as diluent.

Selection of wavelength: The sample was scanned over the wavelength range of 200nm to 400nm through PDA detector and it was found that wavelength 275nm is suitable as both the drugs GBP & MCB showed good response. Therefore 275nm was selected as the suitable wavelength.

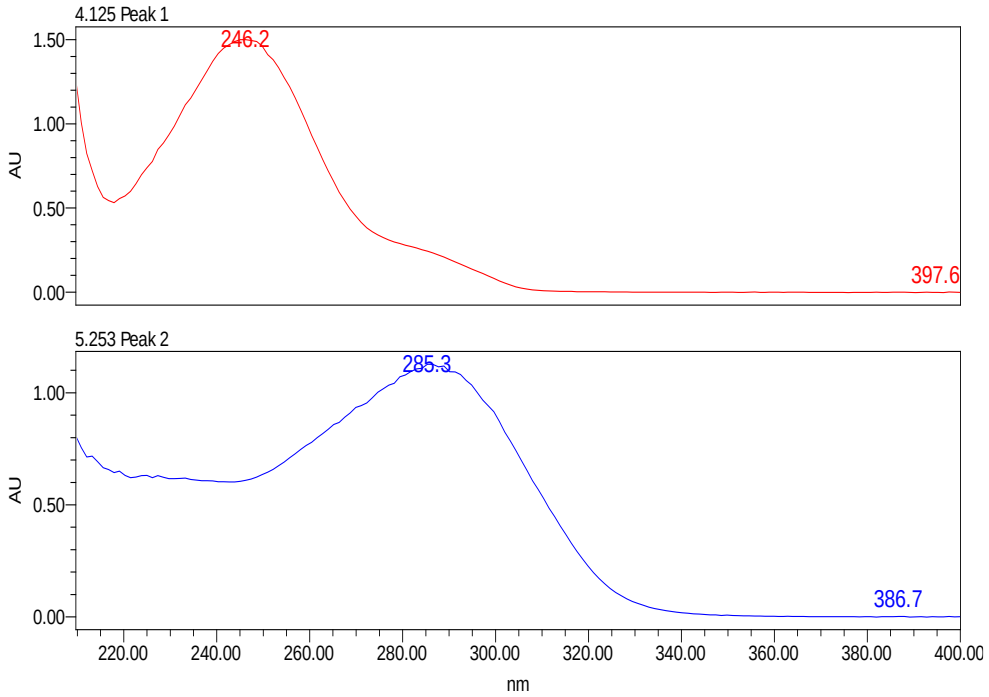


Fig 3.1.wavelength scan over 200-400nm range

Note: All the materials used within the expiry date and were stored at recommended experimental condition.

METHOD DEVELOPMENT TRIALS:

Principle: Reversed phase liquid chromatography with isocratic elution and PDA detection.

TRAIL:I

Procedure: Trail I was conducted using column: (C18,250x4.6, 5µ P.S),wavelength 275nm, mobile phase 600:400 (K2HPO4: methanol) at the flow rate of 1.0ml/ min, maintaining column temperature at 25^0C & injection volume of 10 µl, run for 30 min.

TRAIL:II

Procedure: Trail II was conducted using column: (C18,250x4.6, 5µ P.S), wavelength 275nm, mobile phase 400:600 (K2HPO4: methanol) at the flow rate of 1.0ml/ min, maintaining column temperature at 25^0C , injection volume of 10 µl, & run time 30 min.

TRAIL:III

Procedure: Trail III was conducted using column: (C18,250x4.6, 5µ P.S), wavelength 275nm, mobile phase

600:400 (KH2PO4: methanol) at the flow rate of 1.0ml/ min, maintaining column temperature at 25^0C, injection volume of 10 µl, & run time 20 min.

TRAIL: IV

Procedure: Trail IV was conducted using column: (C18,250x4.6, 5µ P.S), wavelength 275nm, mobile phase 400:600 (KH2PO4: methanol) at the flow rate of 1.0ml/ min, maintaining column temperature at 25^0C, injection volume of 10 µl, & run time 10 min.

TRAIL:V

Procedure: Trail V was conducted using column: (C18,250x4.6, 5µ P.S), wavelength 275nm, mobile phase 400:600 (OPA: methanol) at the flow rate of 0.8ml/ min, maintaining column temperature at 25^0C , injection volume of 5µl, & run time 10 min.

TRAIL:VI

Procedure: Trail VI was conducted using column: (C18,250x4.6, 5µ P.S), wavelength 275nm, mobile phase 500:500 (OPA: methanol) at the flow rate of 0.8ml/ min, maintaining column temperature at 25^0C , injection volume of 5µl, & run time 10 min.

TRAIL:VII

Procedure: Trail VII was conducted using column: (C18,250x4.6, 5µ P.S), wavelength 275nm, mobile phase 500:500 (OPA: methanol) at the flow rate of 0.8ml/ min, maintaining column temperature at 25^0C, run time set for 6 min & injection volume of 10µl.

OPTIMIZED TRAIL:

The trail for which all system suitability parameters qualify well is considered as the optimized trail & these conditions at which the column was run are said to be optimum conditions, i.e.column: (C18,250x4.6, 5µ P.S), wavelength 275nm, mobile phase 500:500 (OPA: methanol) at the flow rate of 0.8ml/ min, maintaining column temperature at 25^0C, run time set for 6 min & injection volume of 10µl.

VALIDATION:

The developed method is validated for the following parameters

1. System suitability
2. Accuracy
3. Precision
4. Linearity
5. Specificity
6. Robustness
7. Limit of detection (LOD)
8. Limit of quantification (LOQ)

1.SYSTEM SUITABILITY: The purpose of the system suitability is to ensure that the complete testing including instrument, method, analyst is suitable for intended application . It is done by using standard solution. SSP is performed to verify that the analytical system is working properly and can give accurate and precise results.

Procedure : The standard solution was prepared as per test method and injected into HPLC and the chromatograms were recorded. Two standard solutions were used. 1ml from standard stock solution was pippetted out and transferred to 25ml volumetric flask. The volume was made up with HPLC grade water. It was filled in two vials and labelled as STD1 and STD2.

2. **LINEARITY:** The linearity was determined at five levels over the range of 50%-150% of standard concentration in a diluents.

Procedure: Accurate aliquots of standard stock solution I were taken in five different 10 ml volumetric flasks. The volume was made up to the mark with diluents to obtain a final concentration of 1200, 1800, 2400, 3000, 3600 µg/ml of gabapentin and 2, 3, 4, 5, 6 µg/ml of mecobalamin. The calibration curves were obtained by plotting the peak area Vs concentration.

3. **ACCURACY :**It is expressed as recovery. Accuracy is determined at three different levels 50%, 100%, 150%.

Procedure: The recovery experiment was carried out by standard addition method. In which the amount of placebo was kept constant and the amount of pure drug was varied that is 404.6 mg, 809 mg, 1213.6 mg for 50%, 100%, 150% respectively. The solutions were prepared in triplicates and the accuracy was indicated by % recovery.

4. **PRECISION:**

Method Precision: In this method a homogenous sample of a single batch is analysed six times. This indicates whether a method is giving consistent results for a single batch. The method precision was performed on GBP and MCB tablet formulation.

Procedure: Precision was estimated by repeatability. It was assessed by preparing six assay preparations as per the test/ proposed method and injected into HPLC system. Results are reported in terms of relative standard deviation (RSD%).

5. **LIMIT OF DETECTION:** The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected. The detection limit is determined by the analysis of sample with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

Procedure: From the stock solutionI flask, 0.5ml was transferred to 10ml volumetric flask and made up the volume with HPLC grade water.

6. **LIMIT OF QUANTIFICATION :**This is carried out in order to determine that lowest concentration of an analyte which it can be estimated with acceptable precision, accuracy under the stated experimental conditions. For this the sample solution can be further diluted & the minimum concentration at which the sample can be reliably quantified should be found.

Procedure: From the above prepared standard solution, about 1ml of solution was transferred to 10 ml volumetric flask. The volume was made up with HPLC grade water.

7. **ROBUSTNESS:** The parameters performed to determine the robustness of the proposed method were; Flow rate variation & column temperature variation.

Procedure: In order to measure the extent of method robustness, the most critical parameters were interchanged while keeping the other parameters unchanged and in parallel, the chromatographic profile was observed and recorded. The studied parameters were: flow rate (± 0.2ml/min) and temperature (±5^{o}C). The flow rate in increased and decreased by 0.2ml/min and temperature is varied by 5^{o}C. The optimized flow rate is 0.8ml/min and temperature of sample compartment is 25^{o}C.

8. **SPECIFICITY:** Peak purity tests: It using (diode array detector) may be useful to show that analyte chromatographic peak is not attributable to more than one component.

Procedure: The assay was performed in duplicate on the weight of placebo, equivalent to the amount present in portion of test preparation and was analyzed as per the test/ proposed method. The chromatogram was run for as atleast three times the R_t of analyte peak i.e. 2.8 min for GBP & 4.4 min for MCB.

9. FORCED DEGRADATION- A STABILITY INDICATING PARAMETER:

The specificity of the method can be demonstrated through forced degradation studies conducted on the sample using acid, alkaline, oxidative, thermal, photolytic degradations. The sample was exposed to these conditions and the main peak was studied for the peak purity.

ACID DEGRADATION

Sample preparation procedure : Accurately weighed 809 mg of sample powder was transferred into50 ml volumetric flask and to this, 10 ml of 0.1M HCL was added, and sonicated for 30 minutes at 60^{o}C, and followed by 10ml addition of 0.1M NaOH, and the volume was made up with diluent. From above solution 5ml was transferred into 25ml volumetric flask, the volume was made up with HPLC grade water.

BASE DEGRADATION

Sample preparation procedure: Accurately weighed 809 mg of sample powder was transferred into 50 ml volumetric flask and to this, 10 ml of 0.1M NaOH was added and sonicated for 30 minutes at 60^{o}C, followed by addition of 10ml of 0.1M HCL making up the volume with diluent. From above solution 5ml was transferred into 25ml volumetric flask, the volume was made up with HPLC grade water.

OXIDATIVE DEGRADATION

Sample preparation procedure: Accurately weighed 809 mg of sample powder was transferred into 50 ml volumetric flask and to this 10 ml of peroxide was added and sonicated for 30 minutes at 60^{o}C and the

volume was made up with diluent. From above solution 5ml was transferred into 25ml volumetric flask making up the volume with HPLC grade water.

THERMAL DEGRADATION

Sample preparation procedure: Before weighing, sample powder was exposed at 105^0C in an oven for 1 hour.

Accurately weighed 809 mg of sample which was exposed to heat was transferred into 50 ml volumetric flask and 15ml of diluent added and sonicated for 30 minutes. The volume was made up with diluent. From above solution 5ml was transferred into 25ml volumetric flask and the volume was made up with HPLC grade water.

PHOTOLYTIC DEGRADATION

Procedure: Before weighing, the sample was exposed in sunlight for 24 hours.

Accurately weighed 809 mg of sample was transferred into 50ml volumetric flask. About 15ml of diluent was added to it and sonicated for 30 minutes. The volume was made up with diluent. From above solution, 5ml was transferred into 25ml volumetric flask & diluted with HPLC grade water.

4. RESULTS & DISCUSSION

Experimental method development trails were performed, finally a reliable, simple method was optimized and was set to validation as per ICH guidelines, the results are shown below.

4.1. Results: The HPLC separation and quantification was achieved on waters C18(250x4.6mm x 5µ). The mobile phase was prepared by mixing orthophosphoric acid and methanol in the ratio of (500:500) v/v that run isocratically at the flow rate of 0.8ml/min. The temperature maintained in sample compartment is 25°C. The injection volume is 10µl. the wavelength at which detector was set is 275nm.

Table4.1 : Optimized method Chromatographic conditions

OPTIMIZED METHOD	
Buffer (A)	OPA-500
Mobile phase (B)	Methanol– 500
Flow rate	0.8ml/min
Injection volume	10 µl
Run time	6 min
Column	waters C18 (250x4.6mm x 5µ)
Column temperature	30°c
Sample temperature	25°c
Wave length (nm)	275 nm

TRAIL-I:

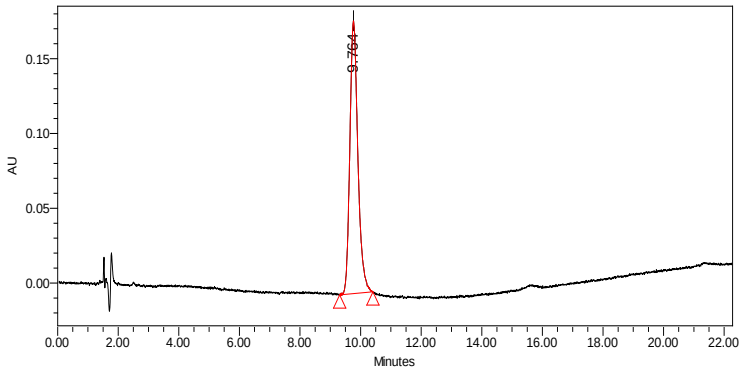


Figure 4.1- Trail 1 for GBP & MCB

Table 4.2. SSP for Trail I of GBP & MCB

s.no	Name	Retention Time	USP Resolution	USP Tailing	USP Plate Count
1	GBP	9.764		1.27	6687

TRAIL-II:

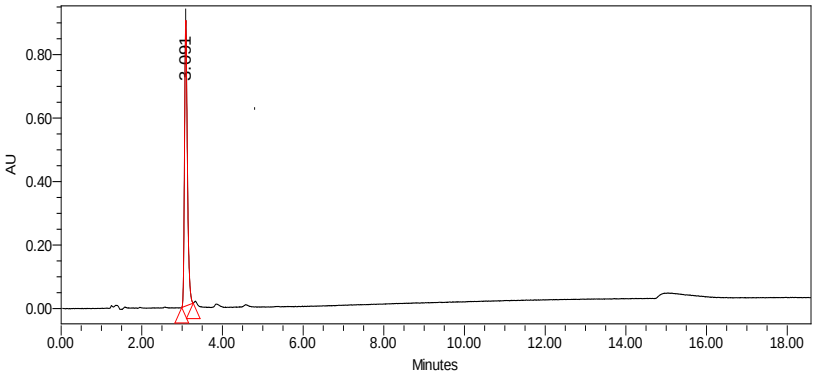


Figure 4.2. Trail 2 for GBP & MCB

Table 4.3. SSP for trail II of GBP & MCB

S.No	Name	Retention Time	USP Resolution	USP Tailing	USP Plate Count
1	GBP	3.091		1.33	9301

TRAIL-III:

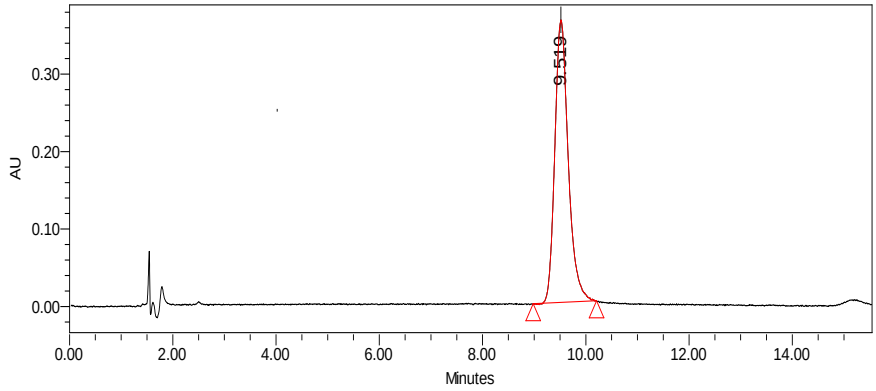


Figure 4.3.Trail 3 for GBP & MCB

Table 4.4 SSP for trail III of gbp& MCB

S.No	Name	Retention Time	USP Resolution	USP Tailing	USP Plate Count
1	GBP	9.519		1.26	6734

TRAIL-1V:

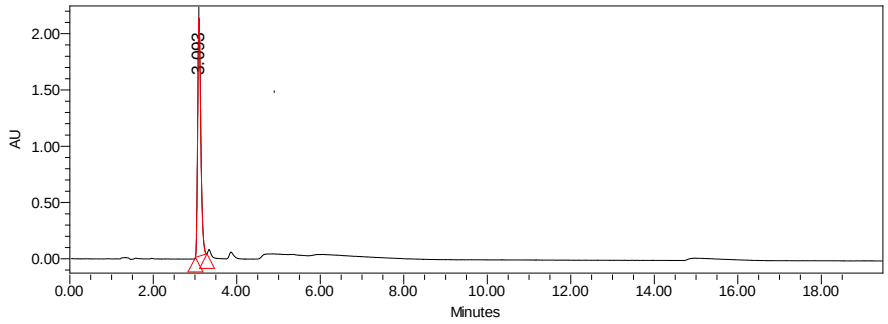


Figure 4.4.Trail 4 for GBP & MCB

Table 4.5.SSP for trail IV of GBP & MCB

S.No.	Name	Retention Time	USP Resolution	USP Tailing	USP Plate Count
1	GBP	3.093		1.40	8641

TRAIL-V:

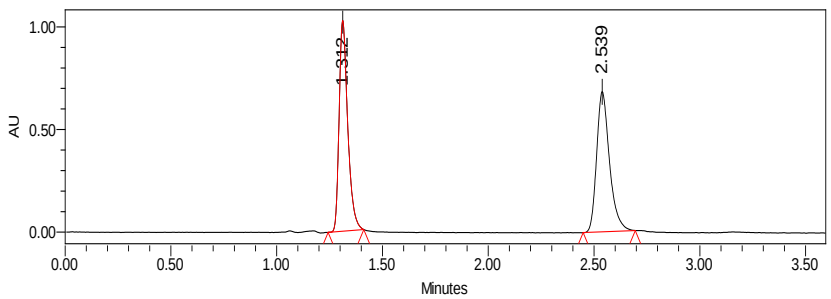


Figure 4.5. Trail 5 for GBP & MCB

Table 4.6.SSP for trail V of GBP & MCB

S.No	Name	Retention Time	USP Resolution	USP Tailing	USP Plate Count
1	GBP	1.312		1.39	2134
2	MCB	2.539	13.03	1.32	8733

TRAIL-VI:

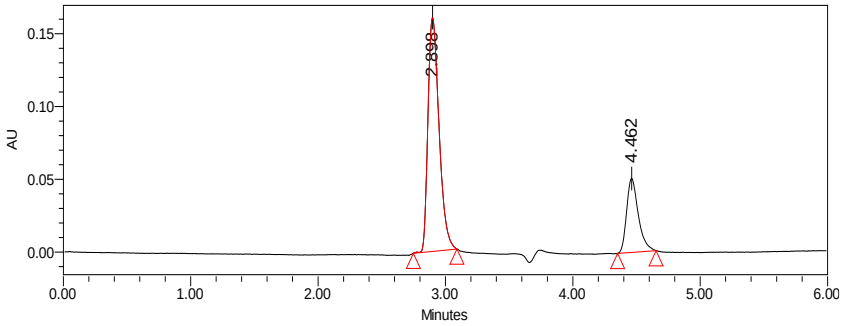


Figure 4.6.Trail 6 for GBP & MCB

Table 4.7.SSP of Trail VI for GBP & MCB

S.No	Name	Retention Time	USP Resolution	USP Tailing	USP Plate Count
1	GBP	2.898		1.42	5349
2	MCB	4.462	9.38	1.35	11836

TRAIL-VII:

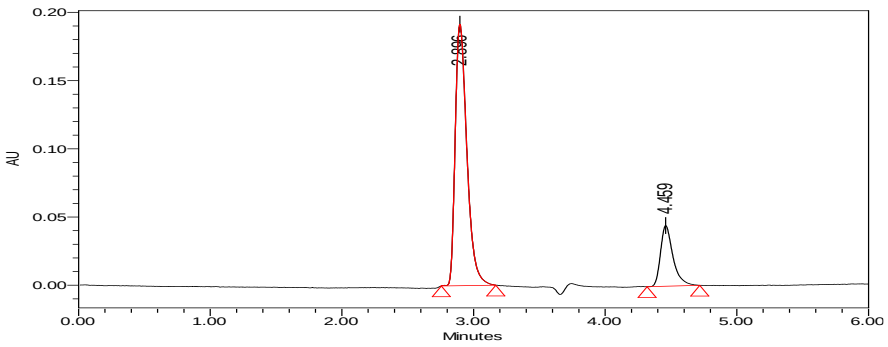


Figure 4.7. Trail 7 for GBP &MCB

Table 4.8.SSP of trail VII for GBP & MCB

S.No	Name	Retention Time	USP Resolution	USP Tailing	USP Plate Count
1	GBP	2.896		1.49	5289
2	MCB	4.459	9.34	1.43	11336

4.2. OPTIMIZED TRAIL:

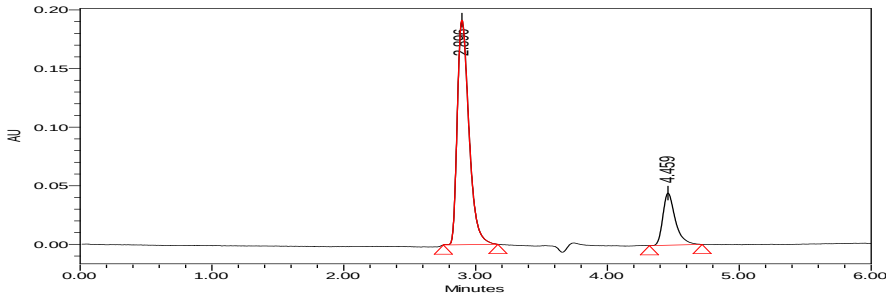


Figure 4.8.Trail 8-optimized trail for GBP & MCB

Table 4.9.SSP for optimized trail of GBP & MCB

S.No	Name	Retention Time	USP Resolution	USP Tailing	USP Plate Count
1	GBP	2.896		1.49	5289
2	MCB	4.459	9.34	1.43	11336

4.3. VALIDATION:

1.System suitability:

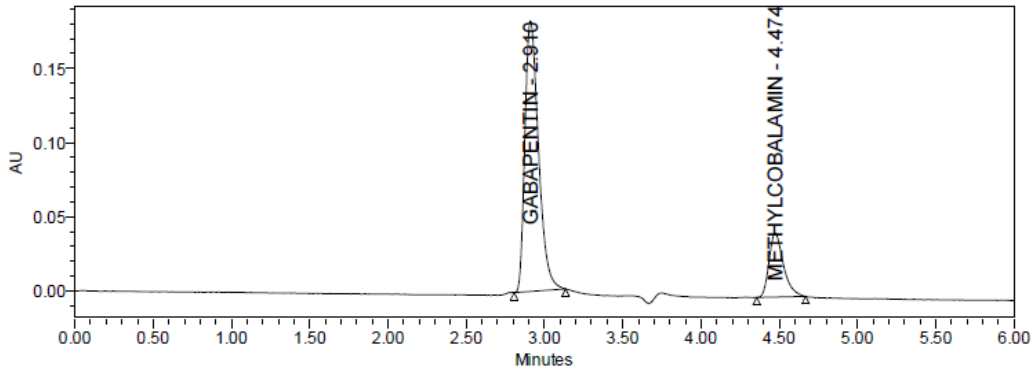
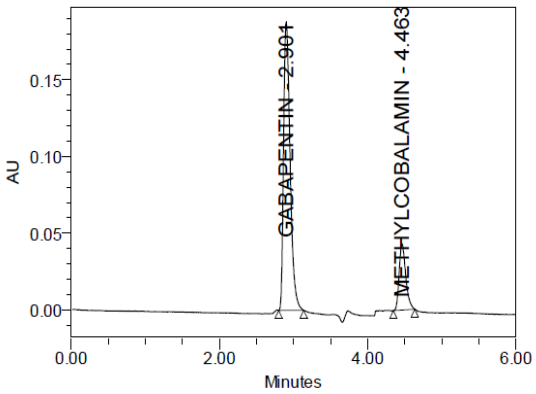
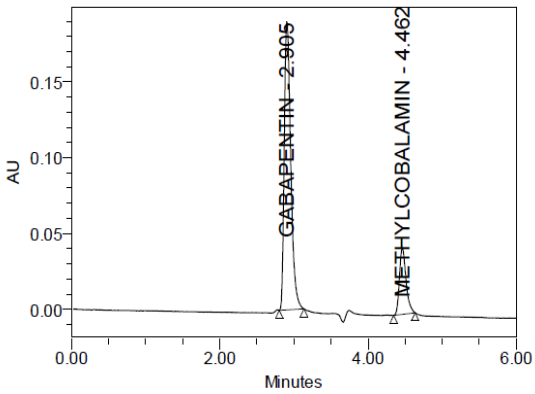


Figure 4.9 Chromatogram of Standard 1 for SSP



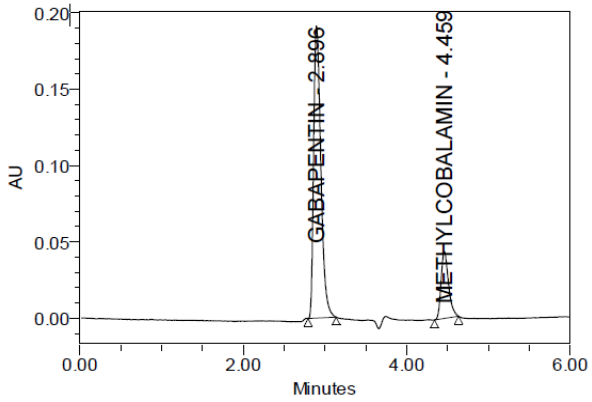
Sample name STD 2, Inj 1

(a)



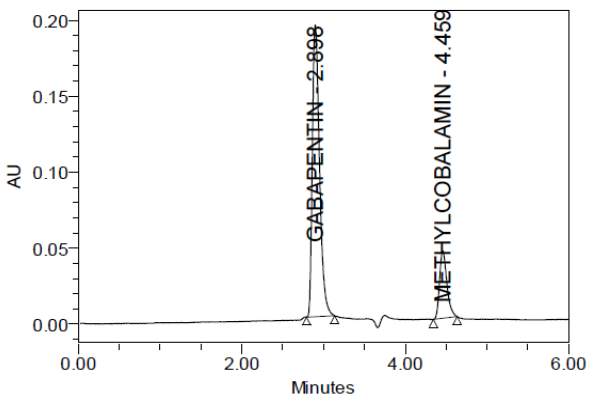
Sample name STD 2, Inj2

(b)



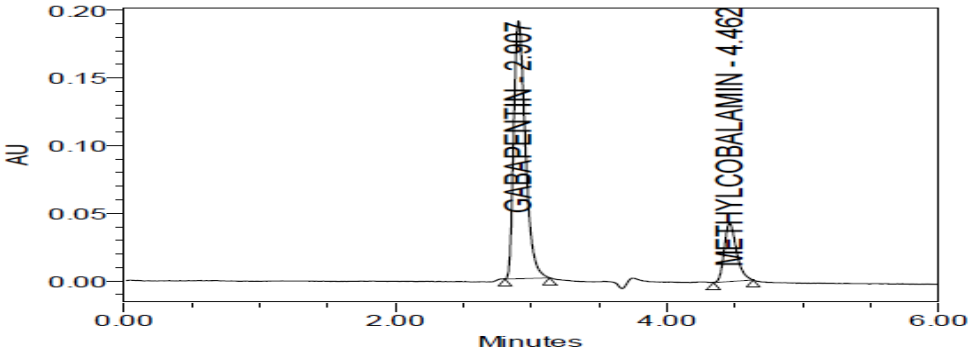
Sample name STD 2, Inj 3

(c)



Sample name STD 2, Inj 4

(d)



Sample name STD 2, Inj 5

(e)

Figure 4.10. (a), (b), (c), (d), (e) – Chromatograms of STD 2 for SSP

Table 4.10.System suitability of STD 1 of GBP & MCB resp.

Peak Name: METHYLCOBALAMIN

	SampleName	Peak Name	RT	Area	USP Plate Count	USP Resolution	USP Tailing	s/n
1	STD1	METHYLCOBALAMIN	4.474	275094	11724	9.44	1.35	4.9
Mean				275094.2				
% RSD								

Peak Name: GABAPENTIN

	SampleName	Peak Name	RT	Area	USP Plate Count	USP Tailing	s/n
1	STD1	GABAPENTIN	2.910	1100759	5429	1.41	2536
Mean				1100759.0			
% RSD							

Table 4.11.System suitability of STD 2 of GBP

Peak Name: GABAPENTIN

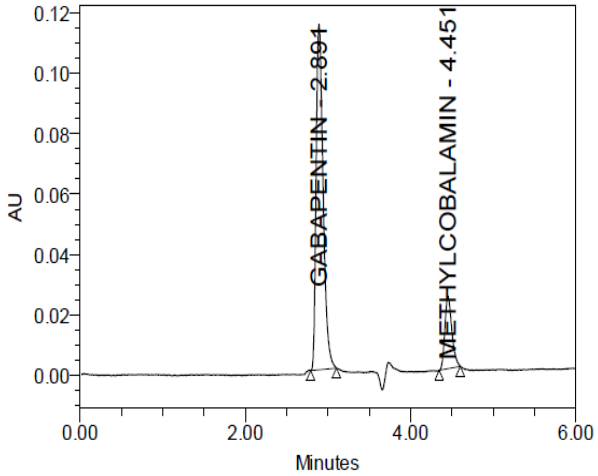
	SampleName	Peak Name	RT	Area	USP Plate Count	USP Tailing
1	STD2	GABAPENTIN	2.901	1154303	5369	1.47
2	STD2	GABAPENTIN	2.905	1152920	5443	1.45
3	STD2	GABAPENTIN	2.896	1149974	5311	1.47
4	STD2	GABAPENTIN	2.898	1153511	5375	1.47
5	STD2	GABAPENTIN	2.907	1161947	5669	1.44
Mean				1154531.1		
% RSD				0.4		

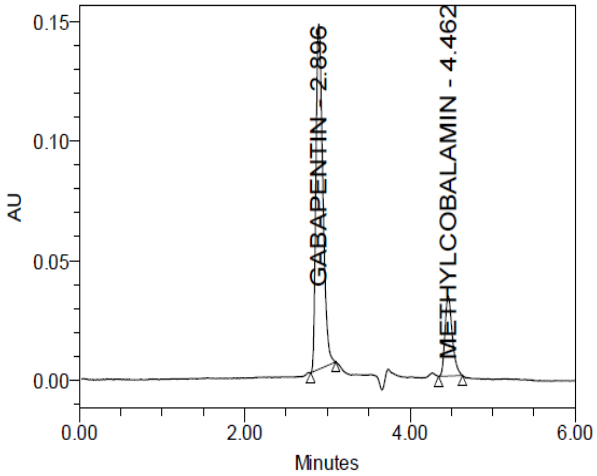
Table 4.12.System suitability of STD 2 of MCB

Peak Name: METHYLCOBALAMIN

	SampleName	Peak Name	RT	Area	USP Plate Count	USP Resolution	USP Tailing
1	STD2	METHYLCOBALAMIN	4.463	270626	12486	9.69	1.31
2	STD2	METHYLCOBALAMIN	4.462	270573	12137	9.52	1.31
3	STD2	METHYLCOBALAMIN	4.459	271395	11685	9.47	1.31
4	STD2	METHYLCOBALAMIN	4.459	271188	11914	9.47	1.31
5	STD2	METHYLCOBALAMIN	4.462	269527	12030	9.55	1.28
Mean				270661.8			
% RSD				0.3			

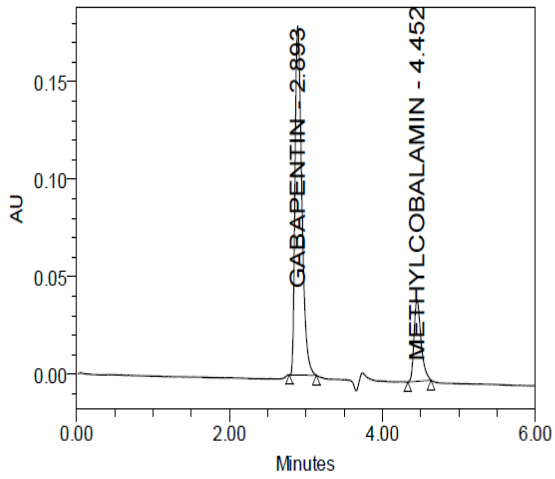
1.**Linearity**:

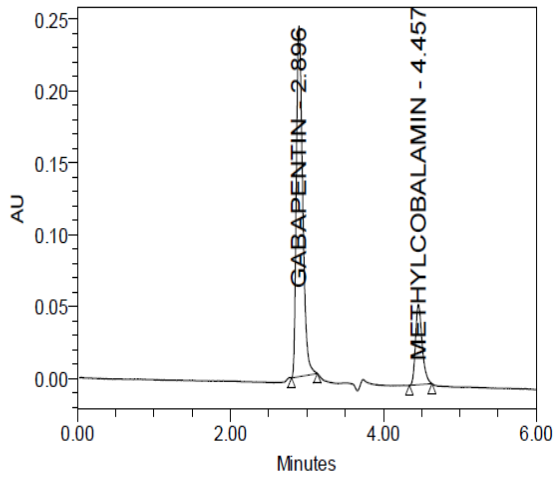




Sample name LINEARITY-50%, Inj 1 Sample name LINEARITY -75%, Inj 1

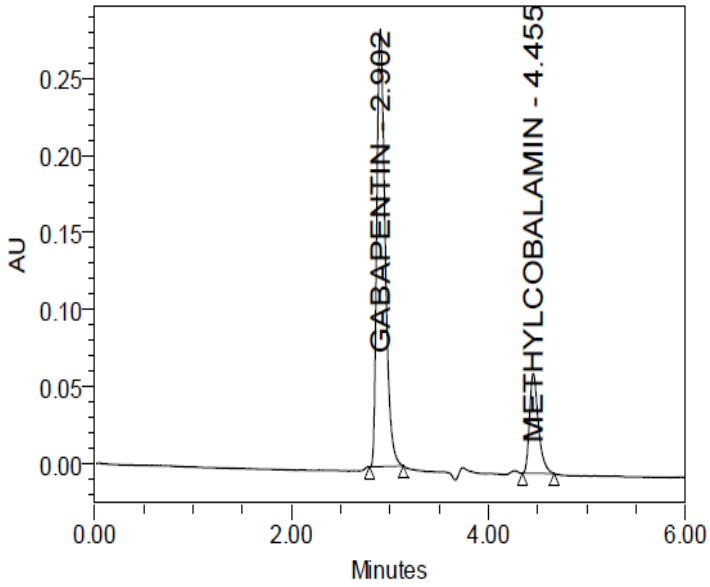
(a) (b)





Sample name LINEARITY-100%, Inj 1 Sample name LINEARITY-125%, Inj 1

(c) (d)



Sample name LINEARITY-150%, Inj 1

(e)

Figure 4.11.(a), (b), (c), (d), (e) - Chromatograms of Linearity 50%,75%, 100%, 125%, 150% resp.

Table 4.13.Linearity of GBP & MCB

GABAPENTIN			**MECOBALAMIN**		
Concentration %	Area	µg/ml	Concentration	Area	µg/ml
50	587801	1200	50	135451	2
75	880166	1800	75	203858	3
100	1174368	2400	100	271112	4
125	1468744	3000	125	339640	5
150	1765133	3600	150	397235	6

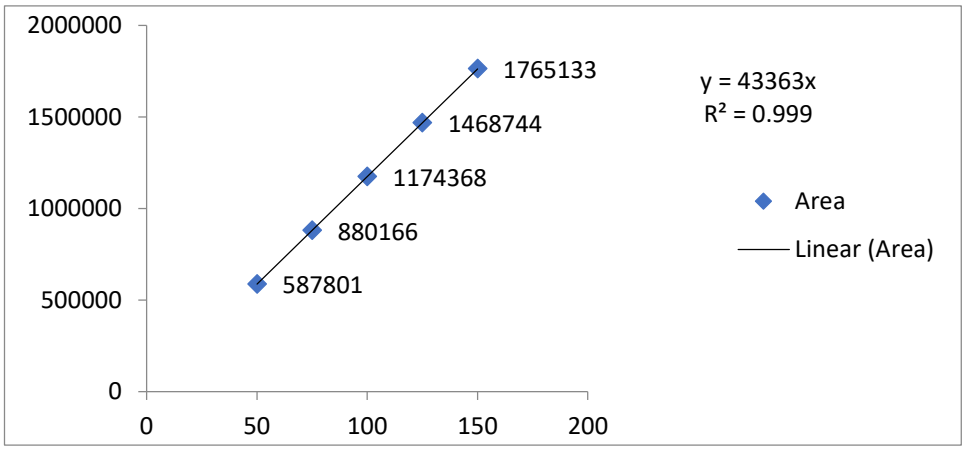


Figure 4.12. Calibration curve of Gabapentin

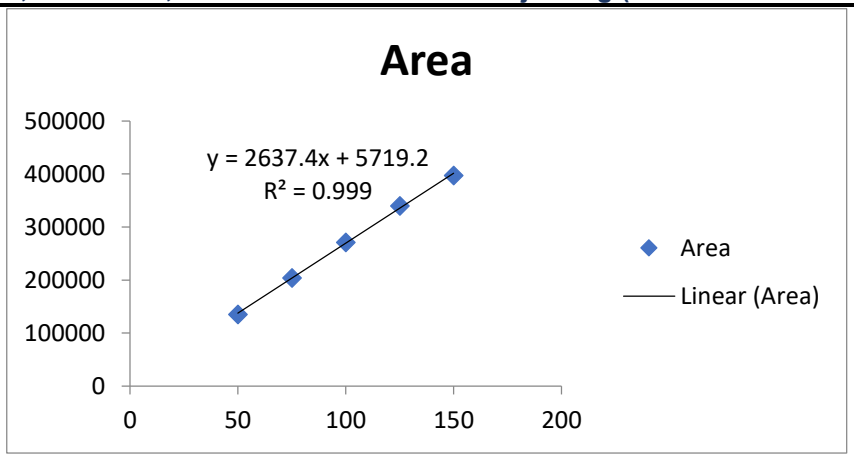
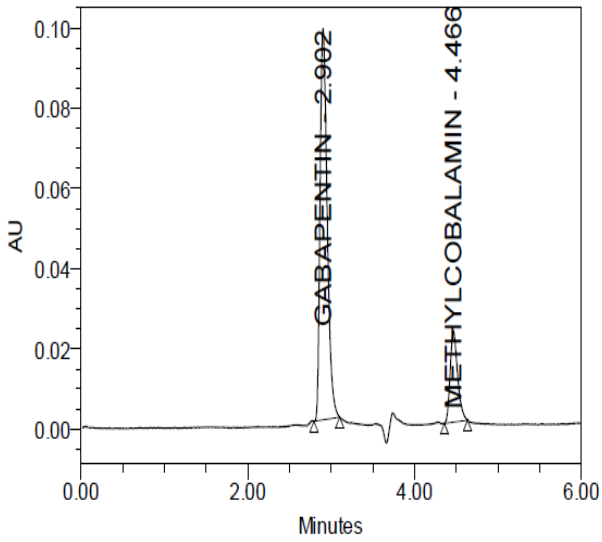
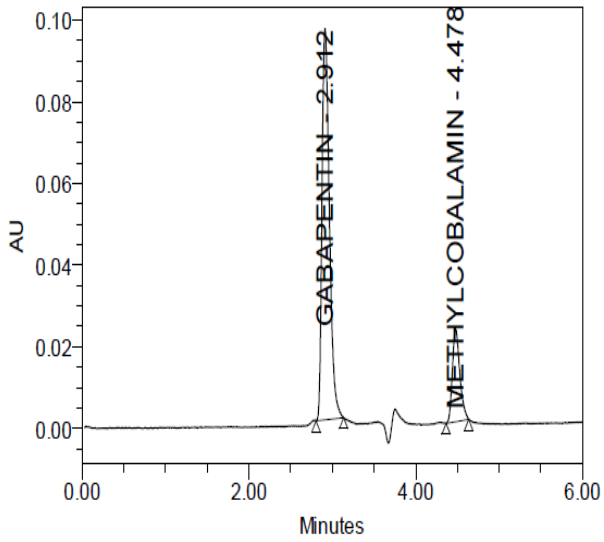


Figure 4.13. Calibration curve of Mecobalamin

1) **Accuracy**:

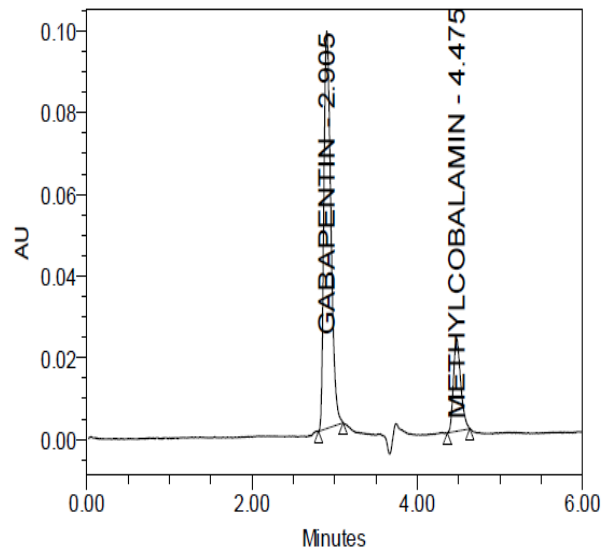
Accuracy 50%:

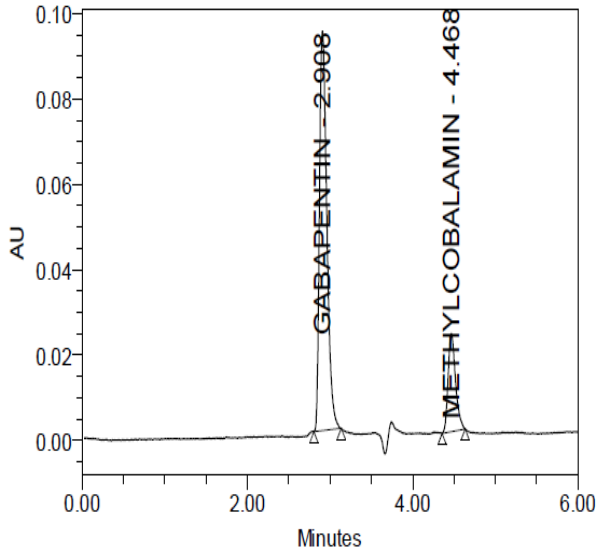




Sample nameACCURACY 50%-1,Inj 1 SamplenameACCURACY 50%-2,Inj 1

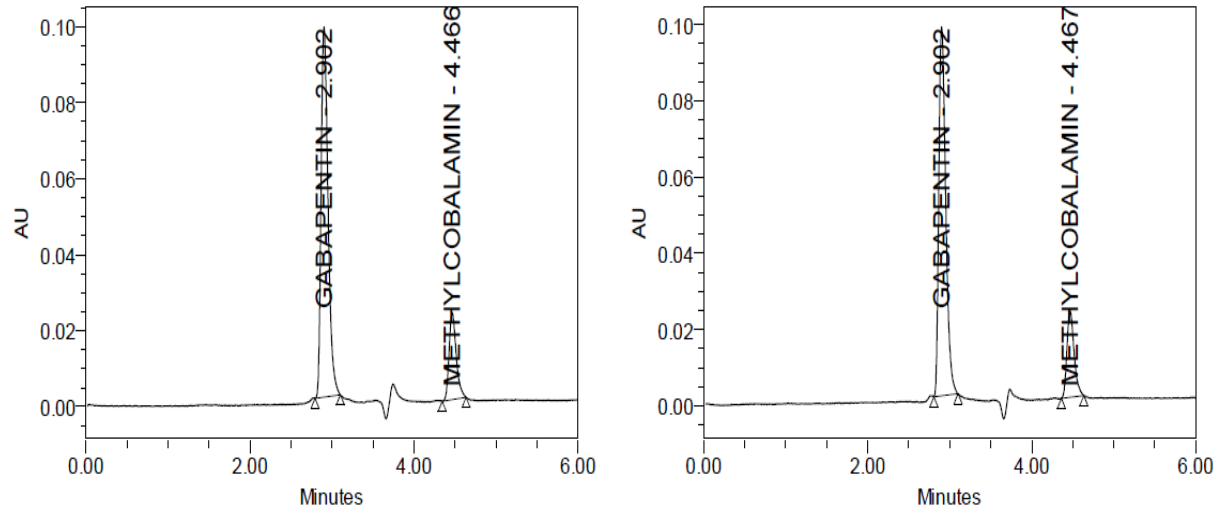
(a) (b)





Sample name ACCURACY 50%-3,Inj 1 Sample name ACCURACY 50%-4,Inj 1

(c) (d)



Sample name ACCURACY 50%-5, Inj1 Sample name ACCURACY 50%-6, Inj1

(e) (f)

Figure 4.14. (a), (b), (c),(d),(e),(f)- Chromatograms of Accuracy 50%

Table 4.12 Accuracy 50% of GBP

Peak Name: GABAPENTIN

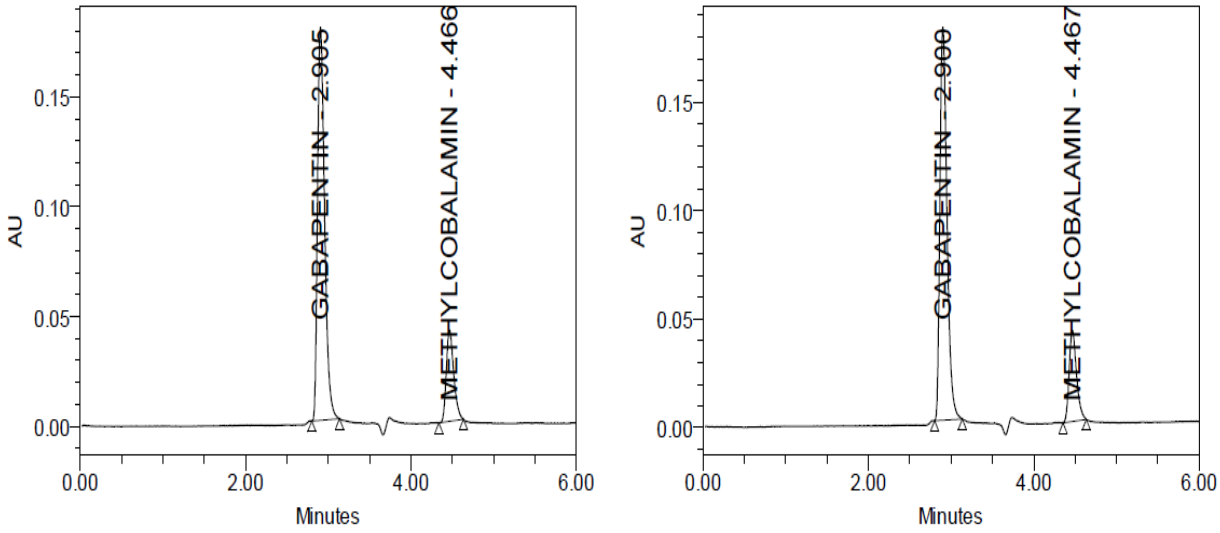
	SampleName	Peak Name	RT	Area
1	ACCURACY-50%1	GABAPENTIN	2.902	577331
2	ACCURACY-50%2	GABAPENTIN	2.912	577845
3	ACCURACY-50%3	GABAPENTIN	2.905	576963
4	ACCURACY-50%4	GABAPENTIN	2.908	577681
5	ACCURACY-50%5	GABAPENTIN	2.902	578037
6	ACCURACY-50%6	GABAPENTIN	2.902	577669

Table 4.13. Accuracy 50% of MCB

Peak Name: METHYLCOBALAMIN

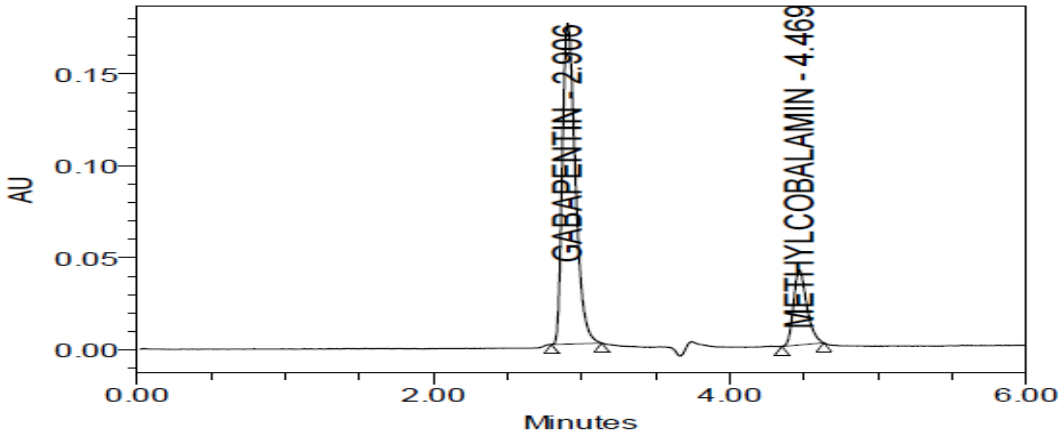
	SampleName	Peak Name	RT	Area
1	ACCURACY-50%1	METHYLCOBALAMIN	4.466	135567
2	ACCURACY-50%2	METHYLCOBALAMIN	4.478	135737
3	ACCURACY-50%3	METHYLCOBALAMIN	4.475	135338
4	ACCURACY-50%4	METHYLCOBALAMIN	4.468	136098
5	ACCURACY-50%5	METHYLCOBALAMIN	4.466	136090
6	ACCURACY-50%6	METHYLCOBALAMIN	4.467	134898

Accuracy 100%:



Sample name ACCURACY 100% -1,Inj 1 Smple name ACCURACY100%-2,Inj 1

(a) (b)



Sample name ACCURACY 100% -3, Inj (c)

Figure 4.15. (a), (b), (c)- chromatograms of Accuracy 100%

Table 4.14. ACCURACY 100% of MCB & GBP

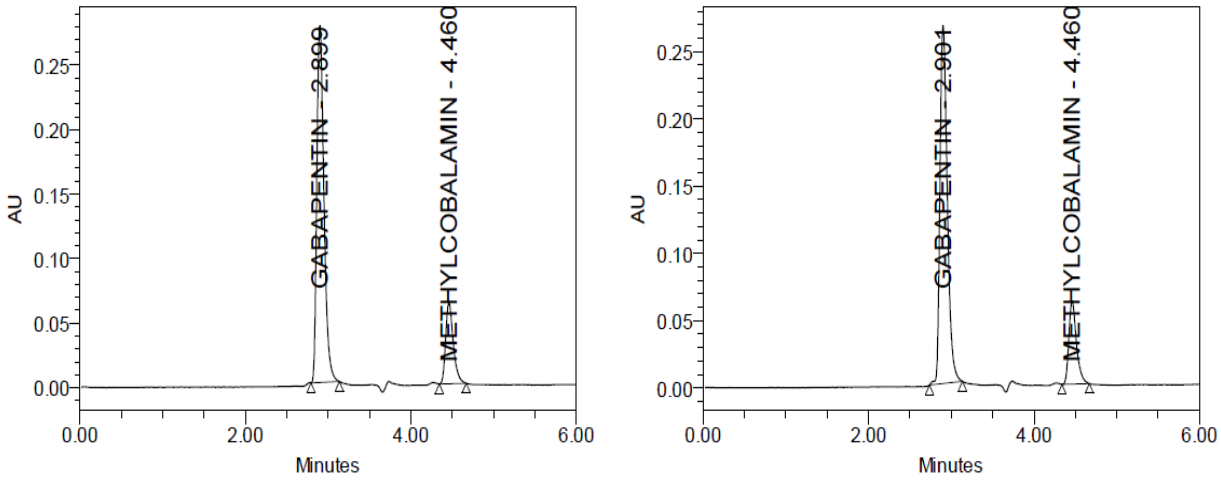
Peak Name: METHYLCOBALAMIN

	SampleName	Peak Name	RT	Area
1	ACCURACY-100%1	METHYLCOBALAMIN	4.466	271079
2	ACCURACY-100%2	METHYLCOBALAMIN	4.467	270887
3	ACCURACY-100%3	METHYLCOBALAMIN	4.469	271297

Peak Name: GABAPENTIN

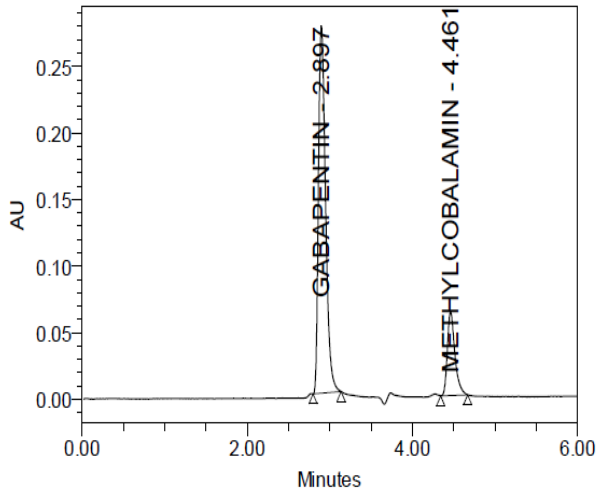
	SampleName	Peak Name	RT	Area
1	ACCURACY-100%1	GABAPENTIN	2.905	1156254
2	ACCURACY-100%2	GABAPENTIN	2.900	1155280
3	ACCURACY-100%3	GABAPENTIN	2.906	1152657

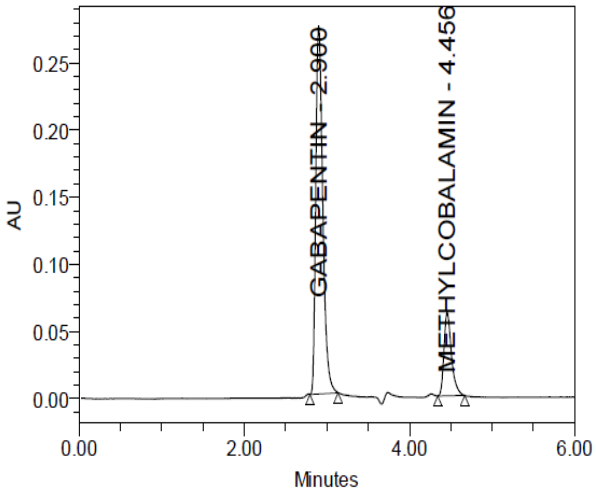
Accuracy 150% :



Sample name ACCURACY 150%-1,Inj1 Sample name ACCURACY 150%-2, Inj

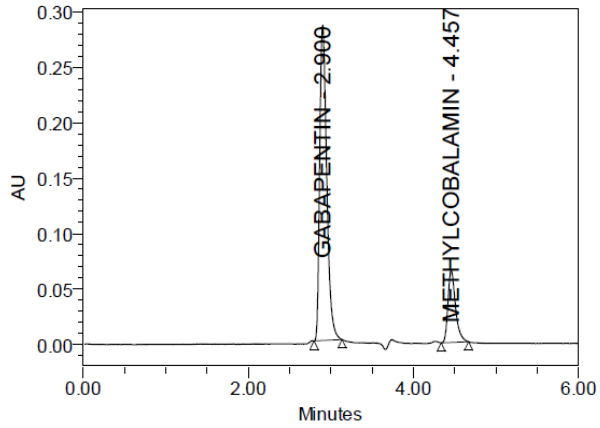
(a) (b)

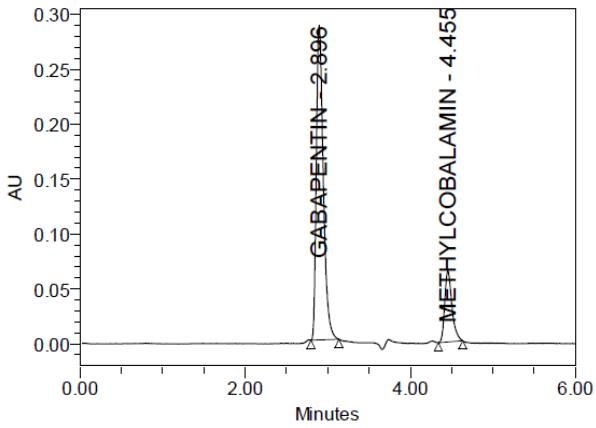




Sample name ACCURACY 150%-3,Inj-1SamplenameACCURACY 150%-4,Inj-1

(c) (d)





Samplename ACCURACY-150%-5,Inj-1SamplenameACCURACY 150%-6,Inj-1

(e) (f)

Figure 4.16.(a) (b),(c),(d)(e),(f):chromatograms of Accuracy 150%

Table 4.15. ACCURACY 150% for GBP

Peak Name: GABAPENTIN

	SampleName	Peak Name	RT	Area
1	ACCURACY-150%1	GABAPENTIN	2.899	1731738
2	ACCURACY-150%2	GABAPENTIN	2.901	1732585
3	ACCURACY-150%3	GABAPENTIN	2.897	1730270
4	ACCURACY-150%4	GABAPENTIN	2.900	1733398
5	ACCURACY-150%5	GABAPENTIN	2.900	1737911
6	ACCURACY-150%6	GABAPENTIN	2.896	1730953

Table 4.16. ACCURACY 150% for MCB

Peak Name: METHYLCOBALAMIN

	SampleName	Peak Name	RT	Area
1	ACCURACY-150%1	METHYLCOBALAMIN	4.460	405556
2	ACCURACY-150%2	METHYLCOBALAMIN	4.460	406218
3	ACCURACY-150%3	METHYLCOBALAMIN	4.461	406114
4	ACCURACY-150%4	METHYLCOBALAMIN	4.456	405672
5	ACCURACY-150%5	METHYLCOBALAMIN	4.457	406119
6	ACCURACY-150%6	METHYLCOBALAMIN	4.455	405907

Table 4.17.Recovery of GBP

GABAPENTIN						
Level	Sample wt	Sample Area	µg/ml added	µg/ml found	% Recovery	%Mean
50%	404.60	587331	237.840	237.87	100	100
50%	404.60	587845	237.840	237.07	100	
50%	404.60	587963	237.840	238.12	100	
50%	404.60	587681	237.840	238.01	100	
50%	404.60	587037	237.840	237.75	100	
50%	404.60	587669	475.562	238.00	100	
100%	809.00	1176254	475.562	476.38	100	100
100%	809.00	1175280	475.562	475.98	100	
100%	809.00	1172657	475.562	474.92	100	
150%	1213.60	1761738	713.402	713.50	100	100
150%	1213.60	1762585	713.402	713.84	100	
150%	1213.60	1760270	713.402	712.90	100	
150%	1213.60	1763398	713.402	714.17	100	
150%	1213.60	1767911	713.402	716.00	100	
150%	1213.60	1760953	713.402	713.18	100	

Table 4.18. Recovery of MCB

METHYLCOBALAMIN				
Sample Area	**µg/ml added**	**µg/ml found**	**% Recovery**	**% Mean**
135567	0.399	0.40	100	100
135737	0.399	0.40	100	
135338	0.399	0.40	100	
135098	0.399	0.40	99	
135090	0.399	0.40	99	
135898	0.399	0.40	100	
271079.00	0.797	0.80	100	100
271887.00	0.797	0.80	100	
271297.00	0.797	0.80	100	
407556	1.196	1.20	100	100
407218	1.196	1.20	100	
407114	1.196	1.20	100	
407672	1.196	1.20	100	
407119	1.196	1.20	100	
407907	1.196	1.20	100	

2) **Precision**:

Method Precision:

Table 4.19. Method Precision of GBP & MCB

S.No	Sample Weight	Sample Area-1	Sample Area-2	% Assay	% Assay
1	809.00	1178398	271098	99	100
2	809.00	1179556	271936	100	100
3	809.00	1171265	271243	99	100
4	809.00	1170530	271474	99	100
5	809.00	1174680	271953	99	100
6	809.00	1178969	271432	99	100
Avarage				99	100
STD				0.34	0.13
%RSD				0.34	0.13

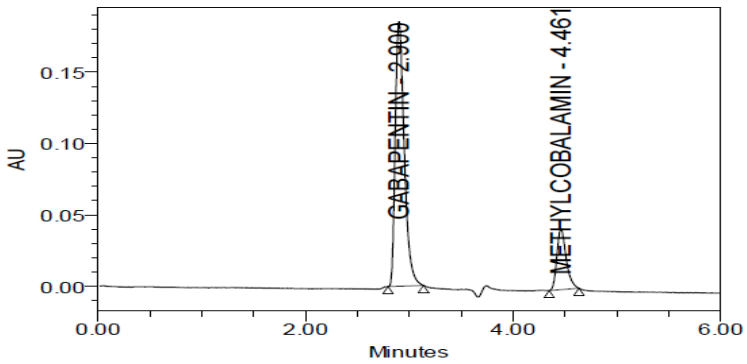


Figure 4.17 Sample name Precision of GBP & MCB, Inj, (a)

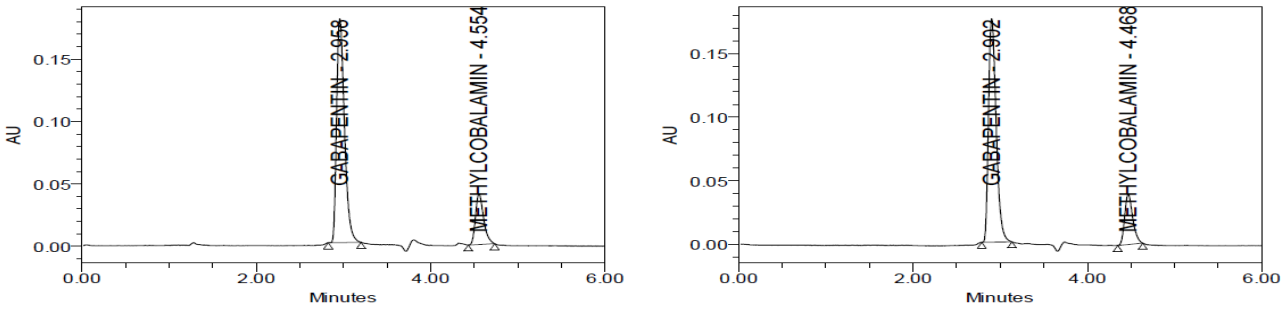
Table 4.20. Precision of GBP & MCB

Peak Name: METHYLCOBALAMIN

	SampleName	Peak Name	RT	Area
1	PRECISION1	METHYLCOBALAMIN	4.461	265098

Peak Name: GABAPENTIN

	SampleName	Peak Name	RT	Area
1	PRECISION1	GABAPENTIN	2.900	1118398



(b) (c)

Figure 4.18. Chromatograms of Precision of GBP & MCB

Table 4.21. Method precision of MCB & GBP

Peak Name: METHYLCOBALAMIN

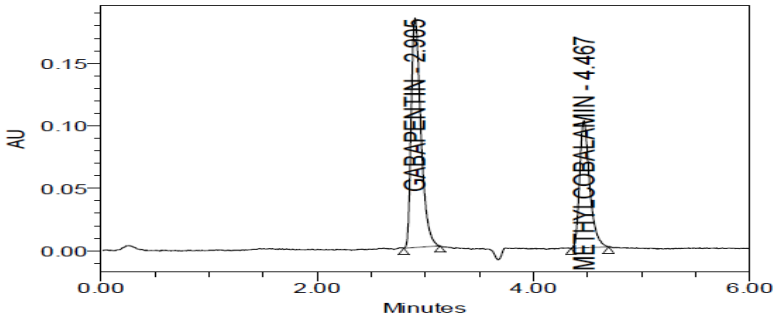
	SampleName	Peak Name	RT	Area
1	PRECISION2	METHYLCOBALAMIN	4.554	270936
2	PRECISION3	METHYLCOBALAMIN	4.468	270243
3	PRECISION4	METHYLCOBALAMIN	4.462	270474
4	PRECISION5	METHYLCOBALAMIN	4.459	270953
5	PRECISION6	METHYLCOBALAMIN	4.465	270432

Peak Name: GABAPENTIN

	SampleName	Peak Name	RT	Area
1	PRECISION2	GABAPENTIN	2.958	1159556
2	PRECISION3	GABAPENTIN	2.902	1151265
3	PRECISION4	GABAPENTIN	2.900	1150530
4	PRECISION5	GABAPENTIN	2.897	1154680
5	PRECISION6	GABAPENTIN	2.901	1158969

3) **Limit of Detection**:

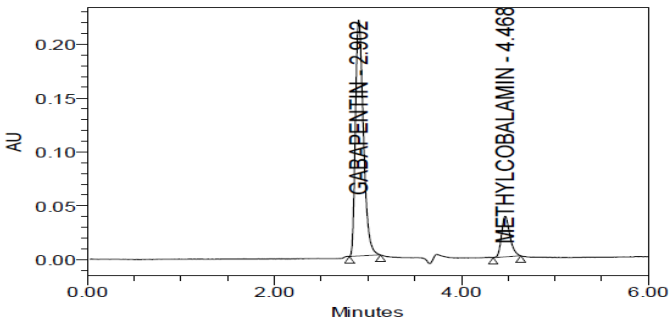
Limit of detection is determined by the standard deviation of the response and the Slope.



Sample name LOD, Inj 1

Figure 4.19.Chromatogram of LOD

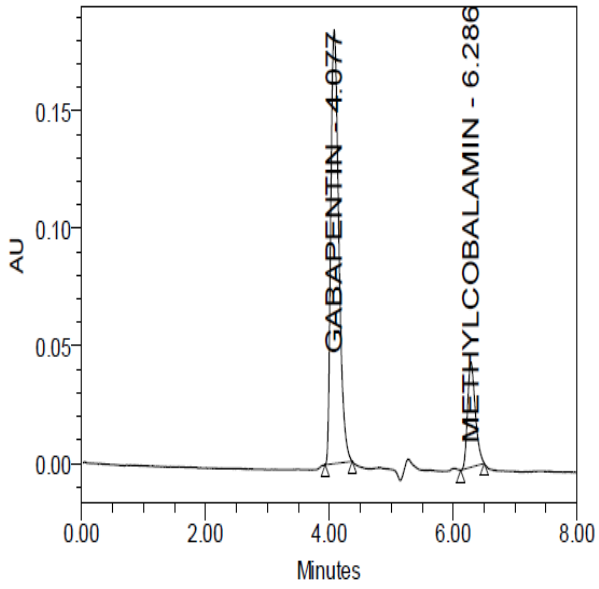
4) **Limit of Quantitation** :



Sample name LOQ, Inj 1

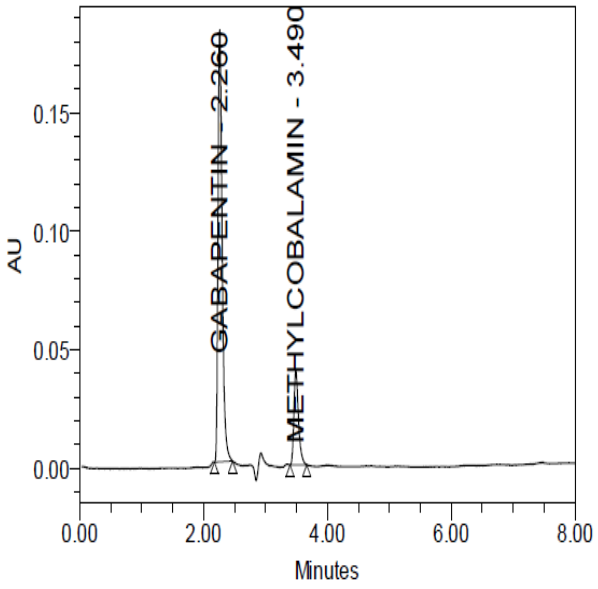
Figure 4.20. Chromatogram of LOQ

5) Robustness:



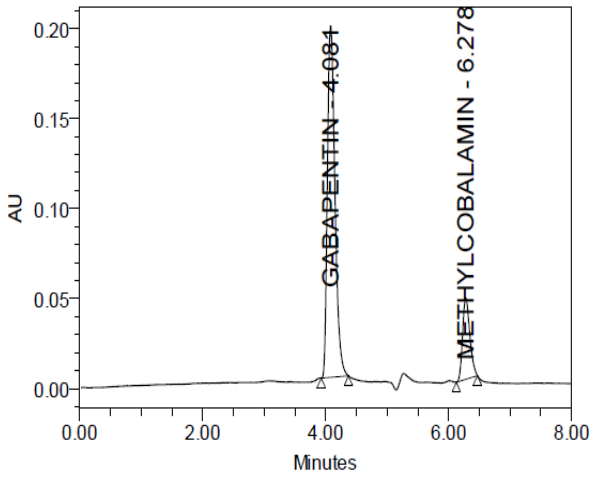
Sample name FLOW 1, Inj 1

(a)



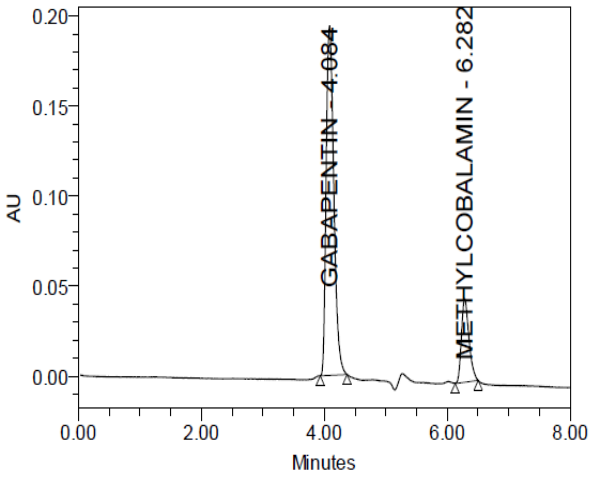
Sample name FLOW 2, Inj 1

(b)



Sample name TEMP 1, Inj 1

(c)



Sample name TEMP 2, Inj 1

(d)

Figure 4.21. (a), (b), (c), (d) –Chromatograms for robustness of GBP & MCB

Table 4.22. Robustness of MCB & GBP

Peak Name: METHYLCOBALAMIN

	SampleName	Peak Name	RT	Area	USP Tailing	USP Plate Count	USP Resolution
1	TEMP1	METHYLCOBALAMIN	6.278	369430	1.23	13848	9.75
2	TEMP2	METHYLCOBALAMIN	6.282	391494	1.31	12780	9.62
3	FLOW1	METHYLCOBALAMIN	6.286	388838	1.27	11932	9.36
4	FLOW2	METHYLCOBALAMIN	3.490	206518	1.38	11110	9.42

Peak Name: GABAPENTIN

	SampleName	Peak Name	RT	Area	USP Tailing	USP Plate Count
1	TEMP1	GABAPENTIN	4.081	1664871	1.37	5407
2	TEMP2	GABAPENTIN	4.084	1667389	1.35	5304

6) Specificity:

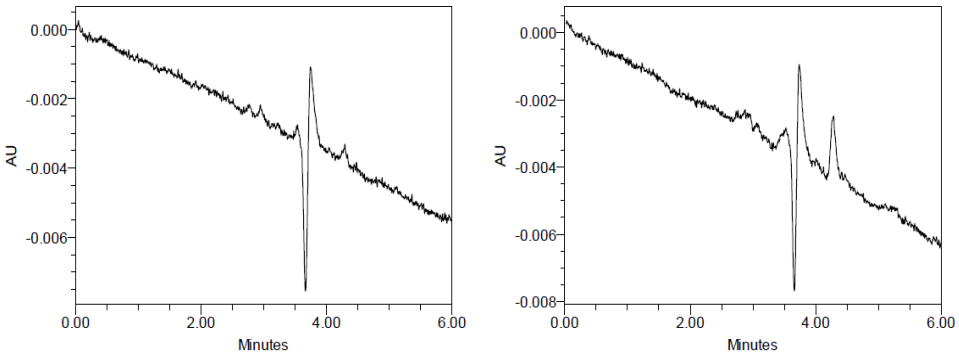


Figure 4.22.Specificity of GBP & MCB

Table 4.23. Specificity of GBP & MCB

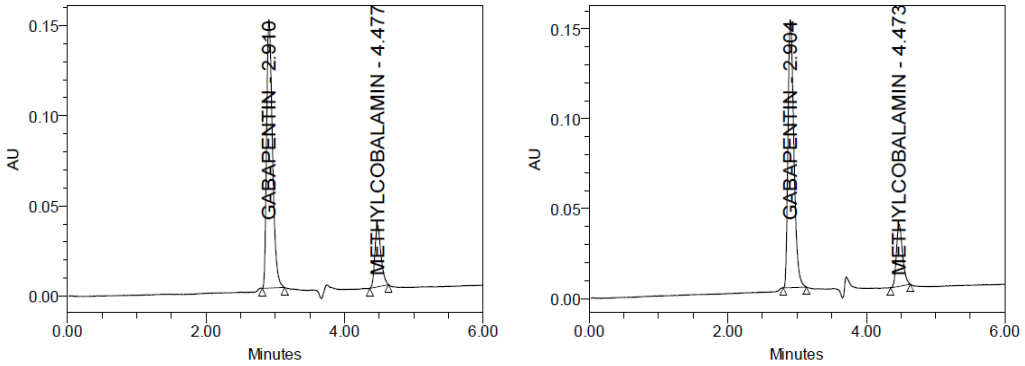
Peak Name: METHYLCOBALAMIN

	SampleName	Peak Name	RT
1	BLANK	METHYLCOBALAMIN	4.400
2	PLACEBO	METHYLCOBALAMIN	4.400

Peak Name: GABAPENTIN

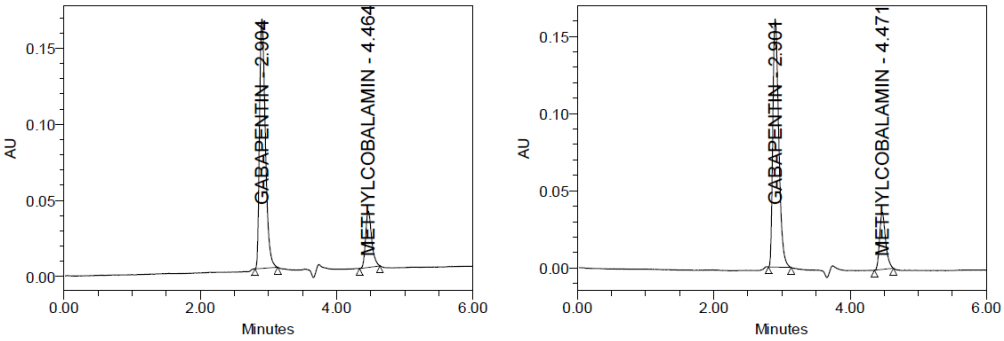
	SampleName	Peak Name	RT
1	BLANK	GABAPENTIN	2.900
2	PLACEBO	GABAPENTIN	2.900

4.4. FORCED DEGRADATION STUDIES – ASTABILITY INDICATING PARAMETER



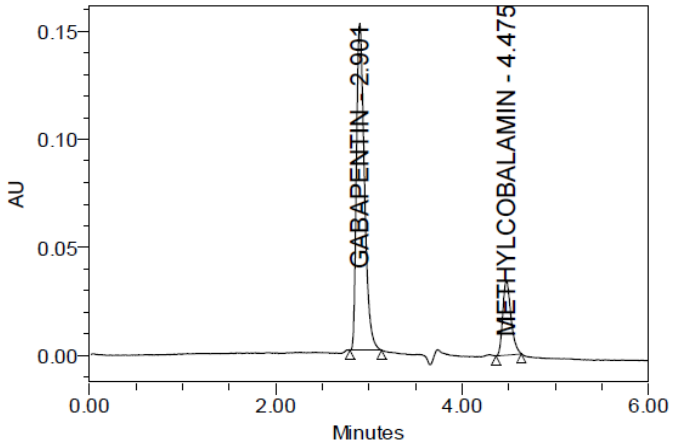
Sample name ACID, Inj 1 Sample name BASE, Inj 2

Figure 4.23. Chromatogram of Acid degradation & Chromatogram of Base degradation



Sample name PEROXIDE, Inj 3 Sample name PEROXIDE, Inj 4

Figure 4.24. Chromatogram of Peroxide degradation & Chromatogram of Heat degradation



Sample name: Sunlight, Inj 5

Figure 4.25. Chromatogram of sunlight degradation

Table 4.24. Forced degradation of GBP & MCB

Parameter	**Area of of GBP**	**Area of MCB**	**% Assay of GBP**	**%Assay of MCB**	**% Degradation of GBP**	**% Degradation of MCB**
Acid	2917192	3109695	67	70	-32	-30
Base	2648221	3231239	61	73	-38	-27
Peroxide	2527496	3227403	58	73	-41	-27
Sunlight	2970691	4110524	68	93	-31	-7
Heat	3968873	4125196	91	93	-8	-7
STD	13285884	1523821				
% RSD	53.0	51.0				

4.5. DISCUSSIONS:

TRAIL-I:

Observation: The second peak did not elute, it might be due to high R_t.

Conclusion : To decrease R_t, the solvent ratio was increased i.e from 400 to 600ml.

TRAIL-II:

Observation: Still there is no appearance of peak

Conclusion: To get a second peak, the basic buffer was changed to acidic buffer i.e. potassium dihydrogen phosphate. (pH4.2).

TRAIL-III:

Observation: The second peak has not eluted, may be due to high R_t.

Conclusion: To decrease Rt, the solvent Ratio was increased i.e from 400 to 600ml.

TRAIL-IV:

Observation: Still there is no peak

Conclusion: To get a second peak ,the basic buffer was changed to more acidic buffer ieortho phosphoric acid (pH 2.2).

TRAIL-V:

Observation: Yes, the second peak has eluted but first peak observed low theoretical plates.

Conclusion: To increase the plate count, the buffer volume was increased (400ml to 500ml).

TRAIL –VI:

Observation: Here the two peaks have eluted with good plate count and low Rt but peak response is very low i.e 0.15.

Conclusion: To increase response better to Change injection volume from 5µl to 10µl.

TRAIL-VII:

Observation: yes, the two peaks are eluted and all system suitability parameters lie within acceptance criteria.

Conclusion: Thus these condition are optimized.

OPTIMIZED TRAIL :

It must pass all the system suitability parameters, acceptance criteria are as

Retention time should be low

Resolution : Not less than 2

Tailing : Not more than 2

Theoretical plates : Not less than 2500

Relative standard deviation (RSD) : Not more than 2

Similarity factor : 0.98-1.02

Observation: yes, the two peaks are eluted and fromthe table 6.1.17of results section , it is seen all system suitability parameters are passed.

Conclusion: Therefore this is the optimized trail.

VALIDATION:

1) **System suitability**:

Acceptance Criteria:

Tailing factor for both GBP &MCB NMT (should not be more than) 1.5.

% RSD for replicate injection for each peak should be NMT 2 %.

Data Interpretation:

It is observed from the data tabulated in the 6.1.20, 6.1.21, 6.1.22of results section; the method complies with system suitability parameters.

Conclusion:

Hence it can be concluded that the system suitability meets the requirements of method validation.

2) **Linearity**:

Acceptance Criteria:

The correlation coefficient should NLT (not be less than) 0.999.

Data Interpretation:

From the graph, fig no's6.1.25 & 6.1.26of concentration vs chromatographic response (area) it can be seen that the correlation coefficient (R^2) is 0.999 for GBP & 0.999 for MCB as well, and the regression line obtained for GBP & MCB are linear. Equation of the line y = 43363x for GBP and y = 2637x + 5719 for MCB.

Conclusion: The proposed method was found to be linear in the concentration range of 1200 to 3600 µg / ml for GBP & 2 to 6 µg / ml for MCB.

3) **Accuracy**:

Acceptance Criteria: The method is considered accurate if individual recovery is between 97.0% to 103%.

Data Interpretation: From the results of accuracy as tabulated in 6.1.35 & 6.1.36 of the results section, the recovery is well within the limit.

Conclusion: Hence the proposed method is accurate.

4) **Precision**:

Acceptance Criteria : The RSD of six replicate assays is NMT (not more than) 2.0%.

Data Interpretation: From the results of precision as tabulated in the 6.1.40 results section, the RSD is well within the limit.

Conclusion: Hence the proposed method is Precise.

5) **Limit of Detection** :

The limit of Detection for GBP was found to be 2.839.

The limit of Detection for MCB was found to be 2.4490.

6) **Limit of Quantitation**:

The limit of Quantitation for GBP was found to be 9.464.

The limit of Quantitation for MCB was found to be 8.1633.

7) **Robustness** :

Acceptance Criteria : The system suitability parameters should pass.

Data Interpretation: From the table no 6.1.44 of results section, it is evident that the SSP (system suitability parameters are passed) even with a flow rate & temperature deliberate variation from the optimized conditions of the same.

Conclusion : Hence the proposed method is robust.

8) **Specificity** :

Acceptance Criteria : There is no 'peak' of blank, placebo or impurity at the analyte retention time.

Data Interpretation: From the data tabulated in 6.1.46 of results section, it is evident that there is appearance of no peak at the analytes' retention time.

Conclusion : Therefore it passes specificity.

FORCED DEGRADATION STUDIES:

Observation : The sample subjected to various stress studies, namely acid, base, peroxide, heat & sunlight, is showing different percent of degradation, so it could be concluded that the sample is very sensitive to degradation.

Conclusion : Hence from these observations as tabulated in 6.1.50 it can be inferred that the proposed method is very specific for GBP & MCB degradation in tablet form.

5. SUMMARY &CONCLUSION

5.1. SUMMARY

- System suitability parameters were calculated which includes efficiency, resolution and tailing factor.
- The reliability and suitability of the method could be seen from recovery studies. Further there is no interference due to excipients.
- Precision of the methods were studied by making repeated injections of the samples and values were determined.
- The method was validated for linearity, accuracy, precision, robustness.
- The method is simple, specific & easy to perform and requires short to analyse the samples.

- Low limit of quantification and limit of detection makes this method suitable for Quality control.
- The method was found to be linear, accurate, precise and robust.
- Hence it was concluded that the RP-HPLC method developed was very much suitable for routine analysis of tablet formulation containing Gabapentin & Methyl cobalamin.

5.2. CONCLUSION

The wide linearity range, Accuracy, Short retention times, & Simple Mobile phase imply that the proposed method can be successfully employed for routine quantification of Gabapentin &Mecobalamin in combined dosage form. The method is economic too as the cost of mobile phase used is less compared to costly solvents that has to be used like acetonitrile for the quantification of GBP & MCB.

Also the forced degradation studies imply that this method is Stability Indicating Method development & validated according to ICH guidelines, one can adopt in an industry confidently for routine analysis.

6. REFERENCES

1. Shetti, P.D. High Performance Liquid Chromatography. 2001; 116.
2. The Merck Index, an encyclopedia of chemicals, drugs and biological.Fourteenth Edn.USA; 2006.
3. Beckett, A.H.; Stenlake, J.B. Practical Pharmaceutical Chemistry, 4thEdn., C.B.S. Publications, 1.
4. Malik, V. Drugs and Cosmetics Act 1940, 16thEdn., Eastern BookCompany, Lucknow, 5.
5. Willard, H.H.; Merit, L.L.; Dean, F.A.; Settle, F.A. Instrumental methods of analysis, 7thEdn., C.B.S. Publishers, New Delhi, 2002.
6. Mendham, J.; Denney, R.C.; Barnes, V; Thomas, M.J.K. Vogel's Textbook of Qualitative Chemical Analysis, 6thEdn., 261-287.
7. Skoog, D.A.; West, D.M. Fundamentals of Analytical Chemistry, 7thEdn.
8. Sharma, B.K., Instrumental methods of Chemical analysis, 19thEdn.2000.
9. Willard,H.H.; Merrit, L.L.; Dean. J.A., Instrumental methods of analysis,7thEdn., CBS Publishers, New Delhi.
10. Khopkar, S.M. Basic concepts of analytical chemistry, 2nd edition, 2005.
11. Tips on Liquid Chromatography, Waters www.waters.com.
12. Validation of Analytical Procedures: Methodology, ICH TripartiteGuidelines 1996.
13. Validation of Analytical Procedures, ICH Harmonized TripartiteGuidelines 1994.
14. ICH, Q2A Text on validation of analytical procedures,Oct,1994www.ich.org.
15. ICH, Q3B Validation of analytical procedures: methodology, Nov, 1996.
16. Ahuja, S.; Scypinsky, S.; Hand book of modern pharmaceutical analysis,2 (4): Academic press-I.

17. "Neurontin, Gralise (gabapentin) dosing, indications, interactions, adverse effects, and more". Medscape Reference. WebMD. Retrieved 6 April 2014.

18. Goa KL; Sorkin, EM (September 1993). "Gabapentin. A review of its pharmacologicalproperties and clinical potential in epilepsy.". Drugs 46 (3): 409–27.

19. Attal N, Cruccu G, Baron R, et al. (September 2010). "EFNS guidelines on the pharmacological treatment of neuropathic pain: 2010 revision". Eur. J. Neurol. 17 (9): 1113–e88.doi:10.1111/j.1468-1331.2010.02999.x.

20. StephenV.Sobel(5November2012). Successful Psychopharmacology: Evidence-Based Treatment Solutions for Achieving Remission. W. W. Norton. p. 124. ISBN 978-0-393-70857-8.

21. Attal N, Cruccu G, Baron R, et al. (September 2010). "EFNS guidelines on the pharmacological treatment of neuropathic pain: 2010 revision". Eur. J. Neurol. **17** (9): 1113–e88.doi:10.1111/j.1468-1331.2010.02999.x. PMID 20402746.

22. Moore, RA; Wiffen, PJ; Derry, S; McQuay, HJ (Mar 16, 2011). "Gabapentin for chronic neuropathic pain and fibromyalgia in adults.". The Cochrane database of systematicreviews (3): CD007938.doi:10.1002/14651858.CD007938.pub2. PMID 21412914.

23. Wiffen, P; Collins S; McQuay H; Carroll D; Jadad A; Moore A (2005-07-20). "Anticonvulsant drugs for acute and chronic pain". In Wiffen, Philip J. Cochrane database of systematic reviews (Online) (3): CD001133.doi:10.1002/14651858.CD001133.pub2. PMID 16034857.

24. Finnerup, NB; Sindrup SH; Jensen TS (September 2010). "The evidence for pharmacological treatment of neuropathic pain". Pain **150** (3): 573–81.doi:10.1016/j.pain.2010.06.019. PMID 20705215.

25. Rada, G; Capurro, D; Pantoja, T; Corbalán, J; Moreno, G; Letelier, LM; Vera, C (Sep 8, 2010). "Non-hormonal interventions for hot flushes in women with a history of breast cancer.". The Cochrane databaseofsystematicreviews (9): CD004923. doi:10.1002/14651858.CD004923.pub2.PMID 20824841.

26. Muncie HL, Jr; Yasinian, Y; Oge', L (Nov 1, 2013). "Outpatient management of alcohol withdrawal syndrome.".American family physician **88** (9): 589–95. PMID 24364635.

27. Jack D. Edinger (2013). Insomnia, an Issue of Sleep Medicine Clinics. Elsevier Health Sciences. p. 339.ISBN 978-0-323-18872-2.

28. Charles M. Morin; Colin A. Espie (2 February 2012). The Oxford Handbook of Sleep and Sleep Disorders. Oxford University Press. p. 544. ISBN 978-0-19-970442-2.

29. "Neurontin packaging insert" (pdf). U.S. Food and Drug Administration (FDA). 2009-05-01. Retrieved 2010-07-16.

30. Jeffrey K Aronson (4 March 2014). Side Effects of Drugs Annual: A worldwide yearly survey of new data in adverse drug reactions. Newnes. p. 137. ISBN 978-0-444-62636-3.

31. Gabapentin Official FDA information, side effects and uses

32. Jeffrey, Susan. "FDA Approves Gabapentin Enacarbil for Postherpetic Neuralgia". Medscape.

33. Kuzminski, A.M., et al. (1998) "Effective treatment of cobalamin deficiency with oral cobalamin." Blood, 92: 1191-1198.

34. Mayer, G., et al. (1996) " Effects of B12 on performance and circadian rhythms in normal subjects." Neuropsychopharm.,15: 456-464.

35. Ohta, T., et al. (1991) "Treatment of persistent sleep-wake schedule disorder in adolescents with methylcobalamin." Sleep, 14: 414-418.

36. Okawa, M. et al. (1990) "Vitamin B12 treatment for sleep-wake rhythm disorders." Sleep, 13: 15-23.

37. Sakane, T., et al. (1982) "Effects of methyl B12 on in vitro immune functions of human T lymphocytes." Experientia, 48: 716-720.

38. Watanaabe, T., et al. (1994) Ultra high dose methylcobalamin promotes nerve regeneration in experimental acrylamide Neuropathy." J. Neurol. Sci., 122: 140-143.

39. B. Lakshmi et al, developed UV VIS Spectrophotometric method for the estimation of Gabapentin in formulations , The Experiment ,August 2012,vol 2, pg no 84-92.

40. Varsha R Galande et al, reported UV VIS Spectrophotometric method for the estimation of Gabapentin & Mecobalamin in bulk & tablet, International Journal of Chemtech research, vol 2, (1), Jan – Mar 2010 , Pg no 695-699.

41.KG. Baheti* and VR.Galande, reported the Validated Simultaneous Estimation of Gabapentin in the Presence of Methylcobalamin in Tablet by HPTLC Method , International journal of research in Pharmaceutical and Biomedical sciences, vol 2 (3), jul-sep 2011 .

42.M. C Sharma et al, reported the Simultaneous Estimation and Validation of Gabapentin and Methylcobalamin in Tablet Dosage form: hydrotropic approach , Drug Invention Today, vol 3,issue 6, june 2011 , pg no 95.

43.Sameer A.M Abdul Rahman et al reported the highly sensitive spectrophotometric method for the determination of gabapentin in capsules using sodium hypochloride, Drug Testing and Analysis, volume 3, issue 10, oct2011, pg no 748-754.

44.Pratik PravubhaiGoti&Parula B Patel reported the Development and validation of ratio derivative spectrophotometric method for simultaneous estimation of Gabapentin, Methylcobalamin and alpha lipoic acid in tablet formulation, Journal of Pharmacy research, vol 6, issue 6, june 2013, pg no 609-614.

45.Maryam Kazemipoura et al reported the Gabapentin Determination in Human Plasma and Capsule by Coupling of Solid Phase Extraction, Derivatization Reaction, and UV-Vis-Spectrophotometry, Iran journal of Pharmaceutical Research, vol 12 (3), pg no 247-253.

46.Ganesan M et al reported a simple spectrophotometric method for the estimation of mecobalamin in injections, International journal of Pharmacy & Pharmaceutical sciences, vol 4, issue 3, 2012, pg no 75-82.

47.Nandini R. Pai and Seema S. Sawant reported a simple and validated RP-HPLC method for the estimation of methylcobalamin and Alphalipoic acid in soft gelatine capsule dosage form,Pelagia research library, vol 4, (5), 2013, pg no 46-53.

48.Rajinder Singh Gujral and S.K Manirul Haque developed a simple HPLC method for thequantitation of Gabapentin in pure form. International journal of biomedical science, vol 5(1) march 2009, pg no 63- 69.

49.Syed Sultan Qasim; Mohammed Mustafa Ali Siddiqui ; Ehab Youssef Abueida; Mohammed AbulKhair, Abudhab reported validation of an isocratic HPLC assay of gabapentin in pharmaceutical formulations and stress test for stabilityof drug substances. Scholars Research library, vol 3(4) 2011, 342-350.

50.Binaya Das developed and validated a simple , sensitive and specific Liquid chromatography-Tandem Mass Spectrometry (LC-MS/MS) quantification of gabapentin in human plasma. Vol 6 suppl 3, july-sept 2013.

51.Narmada P, Vijaya Lakshmi G, Nalini G, Gowtham Y, Suhasini B, Jogi K.V reported a simple, preciseand accurate RP-HPLC method for estimation of pregablin and mecobalamin in capsule formulation. International journal of research in pharmaceutical science, vol4 (1), 2013, pg no 25-29.

ABBREVIATIONS

HPLC	-	High Performance liquid chromatography
UV	-	Ultra violet spectroscopy
Wt	-	Weight
LOD	-	Limit of Detection
LOQ	-	Limit of Quantitation
S.D	-	Standard Deviation
%RSD	-	Percentage Relative Standard Deviation
RS	-	Peak Resolution
M.P	-	Mobile Phase
mg	-	milligrams
µg	-	Micrograms
ml	-	Milliliters
%	-	Percentage
w/w	-	Weight/weight
v/v	-	volume/volume
µg/ml	-	micrograms per milliliter
nm	-	Nanometer
Rt	-	Retention Time
Min	-	Minutes
ICH	-	International conference on Harmonization