Methods for Determination of Drugs, Drug release and its Impurities

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Determination of exact quantity of drug in the drug formulation requires accurate and robust analytical method. Analytical method development and validation plays an important role in the drug discovery, development, and quality control of pharmaceutical products. The method should be simple and fast so that it can be adopted by any laboratory easily. The methods which are used in analysis of stability studies for assay and related substances should be stability indicating analytical methods. The peaks of solutes and impurities under determination should be pure and hence peak purity in the methods for determination of assay and related impurities should be passed. The developed methods need to be validated as per current regulatory guidelines. All the validated methods should pass the acceptance criteria as per industrial and regulatory norms.Due to these high expectations, pharma industry is suffering, since many of the firms are using regular conventional techniques to determine the drug quantities and their impurities. To keep the pace with rapid improvement in quality standards of drugs all over the world and keeping in view the essential requirements.

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METHODS FOR DETERMINATION OF DRUGS

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RESEARCH AND DEVELOPMENT OF NEW ANALYTICAL METHODS FOR THE DETERMINATION OF DRUGS, DRUG RELEASE AND IMPURITIES IN DIFFERENT PHARMACUETICAL DOSAGE FORMS USED IN TREATMENT OF ALZHEIMERS, DIPRESSION AND LIVER DISEASES

SUBMITTED TO

Dr. BABASAHEB AMBEDKAR MARATHWADA UNIVERSITY
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FOR THE AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN CHEMISTRY

BY

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UNDER THE GUIDENCE OF

Prof. Dr. DHANANJAY MANE

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ABBREVIATIONS:

BP= British pharmacopoeia,

ELSD=Evaporative Light Scattering Detector,

FDA= Food and Drug Administration,

USFDA= United States Food and Drug Administration,

HPLC=High Performance Liquid Chromatography,

ICH= International Conference on Harmonization,

IP= Indian pharmacopoeia,

AD = Alzheimer's Disease,

MEM = Memantine HCl,

R&D = Research and Development,

LOD= Limit of detection,

LOQ= Limit of quantification,

PDA=Photodiode array detector,

RI=Refractive Index detector,

USP=United State pharmacopoeia,

UV=Ultra Violet,

VIS=Visible

GPC=Gradient Program

SEC=Size Exclusion Chromatography

ppb= parts per billion

WHO=Word Heath Organization,

TCAs = Tricyclic Antidepressants,

SUMMARY :

Determination of exact quantity of drug in the drug formulation requires accurate and robust analytical method. Analytical method development and validation plays an important role in the drug discovery, development, and quality control of pharmaceutical products. The method should be simple and fast so that it can be adopted by any laboratory easily. The methods which are used in analysis of stability studies for assay and related substances should be stability indicating analytical methods. The peaks of solutes and impurities under determination should be pure and hence peak purity in the methods for determination of assay and related impurities should be passed. The developed methods need to be validated as per current regulatory guidelines. All the validated methods should pass the acceptance criteria as per industrial and regulatory norms.

In the last few years many pharmaceutical regulatory authorities have found major non-compliances in analytical method validations. The regulatory requirements by different regulatory authorities (Indian FDA, USFDA, European agencies) have increased tremendously.

Due to these high expectations, pharma industry is suffering, since many of the firms are using regular conventional techniques to determine the drug quantities and their impurities. Many methods are complex in nature and time consuming. The validations of those methods are also challengeable due to its complex nature. In last few years the analytical technique has improved and use of chromatographic technique (HPLC/GC) has become necessity.

To keep the pace with rapid improvement in quality standards of drugs all over the world and keeping in view the essential requirements for harmonization of analytical methods with those accepted internationally, the Indian Pharmacopeia Commission (IPC) have been taken steps for monitoring the drug standards.

General chemical tests Titrimetric, TLC and UV for determination of assay (potency), dissolution and related substances have been almost eliminated and more specific methods by High Performance Liquid Chromatography and Gas Chromatography tests have given emphasis. The use of chromatographic methods has been greatly extended to cope with the need for more specificity in assays and in particular in assessing the nature and extent of impurities in drugs and drug products.

Most of the current assay and related substances test method in Indian Pharmacopeia (IP) are upgraded by liquid chromatography in view to improve the quality and harmonize the quality standards. Hence this research work was focused on development of new, simple and fast stability indicating, specific analytical methods. Also, it was focused to validate the developed methods to meet the international regulatory guidelines. This research work will help the pharmaceutical industry to meet the new regulatory requirements.

PRESENT WORK:

The thesis entitled **“Research and Development of New Analytical Methods for the Determination of Drugs, Drug Release and Impurities in Different Pharmaceutical Dosage Forms Used in Treatment of Alzheimer’s, Depression and Liver Diseases”** has been divided into four chapters which includes general information of drug products, method development and method validation for different methods like Assay, Related substances and Dissolution for the drug products used in the treatment of above mentioned diseases.The present research work is helpful to the common pharmaceutical industry. This research work will reduce the economic burden on the laboratories by saving time of analysis, chemicals and manpower.

Chapter I: General Introduction and Literature Survey

This chapter is further divided into five sections;

Section A: General Introduction

This section discusses the general introduction to the analytical methoddevelopment and its validation. The selection of analytical technique High Performance Liquid Chromatography is It also discusses the current requirement and expectations of the regulatory bodies like Indian FDA. The scope of this research work is discussed in this section.

This section further divided into two parts as Part -I is about analytical method development and Part -II is about analytical method validation.

Part I includes the literature survey on analytical methods for the selected analytical technique (HPLC). The steps of HPLC method development like selection of HPLC method, type of chromatography, selection of HPLC column, type of detectors and selection of detector, optimization of HPLC conditions, sample preparation etc.

Part II includes the literature survey for current regulatory guidelines on analytical method validations and other information about the analytical method validation. The steps of analytical method validation like Specificity, Accuracy, Linearity, Range, Detection Limit, Quantification Limit, Precision, Repeatability, Intermediate Precision etc. are discussed in detail this part. The definitions of the above-mentioned validation parameters are also discussed.

Section B: ALZHEIMER’S DISEASE (MEMANTINE HCl)

This section discusses the details about Alzheimer’s disease (AD). It includes the detailed literature survey of this disease and the drugs used to cure this disease. The symptoms of this disease and the drugs used to cure are covered. The population affected every year due to AD is also discussed in this section. The Memantine HCl is one of the commonly used drug in the treatment of AD. The literature survey details include the information about the Memantine HCl. The structural formula and other chemical properties of Memantine HCl are discussed. Further it discusses about the current available analytical methods for determination of quantity Memantine HCl (Assay and Dissolution) in its formulations like tablets and capsules. Memantine HCl lacks the chromophore, due to this limitation, detectivity of drug becomes the issue. Hence there very few methods available for quantification of drug. All the available methods are developed using the derivatization method which is complex and time consuming. Hence this research was focused to develop simple and fast analytical method by using conventional detector like refractive index. The aim of the method development, the analytical technique selected and the guidelines used for method validation are also mentioned in this section.

SECTION C: LIVER DISEASE (URSODEOXYCHOLIC ACID)

This section discusses the details about Liver Disease. This includes details of disease like its symptom, the causes of the disease etc., It also discusses about the liver function in the body and its importance. Literature survey shows that the gallstones formed in the gallbladder obstruct the flow of bile acid which causes the liver diseases. The details of drugs used in the treatment of the liver diseases is covered in this section. Ursodeoxycholic acid is used very commonly in the treatment of liver diseases. This section also deals with the literature survey to find the details of the chemical properties of the Ursodeoxycholic acid drug, the current published methods for determination of Ursodeoxycholic acid and its impurities (Assay, Related substances and Dissolution) in its formulations like tablets or capsules etc. The aim of the method development, the analytical technique selected and the guidelines used for method validation are also mentioned in this section.

SECTION D: DEPRESSION DISEASE (NORTRIPTYLINE HCL)

This section discusses the details about Depression Disease, its impacts and the population affected. This includes details of literature survey of this disease and the antidepressants used to cure the depression.Nortriptyline HCl is one of the commonly used tricyclic antidepressant in the treatment of depression diseases. This section also deals with the literature survey to find the details of the chemical properties of the Nortriptyline HCl drug, the current published methods for determination of Nortriptyline HCl and its impurities (Assay and Related substances) in its formulations like tablets or capsules etc. The aim of the method development, the analytical technique selected and the guidelines used for method validation are also mentioned in this section.

SECTION E: OBJECTIVE AND SCOPE OF RESEARCH WORK

The aim of this research was to develop new simple, accurate, precise, specific and robust analytical methods for determination of drug (assay), drug quantity in dissolution samples (dissolution), and drug impurities in drug formulations in different drug formulations which are used in treatment of Alzheimer, Liver and depression disease. The developed methods validation as per current regulatory guidelines was also aimed.

The drug formulations considered under the scope of this research work are Memantine HCl tablets, Ursodeoxycholic Acid tablets and Nortriptyline Tablets. The methodology selected for development of work is chromatographic methodology. The high-performance liquid chromatographic (HPLC) technique is used for development of new methods.

Chapter II: Development and Validation of Analytical Methods for drugs used in treatment of Alzheimer's (Memantine HCl) and Depression Disease (Nortriptyline HCl)

This chapter is divided in to three sections;

Section A: Development and Validation of Novel, Simple HighPerformance Liquid Chromatographic Method with Refractive Index Detector for Quantification of Memantine Hydrochloride in Dissolution Samples.

Chromatographic Method:

The chromatographic separation was achieved on C18 (250 × 4.5 mm, 5µ) column using isocratic mobile phase comprises of buffer (pH-5.2): methanol (40:60 v/v) pumped at a flow rate of 1.0 mL/min. The column effluents were monitored using RI detector. The retention time of MEM was found to be about 6.5±0.3 min.

Result: The developed chromatographic method was validated and found to be linear over the concentration range of 5.0 - 45.0µg/mL for MEM. Mean recovery of MEM was found to be 99.2±0.5%w/w. The method was found to be simple, fast, precise and accurate which can be utilized for the quantification of MEM in dissolution samples.

Conclusion: The proposed liquid chromatographic method provides simple, accurate and reproducible methodology for quantitative measurement of memantine hydrochloride in dissolution sample using refractive index detector without any interference from the excipients. The proposed method is very simple and can be used without derivatization of compound under analysis. This saves time and also reduces economic burden of laboratories.

Section B: To develop and validate a chromatographic method to determine the amount of drug (assay) in the tablets of Memantine hydrochloride (MEM) using high performance liquid chromatography with refractive index (RI) detector.

Chromatographic Method: The chromatographic separation was achieved on C18 (250 × 4.5 mm, 5µ) column using isocratic mobile phase comprises of buffer (pH-6.0): Methanol (45:55 v/v) pumped at a flow rate of 1.0 ml/min. The detection of effluent was monitored using RI detector.

Result: The developed chromatographic method was validated and found to be linear over the concentration range of 5.0 - 45.0µg/mL for MEM. Mean recovery of MEM was found to be 99.2±0.5%w/w. The method was found to be simple, fast, precise and accurate which can be utilized for the quantification of MEM in dissolution samples.

Conclusion: The method validation results proved that the method is precise, accurate and robust which can be utilized for the determination of assay of MEM in tablets and capsules.This method was found to be simple and commercial since it doesn't require derivatization of drug. It saves analysis time.

Section C: The Development and Validation of Stability Indicating Analytical Method for Determination of Nortriptyline HCl in Nortriptyline Tablets by Liquid Chromatography

Chromatographic Method: The separation of impurities and Nortriptyline HCl drug was achieved by an isocratic liquid chromatographic method using Inertsil, C18, 250mm x 4.6mm, 5µm column. The mobile phase consists of 70% Methanol and 30% phosphate buffer of pH-7.5 pumped at a flow rate of 1.0 ml/min. The detection was carried out at a wavelength 220 nm.

Results: The proposed chromatographic method was validated and found to be linear over the concentration range of 50 - 150.0µg/ml. Mean recovery of Nortriptyline HCl was found to be 100.1±0.1%w/w.

Conclusion: The method validation results shows that the method is specific, stability indicating, precise, accurate and robust which can be utilized for the determination of assay of Nortriptyline HCl in Nortriptyline tablets. The method is linear over the range of 50 - 150.0µg/ml drug concentration. This method can be used by any common laboratory for determination of Nortriptyline HCl in Nortriptyline tablets.

Chapter III: The Development and Validation of an Analytical Methods for Assay, Related Substances and Dissolution of Ursodeoxycholic Acid Tablets by Liquid Chromatography

This chapter is divided in three sections

Section A: Development of an Analytical Method for Determination of Related Substances in Ursodeoxycholic Acid Tablets

This section discusses about chromatographic (HPLC) method development and validation of analytical method for determination related substances in ursodeoxycholic acid tablets.

Purpose: To develop rapid, rugged, precise and an accurate stability indicating analytical method for estimation of related substances in Ursodeoxycholic Acid tablets.

Method: The separation of impurities and Ursodeoxycholic Acid drug is achieved by an isocratic chromatographic method on C18, 250 mm x 4.6 mm, 5µm column. The mobile phase consists of buffer, acetonitrile and methanol in the ratio of 35:28:37v/v/v pumped at a flow rate of 1.0 ml/minute. The detection was carried out by using refractive index (RI) detector.

Results: Overall mean recovery of Chenodeoxycholic acid impurity was found to be 100.6±4.0%w/w. The proposed chromatographic method was validated and found to be linear over the concentration range from LOQ to 150.0% of impurity limit level.

Conclusion: The method validation results shows that the method is specific, stability indicating, precise, accurate and robust. This method can be used by any common laboratory for determination of related substances in Ursodeoxycholic Acid tablets.

Section B: Chromatographic Method Development and Validation for Quantitative Determination (Assay) Of Ursodeoxycholic Acid in Ursodeoxycholic Acid Tablets.

Method: The stationary phase used in separation of known impurities and Ursodeoxycholic Acid drug was C18, 250mm X4.6mm, 5µm. The mobile phase was prepared by mixing of buffer and acetonitrile in the ratio of 50:50v/v pumped at a flow rate of 1.5ml/minute.

Results: The developed chromatographic method is validated. The specificity of the method is proved by injecting known impurities and also by performing the forced degradation studies. The Ursodeoxycholic Acid peak was found pure. The method is accurate, precise and linear over the concentration range of 50 – 150 µg/ml. Mean recovery of Ursodeoxycholic Acid tablets is found 99.7 ± 0.4.

Conclusion: The method was found to be simple, stability indicating, precise, accurate and robust which can be utilized for the determination of assay of Ursodeoxycholic Acid tablets. This method can be used in quality control laboratories for drug product release and for doing the stability studies.

Section C: Dissolution Method Development with Chromatographic Method for Determination of Drug Release in Dissolution Samples of Ursodeoxycholic Acid Tablets.

Aim: Chromatographic Method Development and Validation for Quantitative Determination of Ursodeoxycholic Acid in Dissolution samples of Ursodeoxycholic Acid Tablets.

Method: Phosphate buffer (0.05M) of pH- 7.5 is used as dissolution medium and paddle (USP-II) as apparatus at 75 rpm. The sample was withdrawn after 60 minutes. The developed HPLC method was used for quantitative estimation of drug release in dissolution samples of Ursodeoxycholic Acid tablet. The HPLC column of Hypersil - C18, 250X4.6 mm, 5µm is used. The phosphate buffer of pH – 6.5 and acetonitrile was mixed in the ratio of 50:50v/v pumped at a flow rate of 1.0 ml/minute. The detection was made at wavelength of 210 nm.

Results : Through method validation it is proved that the method is accurate, rugged, precise and robust. The method is linear over the concentration range of 50% – 150%. The method also found very precise. Mean recovery of Ursodeoxycholic Acid was found 99.6 ±38%.

Conclusion: The proposed HPLC method is accurate, linear, precise and rugged methodology for quantitative determination of Ursodeoxycholic Acid in dissolution samples of Ursodeoxycholic Acid tablets of different strengths. The results of the method validation study show that the method is accurate and linear in the concentration range of 50%-150 % of Ursodeoxycholic Acid. The analytical method validation was done as per ICH Q2(R1) and USP guidelines. This method can be used by quality control or research laboratories for quantitative determination of the content (assay) of Ursodeoxycholic Acid tablets of different strength.

CHAPTER IV: SUMMARY AND CONCLUSION

In this research we have developed and validated six chromatographic analytical methods for determination of assay, dissolution and impurities in drug formulations (chapter 2 and 3). In this chapter summary of research work for all methods is discussed in detail. The conclusion about the results of the thesis is also covered in this chapter. The applications of method arealso discussed.

CHAPTER I

1.0 INTRODUCTION

1.1 SECTION A: GENERAL INTRODUCTION

Determination of exact quantity of drug in the drug formulation requires accurate and robust analytical method. Analytical method development and validation plays an important role in the drug discovery, development, and quality control of pharmaceutical products. The method should be simple and fast so that it can be adopted by common laboratory easily. The methods which are used in analysis of stability studies for assay and related substances should be stability indicating analytical methods. The peaks of solutes and impurities under determination should be pure and hence peak purity in the methods for determination of assay and related impurities should be passed. The developed methods need to be validated as per current regulatory guidelines. All the validated methods should pass the acceptance criteria as per industrial and regulatory norms.

In the last few years many pharmaceutical regulatory authorities have found major non-compliances in analytical method validations. The regulatory requirements by different regulatory authorities (Indian FDA, USFDA, European agencies) have increased tremendously.

Due to these high expectations, pharma industry is suffering since many of the firms are using regular conventional techniques to determine the drug quantities and their impurities. Many methods are complex in nature and time consuming. The validations of those methods are also challengeable due to its complex nature. In last few years the analytical technique has improved and use of chromatographic technique (HPLC/GC) has become necessity.

To keep the pace with rapid improvement in quality standards of drugs all over the world and keeping in view the essential requirements for harmonization of analytical methods with those accepted internationally, the Indian Pharmacopeia Commission (IPC) have been taken steps for monitoring the drug standards.

General chemical tests like Titrimetric, TLC and UV for determination of assay (potency), dissolution and related substances have been almost eliminated and more specific methods by High Performance Liquid Chromatography and Gas Chromatography tests have given emphasis. The use of chromatographic methods has been greatly extended to cope with the need for more specificity in assays and in particular in assessing the nature and extent of impurities in drugs and drug products.

Most of the current assay and related substances test method in Indian Pharmacopeia (IP) are upgraded by liquid chromatography in view to improve the quality and harmonize the quality standards. Hence this research work was focused on development of new, simple and fast stability indicating, specific analytical methods. Also, it was focused to validate the developed methods to meet the international regulatory guidelines. This research work will help the pharmaceutical industry to meet the new regulatory requirements and to improve the quality of drugs and drug formulations.

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION

From method development to qualification to validation and transfer, it is a continuous yet inter-dependent process from early research to development of pharmaceutical drugs and their products. Analytical method is also important in quality control during drug release. During drug development stage analytical method may or may not need to be robust, qualified or validated. But at the stage of quality control during release of commercial drugs and their formulations the method needs to be validated. The objective is to prove that methods selected are fit for their intended purpose.

With an expanded portfolio of newer formats in the pipelines, knowledge, experience and advanced techniques are critical to establish efficient, accurate and fit-for-purpose analytical methods that can be later qualified, validated and transferred to release testing, thereby improving efficiency and reducing cost, time and resources [1-3].

PART I ANALYTICAL METHOD DEVELOPMENT

Method development usually requires selecting the method requirements and deciding on what type of instrumentation to utilize and why. The wide variety of equipment, columns, eluent and operational parameters involved makes HPLC method development. There are several reasons for developing new methods of analysis: A suitable method for particular analyte in the specific matrix is not available. Existing methods may be unreliable (have poor accuracy or precision) or may be too expensive and time consuming. HPLC method development generally follows the following steps;

Step 1- Selection of the HPLC method and initial system.

Step2- Selection of optimum conditions.

Step3- Selectivity optimization.

Step4- System parameter optimization.

Step5- Method validation.

The details of each step are discussed as below;

STEP1-SELECTION OF HPLC METHOD AND INITIAL CONDITIONS:

When selecting an HPLC system it must have a high probability of actually being able to analyze the sample. For example, if the sample includes polar analytes then RP-HPLC would offer both adequate retention and resolution. Following points shall be considered; sample preparation Types of chromatography, Column selection, Detector selection and selection of mobile phase composition [1-5].

A) SAMPLE PREPARATION:

Sample preparation is an essential part of HPLC analysis, to provide a reproducible and homogenous solution i.e. suitable for injection on to the column. The aim of sample preparation is a sample that: It is relatively free of interferences Will not damage the column Should compatible with the intended HPLC method.

B) TYPES OF CHROMATOGRAPHY:

Reversed phase is the choice for the majority of the samples. But if acidic or basic analytes are present reversed phase ion suppression (for weak acids and bases) or reversed phase ion pairing (for strong acids and bases) should be used. For low or medium polarity analytes normal phase HPLC is used, particularly if the separation of isomers is required. For inorganic anion or cation analysis ion exchange chromatography is best.

C) COLUMN SELECTION:

A column is chosen based on the Knowledge of sample on the expectation of how its components will interact with the packing material. the properties of column packing material.

1.Knowledge of the Sample: which influences the choice of Column Bonded Phase Characteristics Knowledge of the sample

- Structure of sample components
- Number of compounds present
- Sample matrix
- pKa values of sample components
- Concentration range
- Molecular weight range
- Solubility
- Other pertinent data Column Chemistry (bonded phase, bonding type, end capping, carbon load).

D) DETECTOR SELECTION:

Below are the few commonly used detectors in HPLC method development;

1) ULTRA VIOLET (UV)-VISIBLE AND PHOTO DIODE ARROW (PDA) DETECTOR:

The UV, Visible and PDA detectors are commonly used detectors and categorized as absorbance detectors. They provide good sensitivity for light-absorbing compounds at ppm level. They are easy to operate and provide good stability. UV detector is a very commonly used detector for HPLC analysis. During the analysis, sample goes through a clear color-less glass cell, called flow cell. When UV light is irradiated on the flow cell, sample absorbs a part of UV light. Thus, the intensity of UV light observed for the mobile phase (without sample) and the eluent containing sample will differ. By measuring this difference, the amount of sample, chromatographic purity of sample can be determined. Since the UV absorbance also differs depend on the wavelength used, it is important to choose an appropriate wavelength based on the type of analyte. Normally UV detector used in the range of wavelength between 195 to 370 nm. Most of the pharmaceutical compounds shows sufficient absorbance at 254 nm. Compared to a UV detector, a Visible detector uses longer wavelength (400 to 700 nm). There are detectors that provide wider wavelength selection, covering both UV and Visible ranges (195 to 700 nm) called UV-Visible detector detects an entire spectrum simultaneously. UV and VIS detectors visualize the obtained result in two dimensions (light intensity and time), but PDA adds the third dimension (wavelength). By using PDA detector, the most suitable wavelength can be determined without repeating analyses.

2) REFRACTIVE INDEX DETECTOR:

RI detector has lower sensitivity compared to UV detector, and that's the main reason why RI is not as commonly used as UV. However there are some advantages over UV detector. It is suitable for detecting all components. For an example, samples which do not have UV absorption, such as sugar, alcohol, or inorganic ions obviously cannot be measured by a UV detector. In contrast, change in reflective index occurs for all analyte, thus a RI detector can be used to measure all analyte. It is applicable for the use with solvent that has UV absorbance. A

UV detector cannot be used with solvent which has UV absorbance. Sometimes the organic solvent used for GPC analysis absorbs UV, and thus UV detector cannot be used. It provides a direct relationship between the intensity and analyte concentration. The amount of UV absorbed depends on each analyte, thus the intensity of UV detector peak does not provide information on the analyte concentration. While intensity observed by a RI detector is comparable to the concentration of analyte. Because of those advantages, RI is often used for the detection of sugars and for SEC analysis.

3) MASSSPECTROMETER:

Analytes are detected based on their molecular weight. The obtained information is especially useful for compound structure identification. However, its use is not limited to structure identification and can be used to quantify very low detection limit of elemental and molecular components.

4) CONDUCTIVITYDETECTOR:

Solutions containing ionic components will conduct electricity. Conductivity detector measures electronic resistance and measured value is directly proportional to the concentration of ions present in the solution. Thus, it is generally used for ion chromatography.

5) FLUORESCENCEDETECTOR:

The advantage of fluorescence method is its high sensitivity for selective groups of compounds at ppb level. By using a specific wavelength, analyte atoms are excited and then emit light signal (fluorescence). The intensity of this emitted light is monitored to quantify the analyte concentration. Most pharmaceuticals, natural products, clinical samples, and petroleum products have fluorescent absorbance. For some compounds which do not have fluorescence absorbance or low absorbance, they can be treated with fluorescence derivatives such as dansyl chloride. The system is easy to operate and relatively stable.

E) MOBILE PHASE SELECTION:

The organic phase concentration required for the mobile phase can be estimated by gradient elution method. Gradient can be started with 5-10 % of the organic phase in the mobile phase and the organic phase concentration can be increased up to 100% within 30-45%. The elution strength of a mobile phase depends upon its polarity, the stronger the polarity higher is the elution.

STEP 2: SELECTION OF INITIAL CONDITIONS

This step determines the optimum conditions to adequately retain all analytes; i.e. Ensures no analyte has a capacity factor of less than 0.5(poor retention could result in peak overlapping). No analyte has a capacity factor greater than 10-15 (excessive retention leads to long analysis time and broad peaks with poor detect ability). Determination of initial conditions: The recommended method involves performing two gradient runs differ in only in the run time. A binary system based on either acetonitrile / water or methanol/water should be used.

STEP 3: SELECTIVITY OPTIMIZATION

The aim of this step is to achieve adequate selectivity. The mobile phase and stationary phase compositions need to be taken in to an account. To select these the nature of the analytes must be considered. Once the analyte types are identified the relevant optimization parameters may be selected. Following points were considered during selectivity optimization;

- Initial HPLC condition
- Sample preparation
- Method goals and chemistry
- Optimize HPLC separation
- Standardization
- Method validation

STEP 4: SYSTEM PARAMETER OPTIMIZATION:

This is used to find the desired balance between resolution and analysis time after satisfactory selectivity has been achieved. The parameters involve include column dimensions, column packing particle size and flow rate. These parameters may be changed without affecting capacity factors or selectivity.

Parameter optimization can be done by two ways; manually and by using software's. Separation then can be optimized by change in the initial mobile phase composition and the slope of the gradient according to the chromatogram obtained from the preliminary run.

PART II- METHOD VALIDATION:

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. Method validation parameters are based on ICH and USP guidelines [6-9].

A brief description of types of tests considered in this document is provided below;

Identification tests are intended to ensure the identity of analyte in a sample. This normally achieved by comparing the properties of sample with that of the reference standard. Testing for impurities can be either a quantitative test or a limit test for the impurity in a sample. Assay procedures are intended to measure the analyte present in a given sample.

Typical validation characteristics which should be considered are listed below;

1.Accuracy

2. Precision

2a. repeatability

2b. Intermediate precision

3. Specificity

4. Detection limit

5. Quantitation limit

6. Linearity

7. Range

ACCURACY:

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true or an accepted reference value and the value found. Accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range.

LINEARITY:

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. For the establishment of linearity, a minimum of five concentrations is recommended.

PRECISION:

The precision of an analytical procedure expresses the closeness of agreement between the measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. Precision is proved in two ways - repeatability precision and intermediate precision (reproducibility).

REPEATABILITY:

Expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra- assay precision. a) a minimum of nine determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each); or b) a minimum of six determinations at 100% of the test concentration.

INTERMEDIATE PRECISION:

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

REPRODUCIBILITY:

Reproducibility expresses the precision between laboratories. The standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision investigated.

DETECTION LIMIT:

The detection limit of individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

BASED ON VISUAL EVALUATION:

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

BASED ON SIGNAL-TO-NOISE:

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

BASED ON THE STANDARD DEVIATION OF THE RESPONSE AND THE SLOPE:

The detection limit (DL) may be expressed as: $DL = 3.3\ \sigma / s$ Where, σ = the standard deviation of the response S = the slope of the calibration curve.

QUANTITATION LIMIT:

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

BASED ON VISUAL EVALUATION:

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

BASED ON SIGNAL-TO-NOISE:

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte. A signal-to-noise ratio between 10:1 is generally considered acceptable for estimating the detection limit.

BASED ON THE STANDARD DEVIATION OF THE RESPONSE AND THE SLOPE:

The quantitation limit (QL) may be expressed as: QL = 10 σ /s Where, σ = the standard deviation of the response S = the slope of the calibration curve.

RANGE:

The range of an analytical procedure is the interval between the upper and lower concentration of the analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The following minimum specified ranges should be considered.

For the assay of a drug substance or a finished (drug) product: normally from 80 to 120 percent of the test concentration; for related substances (impurity profile):LOQ to 200 percent of the impuritylimitconcentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers), is justified; for dissolution testing: +/-20 % over the specified range.

ROBUSTNESS:

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

1.2 SECTION B:ALZHEIMER’S DISEASE (MEMANTINE HCl)

Alzheimer’s disease (AD) is the most common type of dementia which results in damaged brain cells or connections between brain cells by various diseases and conditions. The number of persons with AD is increasing as populations age and presently about 46.8 million peoples are affected globally in 2015. Major concerns in AD are progressive impairment in both cognitive and functional capacities. Some of the symptoms in AD can be due to depression, delirium, drug interaction, thyroid problems, excess use of alcohol, or certain vitamin deficiencies [12].

Cholinesterase inhibitors (ChEI) are commonly used as treatment strategies for AD which includes donepezil/galantamine/rivastigmine/tacrine. The mentioned drugs were also combined with memantine (MEM) or N-methyl-D-aspartate (NMDA) receptor antagonist.

Alzheimer’s disease (AD), a debilitating neurodegenerative disorder is a growing problem of aging world. It is associated with a deficiency of acetylcholine (ACh) in the forebrain that correlates with brain pathology and cognitive dysfunction. The most promising approach to enhancing central ACh neurotransmission has been the utilization of agents that inhibit cholinesterase’s which block its catabolism. Initially, the success of this strategy was limited by sub therapeutic levels of acetylcholinesterase (AChE) inhibition, tolerability problems and toxicity of the first agents.

Alzheimer’s disease first described by German physician Dr. Aloi Alzheimer in 1906. Alzheimer’s disease is a devastating neuro generative disorder manifested by;

- Deterioration in Memory and cognition
- Impairment in performing activities in daily living
- Behavioral and neuropsychiatric disturbances
- Most common form of dementia in old age

Risk of AD increases with age but AD is not a part of normal aging. Below are few symptoms of AD;

Language problem – Cannot find right word or name for familiar person, object, place etc.

- Loss of recent memory
- Loss of sense of time and place
- Decline in activities of daily living
- Personality changes

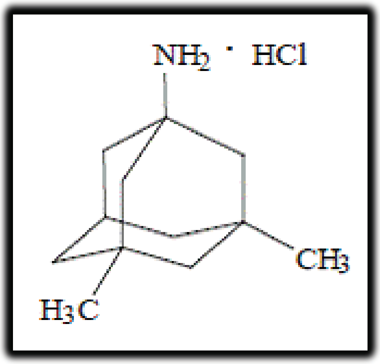
Different drugs used in in Alzheimer's disease are;

1) Memantine Hydrochloride
2) Galantamine
3) Donepezil Hydrochloride
4) Rivastigmine
5) TacrineAmino acridine

Memantine HCl (MEM) is often used in the treatment of Alzheimer's disease (AD). MEM is used as option in a new class of AD treatment and showed very good results in terms of efficacy and safety for patients with moderate to severe symptoms. It has been recently approved by European Union and Australia for the treatment of moderately severe to severe AD.

Chemical name of Memantine HCl is 1-amino-3,5-dimethyladamantane hydrochloride.

Fig. 1 Structure of Memantine HCl



It is an antialzheimer's, uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist with strong voltage dependency and rapid blocking/unblocking kinetics. These pharmacological features appear to allow memantine to block the sustained activation of the receptor by glutamate that may occur under pathological conditions and to rapidly leave the NMDA receptor channel during usual physiological activation [13-16].

The drugs which are used in treatment of AD need to be given in appropriate dosage regimen. Hence it is very important to estimate the amount of drug release from dosage forms. For quantification of amount of drug release there is a need of simple, rapid and economical analytical method.

Chemical structure of MEM reveals that, it doesn’t have chromophore hence does not show the UV absorption. This limitation of the MEM necessitates, selecting specialized analytical techniques for its quantification. Number of HPLC methods were reported for quantification of MEM in pharmaceutical dosage forms with specific techniques such as pre-column derivatization with UV detector [17-19], with charged aerosol detector [20]. The MEM quantification in biological fluids such as plasma and vitreous humour were also performed using pre-column derivatization and fluorescence detection [21-25]. The reported methods are cumbersome and time consuming with tedious sample preparation process.

This research was focused on development of a simple, precise, accurate, specific and robust reverse phase chromatographic method using refractive index detector for quantification of MEM in its different pharmaceutical dosage forms (Assay and Dissolution) [26]. In these newly developed methods no derivatization required; hence more number of samples can be tested in short span of time. This method can be used in quality control and R&D. The simplicity of proposed method is economical and can be utilized by common laboratories. The developed methods are validated as per International Conference on Harmonization (ICH) Q2(R1) guideline and United State of Pharmacopoeia (USP) 38 chapter <1225> and chapter <1092> [6-9, 26]. The method found to be specific, precise, accurate and robust with compliance to acceptance criteria of ICH and USP 38.

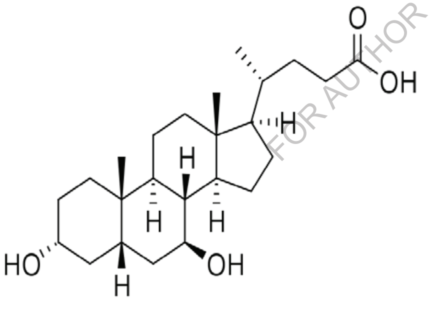
1.3 SECTION C: LIVER DISEASE (URSODEOXYCHOLIC ACID)

Liver disease is any disturbance of liver function that causes illness. The liver is responsible for many critical functions within the body and should it become diseased or injured, the loss of those functions can cause significant damage to the body. Liver disease is also referred to as hepatic disease. Liver disease is a broad term that covers all the potential problems that cause the liver to fail to perform its designated functions. Usually, more than 75% or three quarters of liver tissue needs to be affected before a decrease in function occurs [27].

The liver is the largest solid organ in the body; and is also considered a gland because among its many functions, it makes and secretes bile. The liver is located in the upper right portion of the abdomen protected by the rib cage. It has two main lobes that are made up of tiny lobules. The liver cells have two different sources of blood supply. The hepatic artery supplies oxygen rich blood that is pumped from the heart, while the portal vein supplies nutrients from the intestine and the spleen. Normally, veins return blood from the body to the heart, but the portal vein allows nutrients and chemicals from the digestive tract to enter the liver for processing and filtering prior to entering the general circulation. The portal vein also efficiently delivers the chemicals and proteins that liver cells need to produce the proteins, cholesterol, and glycogen required for normal body activities. As part of its function, the liver makes bile, a fluid that contains among other substances, water, chemicals, and bile acids (made from stored cholesterol in the liver). Bile is stored in the gallbladder and when food enters the duodenum (the first part of the small intestine), bile is secreted into the duodenum, to aid in the digestion of food. The liver is the only organ in the body that can easily replace damaged cells, but if enough cells are lost, the liver may not be able to meet the needs of the body. Normally, bile flows from the liver into the gallbladder and ultimately into the intestine to help with the digestion of food. If bile flow is obstructed, it can cause inflammation within the liver. Most commonly, gallstones can cause an obstruction of the ducts that drains bile from the liver[28-30].

Ursodeoxycholic acid (UDCA), is generally used in the treatment of liver diseases. UDCA is a naturally occurring bile acid with multiple hepatoprotective activities, improves liver condition in patients with a wide range of chronic liver diseases. UCDA is increasingly used for the treatment of cholestatic liver diseases[27-30].. Studies done on UDCA suggests different mechanisms of action like: (1) protection of cholangiocytes against cytotoxicity of hydrophobic bile acids, resulting from modulation of the composition of mixed phospholipid-rich micelles, reduction of bile acid cytotoxicity of bile and, possibly, decrease of the concentration of hydrophobic bile acids in the cholangiocytes; (2) stimulation of hepatobiliary secretion, putatively via Ca2+- and protein kinase C-a-dependent mechanisms and/or activation of p38MAPK and extracellular signal-regulated kinases (Erk) resulting in insertion of transporter molecules (e.g., bile salt export pump) into the canalicular membrane of the hepatocyte and, possibly, activation of inserted carriers; (3) protection of hepatocytes against bile acid-induced apoptosis, involving inhibition of mitochondrial membrane permeability transition (IMPT), and possibly, stimulation of a survival pathway [32-33].

Fig. 2 - Structure of Ursodeoxycholic Acid



Since UDCA shows moderate absorption only in short wavelength region i.e. 200-210 nm, quantification of the amount of the UDCA in its formulations along with excipients and solvents by simple UV spectrophotometry is hampered by possible interferences [34,44-46]. High performance liquid chromatography (HPLC) of its assay utilizing refractive index (RI) detector has been reported in United States Pharmacopoeia [4]. Moreover, there are few analytical methods for the quantification of UDCA in biological fluids and few pharmaceutical dosage forms have been developed. Most of them describe HPLC methods coupled with MS and evaporative light scattering mass detection in biological matrix [35-47].

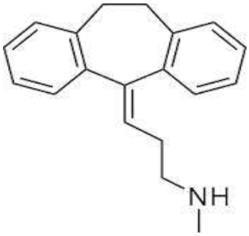
This research work was done to develop stability indicating, precise, accurate, specific and robust chromatographic method for quantification of UDCA in its pharmaceutical dosage forms, method for determination of impurities in UDCA tablets and drug release quantification in dissolution samples. These methods can be used in the quality control or in research laboratories of pharmaceutical companies for assay determination of Ursodeoxycholic Acid tablet. The developed methods are validated as per International Conference on Harmonization (ICH) Q2 (R1) guideline and United State of Pharmacopoeia (USP) 38 chapter <1225> and chapter <1092> [6-11]. The methods found to be specific, precise, accurate, robust and stability indicating. The method validation is compliant with method validation criteria of ICH Q2 (R1) and USP guidelines.

1.4 SECTION D: DEPRESSION DISEASE (NORTRIPTYLINE HCL)

Depression is a psychiatric condition that affects about 120 million people worldwide and can interfere with independence and productivity in essentially all aspects of daily life. Depression is also associated with risk of self-harm, and ultimately suicide. Antidepressant medications are widely used to treat symptoms of depression. While there are several classes of antidepressants, therapeutic drug management (TDM) is most common for the tricyclic antidepressants (TCAs). TDM of TCAs is important due to wide inter-individual variability in pharmacokinetics, production of active metabolites, and a high risk of drug-drug interactions. In addition, TDM of some TCAs can be used to optimize dose, wherein concentration relationships are recognized for both therapeutic response and potentially life-threatening toxicity. In many clinical scenarios, TDM of TCAs is accomplished by currently available point of care or automated immunoassays that provide a total TCA concentration. However, these assays may not be adequately specific to meet the needs of all clinical scenarios and hence chromatographic separation and quantification of individual TCA parent drugs and active metabolites that may contribute to the total TCA concentration is sometimes required. This chapter describes an analytical method designed to detect and or quantify clinically significant concentrations of Nortriptyline HCl [48-49].

Nortriptyline hydrochloride, the N-demethylated active metabolite of amitriptyline, is a dibenzocycloheptene-derivative tricyclic antidepressant (TCA) [1]. TCAs are structurally similar to phenothiazines 3-(10, 11-dihydro-5H-dibenzo [a, d] cyclohepten-5-ylidene)-N-methyl-1-propanamine (Fig. 3).

Fig. 3 Structure of Nortriptyline Hydrochloride



Nortriptyline hydrochloride contain a tricyclic ring system with an alkyl amine substituent on the central ring. Nortriptyline Base is a tricyclic antidepressant agent used for short-term treatment of various forms of depression since it is a non-selective serotonine uptake inhibitor [49]. Nortriptyline blocks the norepinephrine presynaptic receptors, thereby blocking the reuptake of this neurotransmitter and raising the concentration in the synaptic cleft in the CNS. Nortriptyline also binds to alpha-adrenergic, histaminergic and cholinergic receptors [50].

This research was focused on development of a simple, precise, accurate, specific and robust stability indicating reverse phase chromatographic method for quantification of Nortriptyline HCl in its pharmaceutical dosage forms.

Literature survey reveals that nortriptyline hydrochloride is official in British Pharmacopeia [11]. Few chromatographic methods for the determination of nortriptyline hydrochloride and fluphenazine hydrochloride in pharmaceutical preparations and/ or with other active ingredients have been reported[50-55]. The proposed method is stability indicating, simple and economical and can be utilized by common laboratories. The developed method was validated as per International Conference on Harmonization (ICH) Q2(R1) guideline [9] and United State of Pharmacopoeia (USP) 38 chapter <1225> and chapter <621> [10-11]. The method found to be specific, precise, accurate and robust with compliance to acceptance criteria of ICH and USP 38. This method can be used in quality control of manufactured and developed dosage forms.

1.5 SECTION E: OBJECTIVE AND SCOPE OF RESEARCH WORK

The aim of this research was to develop new simple, accurate, precise, specific and robust analytical methods for determination of drug (assay), drug quantity in dissolution samples (dissolution), and drug impurities in drug formulations in different drug formulations which are used in treatment of Alzheimer, Liver and depression disease. Thevalidation of the developed methods as per current ICH and USP guidelines was also aimed.

The drug formulations considered under the scope of this research work are Memantine HCl tablets, Ursodeoxycholic Acid tablets and Nortriptyline Tablets. The methodology selected for development of work is chromatographic methodology. The high-performance liquid chromatographic (HPLC) technique is used for development of new methods.

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CHAPTER II

2.0 DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR DRUGS USED IN TREATMENT OF ALZHEIMER'S (MEMANTINE HCl) AND DEPRESSION DISEASE (NORTRIPTYLINE HCl)

2.1 SECTION A:

THE DEVELOPMENT AND VALIDATION OF NOVEL, SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD WITH REFRACTIVE INDEX DETECTOR FOR QUANTIFICATION OF MEMANTINE HYDROCHLORIDE IN DISSOLUTION SAMPLES

INTRODUCTION:

Alzheimer’s disease (AD) is the most common type of dementia which results in damaged brain cells or connections between brain cells by various diseases and conditions. The number of persons with AD is increasing as populations age and presently about 46.8 million peoples are affected globally in 2015. Major concerns in AD are progressive impairment in both cognitive and functional capacities. Some of the symptoms in AD can be due to depression, delirium, drug interaction, thyroid problems, excess use of alcohol, or certain vitamin deficiencies [1, 2].

Cholinesterase inhibitors (ChEI) are commonly used as treatment strategies for AD which includes donepezil/galantamine/rivastigmine/tacrine. The mentioned drugs were also combined with memantine (MEM) or N-methyl-D-aspartate (NMDA) receptor antagonist.

Memantine HCl (Fig. 1) chemically is 1-amino-3,5-dimethyladamantane hydrochloride. It is an antialzheimer's, uncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist with strong voltage dependency and rapid blocking/unblocking kinetics. These pharmacological features appear to allow memantine to block the sustained activation of the receptor by glutamate that may occur under pathological conditions and to rapidly leave the NMDA receptor channel during usual physiological activation [3, 4].

MEM is used as option in a new class of AD treatment and showed very good results in terms of efficacy and safety for patients with moderate to severe symptoms. It has been recently approved by European Union and Australia for the treatment of moderately severe to severe AD [5].

The drugs which are used in treatment of AD need to be given in appropriate dosage regimen. Hence it is very important to estimate the amount of drug release from dosage forms.

For quantification of amount of drug release there is a need of simple, rapid and economical analytical method.

Chemical structure of MEM reveals that, it doesn't have chromophore hence does not show the UV absorption. This limitation of the MEM necessitates, selecting specialized analytical techniques for its quantification. Numbers of HPLC methods were reported for quantification of MEM in pharmaceutical dosage forms with specific techniques such as pre-column derivatization with UV detector [6-8], with charged aerosol detector [9]. The MEM quantification in biological fluids such as plasma and vitreous humour were also performed using pre-column derivatization and fluorescence detection [10-14]. The reported methods are cumbersome and time consuming with tedious sample preparation process.

The production of counterfeit and poor-quality medicines is a major community health concern; mainly in developing countries which lack resources for monitoring drug quality. Unavailability of advanced techniques in common laboratories create need to develop simple, accurate, specific and affordable methods to provide a practical means of speedily monitoring drug quality.

Hence our research was focused on development of a simple, accurate and specific reverse phase chromatographic method using refractive index detector for quantification of MEM in drug release profile studies of pharmaceutical dosage forms. The developed method is having advantage of short run time, no derivatization required; hence more number of samples can be tested in short span of time. The reported method can be utilized during dissolution testing in quality control of manufactured and developed dosage forms. The simplicity of proposed method is economical and can be utilized by common laboratories.

The developed method was validated as per International Conference on Harmonization (ICH) Q2(R1) guideline and United State of Pharmacopoeia (USP) 38 chapter <1225> and chapter <1092> [15-17]. The method found to be specific, precise, accurate and robust with compliance to acceptance criteria of ICH and USP 38.

EXPERIMENTAL:

MATERIALS AND REAGENTS:

Memantine hydrochloride pure drug (100.0% w/w) and tablets with label claim 30 mg of Memantine hydrochloride per tablet were provided by Wockhardt Ltd Aurangabad, Maharashtra India.

HPLC grade methanol was purchased from Merck Chemicals, Mumbai, India. Ultrapure water was generated from Milli-Q water purifier. Diethylamine was purchased from Spectrochem, Mumbai, India. Glacial acetic acid and Hydrochloric acid were purchased from Merck Ltd, Mumbai, India. Sodium chloride was purchased from Merck Chemicals, Mumbai, India.

INSTRUMENTS AND METHODS:

INSTRUMENTATION:

Thermo dionex –Ultimate 3000 High performance liquid chromatography (HPLC) system with Shodex DEX RI 101 Refractive Index Detector, Dissolution equipment -Electrolab 88DT.

CHROMATOGRAPHIC PARAMETERS:

The chromatographic column used was a Kromasil 100, C18 (250.0 × 4.6mm; 5 µm) which was maintained at 35º C. The mobile phase was prepared by mixture of ultrapure water containing 1.0% diethylamine (pH 5.2 adjusted with dilute glacial acetic acid) – methanol in the ratio of 40:60 v/v. The flow rate of the mobile phase was 1.0 mL/min. The injection volume was 100.0 µL. The column effluents were monitored by refractive index detector. The detector temperature was set at 35.0º C with sensitivity of 512. The sample and reference cell were purged with mobile phase for 30min each with flow rate 1.0 mL/min.

DISSOLUTION PARAMETERS AND PROCEDURE:

The dissolution test was performed in an Electrolab multi-bath (n = 7) dissolution test system (Mumbai, India) using dissolution apparatus of USP Type II in accordance with USP general methods.

The dissolution method was used according to USP 38 monographs for memantine hydrochloride tablets. The dissolution medium was composed of 0.1M HCl with sodium chloride (2 g/L of sodium chloride in water) of pH 1.2 (adjusted with hydrochloric acid). The volume of dissolution medium was 900mL maintained at 37°C. The paddle was rotated with speed of 75 rpm. The dissolution study was performed at single point with sample aliquot drawn after 30 mins.

PREPARATION STANDARD SOLUTIONS:

Weighed accurately about 50 mg of MEM working standard and transferred to 100 mL volumetric flask. The content of the flask was dissolved with mobile phase with sonication and volume was made up to mark with mobile phase as primary stock solution. Further 3.0 mL of prepared standard primary stock solution was pipetted and transferred to 50 mL volumetric flask and made up to the mark with dissolution media to get nominal concentration about 30 µg/mL.

METHOD VALIDATION:

SPECIFICITY:

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities or expected to present. The specificity of the developed LC method for MEM was carried out by dissolving 10mg placebo in 10 mL of volumetric flask in dissolution medium and sonicated. The volume was made up to the mark. The resulting solution was filtered through 0.45µ syringe filter.

The mobile phase, dissolution medium, placebo solution and standard drug solution (30µg/mL) were injected in sequence for evaluation of specificity of proposed method. The chromatograms were monitored for any peak eluted at the retention time of drug.

PRECISION:

Precision express the measure of how close the analytical results are to each other from a set of measurements under controlled analytical conditions. Precision proves random errors of the measurement.

Precision is a measure of the degree of repeatability (Intra-day), intermediate precision and reproducibility (inter-day) of the analytical method under normal operating circumstances.

Precision is usually measured as the coefficient of variation or relative standard deviation of analytical results acquired from independently prepared quality control standards.

Chromatographic method precision was evaluated by carrying out dissolution on six tablets of MEM (30mg/tablet) test sample and calculated % released for six tablets. The % RSD for set of six tablets for their % drug released was calculated.

The intra-day precision was evaluated by analyzing six dissolution sample solutions of MEM (n = 6) in two different set in a day. The acceptance criteria for % RSD is not more than 5%.

The intermediate precision of the method was also evaluated using different analyst and a different instrument in the same laboratory by carrying out dissolution test on six tablets of MEM sample and calculated % released for six tablets. Calculated the %RSD for 12 results. The acceptance criteria for % RSD is not more than 5%.

RECOVERY (ACCURACY):

Accuracy is extremely important in analytical method validation as it assures the closeness of agreement between a test result and the accepted reference value. Accuracy is expressed as trueness and involves a combination of random components and a common systematic error or bias component. The accuracy of the method was performed by recovery studies.

In order to evaluate the accuracy of the proposed methods, a recovery test was performed by adding known amounts of standard solutions to the placebo formulation sample followed by dissolution, followed by analysis using the proposed method.

The recovery studies were done for three different levels at 80%, 100% and 120% with three determination of working level concentration using standard spiking method.

The placebo was accurately weighed about 120mg for all level. For 80% (26.7µg/mL) level of recovery studies 24.0 mg of MEM standard was spiked along with 120mg placebo in 900mL of dissolution media.

In same manner for 100% and 120% recovery studies 30mg (33.3 µg/mL) and 36mg (40.0 µg/mL) of MEM was spiked respectively. The dissolution test was performed and samples were analyzed by proposed chromatographic method. The recovery at each level was calculated by using the theoretical value from exact weight taken for spiking. The prepared samples were analyzed using proposed chromatographic condition. The % recovery was calculated with respect to amount added. The acceptance criteria for % recovery is in the range of 95 - 105%.

LINEARITY:

The linearity of an analytical method is its ability to elicit test results that are directly, or by means of well-defined mathematical transformations, proportional to the concentration of analytes in the samples within a given range. The linearity plot was constructed for MEM in the range of 5 to 45.0µg/mL. The primary stock solution of 500µg/mL of MEM was prepared in mobile phase.

From the primary stock solution, secondary stock solution was prepared to get the concentration of 100.0 µg/mL of MEM in mobile phase. Appropriate dilution of the primary and secondary stock solution was carried in dissolution medium to get concentration of 5.0, 10.0, 15.0, 20.0, 30.0 and 45.0µg/mL for MEM in dissolution media. The calibration curve was plotted as concentration of the respective drug solutions versus the peak area at each level. The correlation coefficient determination (r2), slope and y-intercept values were calculated and statistically evaluated.

ROBUSTNESS:

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. One consequence of evaluation of robustness is that a series of system suitability parameters is established to ensure that the analytical procedure is maintainedwhenever used. In the present study the working concentration 30.0µg/mL of MEM was used for the determination of the robustness of the method. The following parameters were considered for the robustness of the proposed chromatographic method.

Effect of pH in the mobile phase (±0.2)

Effect of mobile phase composition (± 2%)

Effect of flow rate (±10%)

Column oven temperature (±2°C)

SOLUTION STABILITY IN DISSOLUTION MEDIA:

The solution stability of MEM standard and samples were performed in dissolution medium to understand stability which will be helpful to understand sample handling using proposed chromatographic method. A solution stability of MEM was carried out for standard and sample solution (30.0µg/mL) in a tightly capped volumetric flask at ambient temperature for 72 hr. The sample and standard solution after preparation were injected immediately to the system considering as an initial at 0 hour as baseline.

SYSTEM SUITABILITY:

The rationale of the system suitability assessment is to make sure that the complete testing system (including instrument, reagents, columns, analysts) is appropriate for the intended application.

System suitability tests (SST) are vital part of liquid chromatographic methods. They are used to verify the reproducibility of the chromatographic parameters and system is satisfactory for the analysis to be done. SST is support on the concept that the equipment, electronics, analytical operations and samples to be analyzed comprise an integral system that can be evaluated as such.

The system suitability test (Table I) was performed in accordance with USP [18].

TABLE ISYSTEM SUITABILITY PARAMETERS OF MEMANTINE HCl DISSOLUTION METHOD

PARAMETER	LIMIT	OBTAINED RESULTS FOR MEMANTINE HCl
Capacity factor	k' > 2	3.6
Injection precision	RSD < 1% for n > 6	0.52%
Tailing factor	T < 2	1.10
Theoretical plates/meter	N > 2000	7178

APPLICATION OF CHROMATOGRAPHIC METHOD FOR QUANTIFICATION OF MEM IN DISSOLUTION SAMPLES:

Dissolution test was performed using methodology as per section 2.2.3 on six tablets. Sample aliquot was withdrawn after 30 mins and filtered through 0.45µ syringe filter and injected (100.0 µL) directly to HPLC system with RI detector. Testing was performed at ambient temperature.

RESULTS:

VALIDATION OF CHROMATOGRAPHIC METHOD

SPECIFICITY:

The overlay chromatogram (Fig. 4) of diluent, placebo and standard solution were revealed that there is no interference at the retention of MEM. The developed chromatographic method was found to be highly specific for quantification of MEM in dissolution samples.

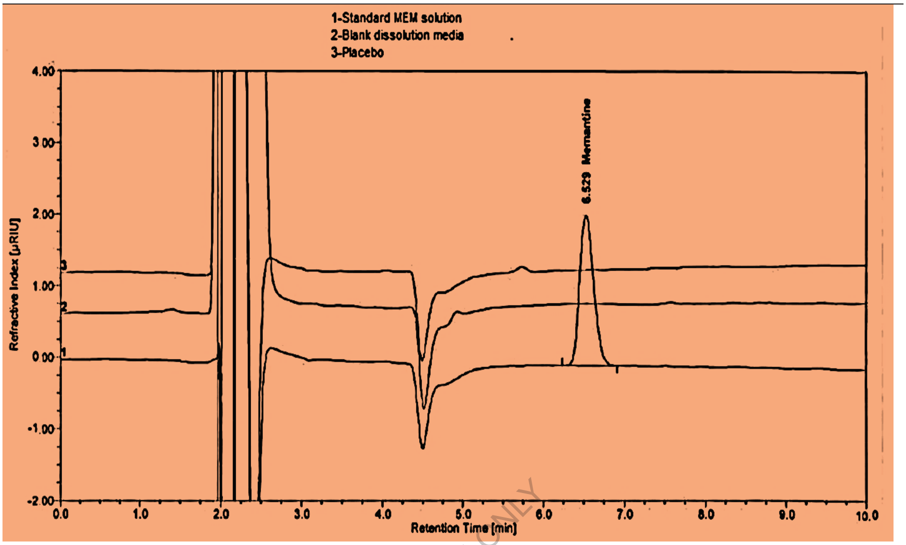

Figure 4 Overlay Chromatograms of Blank, Placebo and MEM Standard

PRECISION:

The intra-day precision was evaluated by analyzing six dissolution sample solutions of MEM (n = 6) in two different set in a day was found to be 0.945%.

The % RSD for inter day precision of MEM for two sets of six tablets (n=12) for their % drug released was found to be 1.8%.

The absolute difference between results for intermediate precision found 3.4% (Table II).

TABLE II The Precision Data Of Mem Dissolution By The Proposed HPLC Method.

Concentration (µg/mL)	**% RSD**		**Absolute % difference in Mean value for intermediate precision study**
	Intra-day(n=6)	**Inter-day (n=12)**	
33	0.945%	1.8%	3.4%

ACCURACY (RECOVERY)

The % recovery at 80, 100 and 120% was found to be 98.8±0.6, 99.2± 0.5, and 99.7±1.5 % respectively (Table III). The overall mean recovery was found to be 99.2± 0.5%. The recovery results were found within acceptance criteria. The developed method found to be accurate for quantification of MEM in dissolution study.

TABLE III. The Accuracy Data of Memantine HCl Dissolution By he Proposed HPLC Method.

Spiked level	Amount added (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean % Recovery
80%	26.35	25.95	98.5	98.8±0.6
	26.35	26.21	99.5	
	26.35	25.91	98.3	
100%	33.38	33.18	99.4	99.2±0.5
	33.38	33.23	99.6	
	33.38	32.95	98.7	
120%	39.6	40.1	101.3	99.7±1.5
	39.6	39	98.5	
	39.6	39.3	99.2	
% Mean Recovery				99.2±0.5

LINEARITY

The linearity of the MEM in dissolution medium was performed in the range of 5-45µg/mL and found to be linear. The representative regression equation was found to be y=1.6072x +1.5924 with lowest correlation coefficient (r2) was found to be 0.997. The linearity was found with in acceptance criteria.

ROBUSTNESS

The robustness parameters for chromatographic method are presented in table IV and found within acceptance criteria.

TABLE IV -Robustness Parameter of The Proposed HPLC Method of MEM HCL Tablets DISSOLUTION

CONDITIONS	PARAMETER		%RSD
HPLC	Flow	0.9 mL/min	0.72
		mL/min	0.66
	pH	5.0	1.10
		5.4	0.76
	Mobile phase composition (± 2%)	62:38	0.52
		58:42	0.34
	Column oven temperature (±2°C)	28°C	0.43
		32°C	0.32
		80	0.98

SOLUTION STABILITY IN DISSOLUTION MEDIA

The MEM found to be stable up to 72h in dissolution medium at ambient temperature. The results after 72h were found to be 99.6±0.7 and 98.8±0.1% for standard and sample respectively.

APPLICATION OF DEVELOPED METHOD:

The proposed chromatographic method was used for quantification of MEM in dissolution samples of memantine hydrochloride 30mg tablets and the results were found within the specification (Table V).

TABLE V- %MEMANTINE IN DISSOLUTION SAMPLES

TAB SR. NO.	% MEM	LIMIT
1	94	≥85% drug release at 30 minutes
2	96	
3	94	
4	103	
5	94	
6	96	

DISCUSSION:

The RI detector is used as universal detector in HPLC. The detection technique is based on measurement of the change in RI of mobile phase. RI detectors consist of two-path cell–sample and reference. The RI of sample path cell is continuously compared with the reference cell. The greater the RI differences between sample and mobile phase, higher the sensitivity. The RI detector is having advantages as a universal detector with low sensitivity to air bubbles in the flow cells; and also covers the RI range from 1.000 to 1.750 RI.

The only limitation is the comparatively low sensitivity (19). In this study, the chromatographic method optimization was carried out by utilizing different stationary phase, with organic modifier and pH. The optimized chromatographic conditions obtained was Kromasil 100, C18, (250 × 4.6 mm, 5 µm) with mobile phase, mixture of ultrapure water containing 1% Diethyl amine (pH 5.2 adjusted with dilute glacial acetic acid)–methanol in the ratio of 40:60 (v/v). The retention time of MEM in the optimized condition was found was 6.5min (Figure 5). For the present the study, the 30 µg/mL of MEM as the standard concentration was injected six times to record the system suitability parameter.

The system suitability was found to be with acceptance criteria (Table I). The representative chromatogram for Limit of Quantification (LOQ) (Figure 6).

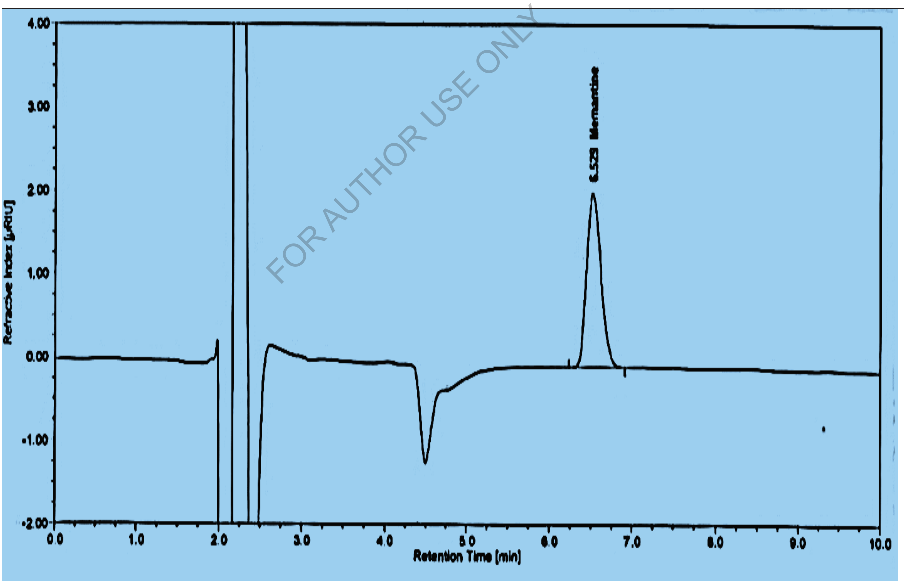

Figure 5 -Reference Chromatogram of MEM Standard of Dissolution Method

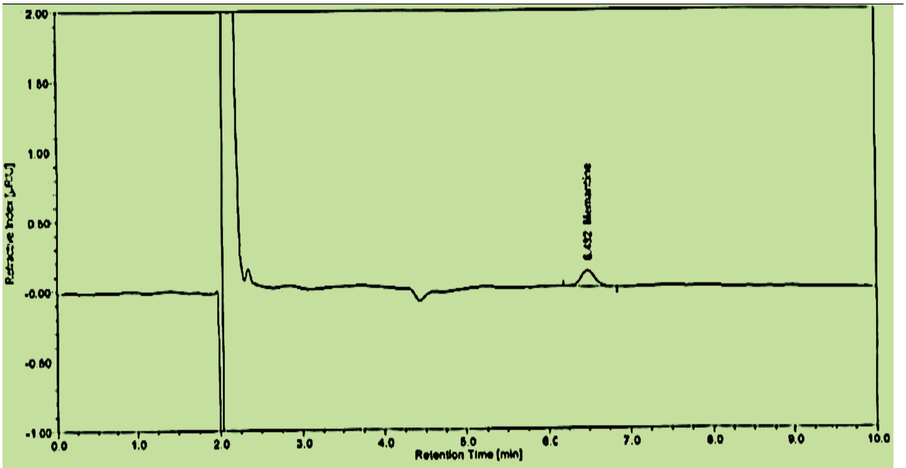

Figure 6- Chromatogram for limit of quantification (LOQ) in dissolution method

CONCLUSION:

The proposed liquid chromatographic method provides simple, accurate and reproducible methodology for quantitative measurement of memantine hydrochloride in dissolution sample using refractive index detector without any interference from the excipients. The proposed method is very simple and can be used without derivatization of compound under analysis. The response of the method was found to be linear in the range of 5.0–45 µg/mL, and it proved to be precise and accurate. The developed chromatographic method was fully validated accordingly to ICH Q2(R1) and USP 38 showing acceptable data for all the method validation parameters tested. The developed method is economical with utilization of commonly used reagents and instruments.

The intended method can be used conveniently by quality control to establish the drug release in dissolution samples in regular memantine hydrochloric production samples and stability samples. The said method also can be used during developmental studies of newer formulations for quantification of memantine hydrochloride during dissolution profile studies.

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2.2 SECTION B:

TO DEVELOP HPLC METHOD FOR THE ASSAY OF MEMANTINE HYDROCHLORIDE TABLETS USING REFRACTIVE INDEX (RI) DETECTOR

INTRODUCTION:

Memantine Hydrochloride is often used in the treatment of Alzheimer's disease (AD).AD can be caused due to depression, delirium, drug interaction, thyroid problems, excess use of alcohol, or certain vitamin deficiencies [1, 2].

Memantine HCl chemically is 1-amino-3,5-dimethyladamantane hydrochloride (Fig. 1).

Memantine hydrochloride occurs as a fine white to off-white powder and is soluble in water. Its molecular weight is 215.76 and molecular formula is C12H21N.HCL.

It's highly basic (pKa 10.42) and lipophilic (log P 3.28) nature suggests that it may show binding with (2-Napthoxy) Acetyl chloride, 9-fluorenylmethyl chloroformate, dansyl chloride etc. due to interaction of its primary amine group.

MEM is used as option in a new class of AD treatment and showed very good results in terms of efficacy and safety for patients with moderate to severe symptoms. It has been recently approved by European Union and Australia for the treatment of moderately severe to severe AD [5].

The drugs which are used in treatment of AD need to be given in appropriate dosage regimen. Hence it is very important to estimate the amount of drug present in the dosage forms. For determination of amount of drug (assay) in different formulations like tablets, capsules etc. a simple, rapid and economical analytical method is required.

MEM doesn't have chromophore. This limitation of the MEM necessitates, selecting specialized analytical techniques for its quantification. Numbers of HPLC methods were reported for

quantification of MEM in pharmaceutical dosage forms with specific techniques such as pre-column derivatization with UV detector [6-8], with charged aerosol detector [9]. The MEM quantification in biological fluids such as plasma and vitreous humour were also performed using pre-column derivatization and fluorescence detection [10-14]. The reported methods are cumbersome and time consuming with tedious sample preparation process.

This research was focused on development of a simple, precise, accurate, specific and robust reverse phase chromatographic method using refractive index detector for quantification of MEM in its different pharmaceutical dosage forms. The developed method is having advantage of short run time, no derivatization required; hence more number of samples can be tested in short span of time. This method can be used in quality control and R&D. The simplicity of proposed method is economical and can be utilized by common laboratories. The developed method was validated as per International Conference on Harmonization (ICH) Q2(R1) guideline and United State of Pharmacopoeia (USP) 38 chapter <1225> and chapter <1092> [15-17]. The method found to be specific, precise, accurate and robust with compliance to acceptance criteria of ICH and USP 38.

EXPERIMENTAL:

MATERIALS AND REAGENTS:

Memantine hydrochloride pure drug (100.0% w/w), tablets with label claim 30 mg of Memantine hydrochloride per tablet were provided by Wockhardt Ltd Aurangabad, Maharashtra India.

HPLC grade methanol was purchased from Merck Chemicals, Mumbai, India. Ultrapure water was generated from Milli-Q water purifier. Diethylamine was purchased from Spectrochem, Mumbai, India. Orthophosphoric acid and hydrochloric acid were purchased from Merck Ltd, Mumbai, India. Sodium chloride was purchased from Merck Chemicals, Mumbai, India.

INSTRUMENTS AND METHODS:

INSTRUMENTATION:

Waters High performance liquid chromatography (HPLC) system with Refractive Index Detector.

CHROMATOGRAPHIC PARAMETERS:

The chromatographic column used was a Hypersil-BDS, C18 (250.0 × 4.6mm; 5 µm) which was maintained at 40º C. The mobile phase was prepared by mixture of ultrapure water containing 0.2% diethylamine (pH 6.0 adjusted with dilute Orthophosphoric acid) – methanol in the ratio of 45:55 v/v. The flow rate of the mobile phase was 1.0 ml/min. The injection volume was 50.0 µL. The column effluents were monitored by refractive index detector. The detector and column oven temperature was set at 40.0º C with sensitivity of 512. The sample and reference cell were purged with mobile phase for 30min each with flow rate 1.0 ml/min.

PREPARATION STANDARD SOLUTIONS:

Weighed accurately about 50 mg of MEM working standard and transferred to 50 ml volumetric flask. The content of the flask was dissolved with mobile phase with sonication and volume was made up to mark with mobile phase as primary stock solution. Further 5.0 ml of prepared standard primary stock solution was pipetted and transferred to 50 ml volumetric flask and made up to the mark with mobile phase to get nominal concentration about100 µg/ml.

METHOD VALIDATION:

SPECIFICITY:

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities or expected to present. The specificity of the developed LC method for MEM was carried out by injecting placebo, known impurities of MEM (Admantamine) The placebo was prepared by dissolving 10mg placebo in 10 ml of volumetric flask in mobile phase and sonicated. The volume was made up to the mark with mobile phase. The resulting solution was filtered through 0.45µ syringe filter.

Each impurity was prepared by weighing 1mg of individual impurity in 10 ml volumetric flask and dissolved in mobile phase by sonication. The volume was made up to the mark with mobile phase. Further diluted 1ml of this solution to 10 ml with mobile phase.

The mobile phase (blank), placebo solution, individual impurities (10µg/ml) and standard drug solution (100µg/ml) were injected in sequence for evaluation of specificity of proposed method. The chromatograms were monitored for any peak eluted at the retention time of drug.

PRECISION:

Precision express the measure of how close the analytical results are to each other from a set of measurements under controlled analytical conditions. Precision proves random errors of the measurement.

Precision is a measure of the degree of repeatability (Intra-day), intermediate precision and reproducibility (inter-day) of the analytical method under normal operating circumstances.

Precision is usually measured as the relative standard deviation (RSD) of analytical results acquired from independently prepared quality control standards.

Method precision was evaluated by six sample preparations of same homogeneous test sample of MEM (30mg/tablet) and calculated % assay for each sample preparation. The % RSD for set of six preparations was calculated.

The intra-day precision was evaluated by analyzing six preparations of MEM (n = 6) in two different set in a day. The acceptance criteria for % RSD is not more than 2%.

The intermediate precision of the method was also evaluated using different analyst and a different instrument in the same laboratory by carrying out six sample preparation of same test sample of MEM tablets and calculated % assay for all preparations. Calculated the %RSD for 12 results. The acceptance criteria for % RSD is not more than 2%.

RECOVERY (ACCURACY) :

Accuracy is extremely important in analytical method validation as it assures the closeness of agreement between a test result and the accepted reference value. Accuracy is expressed as trueness and involves a combination of random components and a common systematic error or bias component. The accuracy of the method was performed by recovery studies.

In order to evaluate the accuracy of the proposed methods, a recovery test was performed by adding known amounts of standard solutions to the placebo formulation sample, followed by analysis using the proposed chromatographic method.

The recovery studies were done for three different levels at 80%, 100% and 120% with three determinations of working level concentration using standard spiking method.

The placebo was accurately weighed about 120mg for all level. For 80% (80µg/ml) level of recovery studies, 40.0 mg of MEM standard was spiked along with 120mg placebo and dissolved in 50ml of mobile phase. Further diluted 5.0 ml of this solution to 50 ml with mobile phase. The resulting solution was filtered through 0.45µ syringe filter. In same manner for 100% and 120% recovery studies 50mg (100µg/ml) and 60mg (120.0µg/ml) of MEM was spiked with 120mg of placebo respectively and prepared the solutions. All the above solutions were prepared in triplicate and were analyzed using proposed chromatographic condition. The recovery at each level was calculated by using the theoretical value from exact weight taken for spiking. The % recovery was calculated with respect to amount added. The acceptance criteria for % recovery is in the range of 98 - 102%.

LINEARITY:

The linearity of an analytical method is its ability to elicit test results that are directly, or by means of well-defined mathematical transformations, proportional to the concentration of analytes in the samples within a given range.

The linearity plot was constructed for MEM in the range of 50 to 150µg/ml. The primary stock solution of 1000µg/ml of MEM was prepared in mobile phase. From the primary stock solution, appropriate dilutions were made to get concentration of 50.0, 70.0, 100.0, 120.0 and 150.0.

The calibration curve was plotted as concentration of the respective drug solutions versus the peak area at each level. The results were statistically evaluated and correlation coefficient determination (r^2), slope and y-intercept values were calculated.

ROBUSTNESS:

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. One consequence of evaluation of robustness is that a series of system suitability parameters is established to ensure that the analytical procedure is maintained whenever used. In the present study the working concentration 100.0µg/ml of MEM was used

for the determination of the robustness of the method. The following parameters were considered for the robustness of the proposed chromatographic method.

- Effect of pH in the mobile phase (±0.2)
- Effect of organic modifier in mobile phase composition (± 2%)
- Effect of flow rate (±0.1)
- Column oven and detector temperature (±2°C)

SOLUTION STABILITY OF SAMPLE AND STANDARD IN MOBILE PHASE:

The solution stability of MEM sample was performed to understand stability which will be helpful to understand sample handling in proposed chromatographic method. The sample and standard solution after preparation were injected immediately to the system considering as an initial at 0 hour as baseline. A solution stability of MEM was carried out for sample solution (100.0µg/ml) in a tightly capped volumetric flask at ambient temperature for 72 hr. The results were calculated against freshly injected standard.

SYSTEM SUITABILITY:

The rationale of the system suitability assessment is to make sure that the complete testing system (including instrument, reagents, columns, analysts) is appropriate for the intended application.

System suitability tests (SST) are vital part of liquid chromatographic methods. They are used to verify the reproducibility of the chromatographic parameters and system is satisfactory for the analysis to be done. SST is support on the concept that the equipment, electronics, analytical operations and samples to be analyzed comprise an integral system that can be evaluated as such.

The system suitability test was performed in accordance with USP [18].

APPLICATION OF CHROMATOGRAPHIC METHOD FOR DETERMINATION OF ASSAY OF MEM TABLETS:

Assay test of MEM tablets was performed using developed method. The results found well within predefined specification.

RESULTS:

VALIDATION OF CHROMATOGRAPHIC METHOD:

SPECIFICITY:

The chromatogram (Fig. 7) of diluent, placebo, known impurities and standard solution were revealed that there is no interference at the retention of MEM. The developed chromatographic method was found to be highly specific for determination of assay for MEM tablets and capsules.

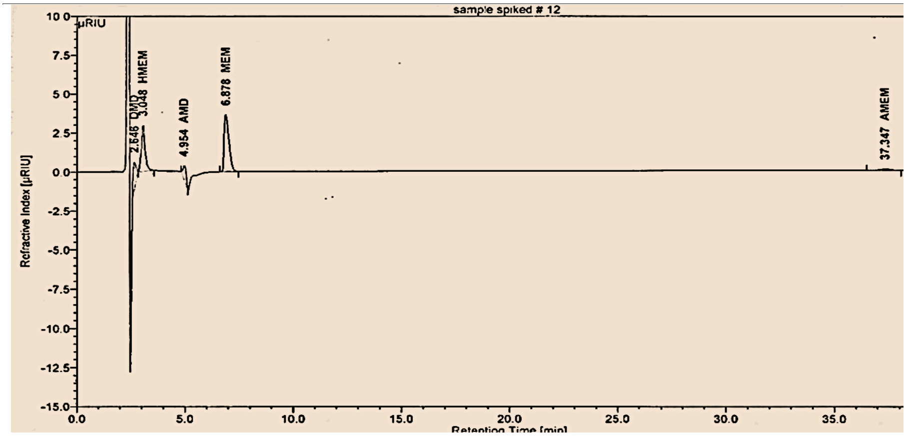

Fig. 7 Chromatogram of Memantine HCl along with diluents, placebo and known impurities in assay.

PRECISION:

The intra-day precision was evaluated by performing six (n = 6) assay determinations on same homogeneous sample of MEM tablets and the % RSD was found to be 0.47%.

The % RSD for inter day precision of assay test for two sets (n=12) for their % assay was found to be 0.53%.

The absolute difference between results for intermediate precision found 0.4% (Table no. VI).

TABLE NO. VI. -The Precision Results of Memantine HCl Tablets Assay Method

Concentration (µg/mL)	**% RSD**		**Absolute % difference in mean assay value**
	Intra-day(n=6)	**Inter-day (n=12)**	
100	0.47%	0.53%	0.4%

ACCURACY (RECOVERY):

The % recovery at 80, 100 and 120% was found to be 99.8±0.5,100.1±0.5, and 100.1±0.1% respectively (Table no. VII). The overall mean recovery was found to be 100.0±0.2%. The recovery results were found within acceptance criteria. The developed method found to be accurate for determination of assay in MEM tablets.

TABLE NO. VII -Accuracy (Recovery) Results of Memantine HCl Tablets Assay Method

Concentration level	% Recovery	Mean % Recovery
80%	100.2	99.8±0.5
	99.8	
	99.3	
100%	99.9	100.1±0.5
	100.6	
	99.7	
120%	100.3	100.1±0.1
	99.9	
	100.2	
% Mean Recovery		100.0±0.2

LINEARITY:

The linearity of the MEM in dissolution medium was performed in the range of 50-150µg/ml and found to be linear. The representative regression equation was found to be y=1.6072x +1.5924 with lowest correlation coefficient (r^2) was found to be 0.999. The linearity was found with in acceptance criteria.

ROBUSTNESS:

The robustness parameters for chromatographic method are presented in table VIII and found within acceptance criteria.

TABLE VIII -Robustness Results Of Memantine HCl Tablets Assay Method

PARAMETER		%RSD
Flow (mL/min)	**0.9**	**0.45**
	1.1	**0.35**
pH	**5.8**	**0.91**
	6.2	**0.68**
Mobile phase composition (change in organic modifier ± 2%)	**53**	**0.85**
	57	**0.64**
Column oven and detector temperature (±2°C)	**38**	**0.43**
	42	**0.32**

SOLUTION STABILITY OF SAMPLE AND STANDARD:

The MEM found to be stable up to 72hour in dissolution medium at ambient temperature. The difference between the initial results and after 72hour was found to be 0.5%.

APPLICATION OF DEVELOPED METHOD:

The proposed validated method was used for determination of assay in MEM tablets and the results were found within the specification (98.5%).

DISCUSSION:

MEM lacs chromophore, due to this limitation, MEM cannot be readily assayed by HPLC-UV techniques and hence refractive index detector was selected for detection. During development of this method, method optimization was carried out by using different HPLC columns, by changing concentration of organic modifier (methanol) and also different pH to achieve the separation between impurity and MEM. The main peak of MEM in the proposed method was found at 7.0 min. (Fig. 8). The sensitivity of standard and sample at concentration 100 µg/mL of MEM was found good.

The method was found economical, simple and robust. The system suitability was found to be with acceptance criteria. The proposed method is more accurate and time saving than the current published methods. It can be used by laboratories to determine the assay of memantine hydrochloride tablets of different strengths. The refractive index detector is a universal detector and easily available. Analyst need not to do derivatization of drug which is complex and time consuming.

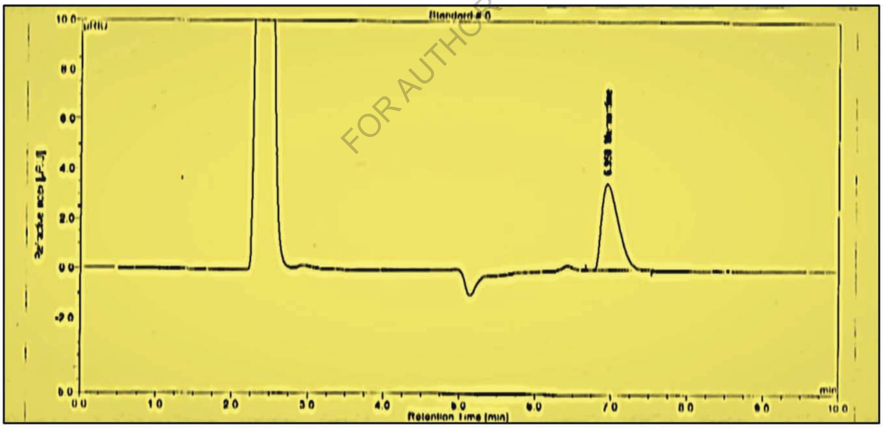

Fig. 8 Representative chromatogram of Memantine HCl standard in assay

CONCLUSION:

The validation of the method proven that the method is linear in the range of 50–150 µg/ml, to be precise and accurate over the range. The validation of the method is done accordingly to ICH Q2 (R1) and USP 38. All the parameters are meeting the acceptance criteria. Since method is simple and time saving hence it can be used conveniently by laboratories to determine the assay of different formulations of MEM.

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2.3 SECTION C:

THE DEVELOPMENT AND VALIDATION OF STABILITYINDICATINGANALYTICAL METHOD FOR DETERMINATION OF NORTRIPTYLINE IN NORTRIPTYLINE HCL TABLETS BY LIQUID CHROMATOGRAPHY

INTRODUCTION:

Nortriptyline hydrochloride, the N-demethylated active metabolite of amitriptyline, is a dibenzocycloheptene-derivative tricyclic antidepressant (TCA). TCAs are structurally similar to phenothiazines 3-(10, 11-dihydro-5H-dibenzo [a, d] cyclohepten-5-ylidene)-N-methyl-1-propanamine (Fig. 3).

Nortriptyline hydrochloride contains tricyclic ring system with an alkyl amine substituent on the central ring. Nortriptyline Base is a tricyclic antidepressant agent used for short-term treatment of various forms of depression since it is a non-selective serotonine uptake inhibitor [1]. Nortriptyline blocks the norepinephrine presynaptic receptors, thereby blocking the reuptake of this neurotransmitter and raising the concentration in the synaptic cleft in the CNS. Nortriptyline also binds to alpha-adrenergic, histaminergic and cholinergic receptors [2].

This research was focused on development of a simple, precise, accurate, specific and robust stability indicating reverse phase chromatographic method for assay [3, 4, 5, 6, 7, 8].

Literature survey reveals that nortriptyline hydrochloride is official in British Pharmacopeia [9].

Few chromatographic methods for the determination of nortriptyline hydrochloride and fluphenazine hydrochloride in pharmaceutical preparations and/ or with other active ingredients [10, 11, 12, 13] have been reported. The proposed method is stability indicating, simple and economical and can be utilized by common laboratories. The developed method was validated as per International Conference on Harmonization (ICH) Q2(R1) guideline[14] and United State of Pharmacopoeia (USP) 38 chapter <1225> and chapter <621> [15-16].

The method found to be specific, precise, accurate and robust with compliance to acceptance criteria of ICH and USP 38. This method can be used in quality control of manufactured and developed dosage forms.

MATERIALS AND METHODS:

MATERIALS:

HPLC grade methanol, potassium dihydrogen phosphate and Orthophosphoric acid was purchased from Merck Chemicals, Mumbai, India. Ultrapure water was generated from Milli-Q water purifier.

INSTRUMENTATION:

Thermo–Ultimate 3000 High performance liquid chromatography (HPLC) system with UV Detector.

METHODS:

CHROMATOGRAPHIC PARAMETERS:

The chromatographic column used was Inertsil, C18, 250mm x 4.6mm, 5µm which was maintained at 45º C. The mobile phase was prepared by mixture of methanol and buffer of pH 7.5 containing 0.05M of potassium dihydrogen phosphate and tetra butyl ammonium hydroxide solution (4ml/lit of 4g/10ml) in the ratio of 70:30 v/v. The flow rate of the mobile phase was 1.0 ml/min. The injection volume was 20.0 µL. The column effluents were monitored by UV detector at 220nm.

PREPARATION OF SOLUTIONS:

STANDARD PREPARATION:

Weighed accurately about 50 mg of Nortriptyline HCl working standard and transferred to 50 ml volumetric flask. The content of the flask was dissolved with diluents (methanol and water in the ratio of 70:30) with sonication and volume was made up to mark with diluents as primary stock solution. Further 5.0 ml of prepared standard primary stock solution was pipetted and transferred to 50 ml volumetric flask and made up to the mark with diluents to get nominal concentration about 100µg/ml.

Sample Preparation: Powder equivalent to 100mg of Nortriptyline HCl was weighed and dissolved in 100 ml volumetric flask with diluents with sonication. Filtered through 0.45µm nylon syringe filter. Further 5.0 ml of filtered solution was pipetted and transferred to 50 ml volumetric flask and made up to the mark with diluents to get nominal concentration about 100µg/ml.

METHOD VALIDATION:

SYSTEM SUITABILITY:

The rationale of the system suitability assessment is to make sure that during the complete testing, system (including instrument, reagents, columns, analysts) is appropriate for the intended application.

System suitability tests (SST) are vital part of liquid chromatographic methods. They are used to verify the reproducibility of the chromatographic parameters and system is satisfactory for the analysis to be done. SST is support on the concept that the equipment, electronics, analytical operations and samples to be analyzed comprise an integral system that can be evaluated as such.

The system suitability test was performed in accordance with USP [18].

SPECIFICITY:

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities or expected to present. The specificity of the developed LC method for all impurities was carried out by injecting placebo, known impurities of Nortriptyline HCl. The placebo was prepared by dissolving 10mg placebo in 10 ml of volumetric flask in mobile phase and sonicated. The volume was made up to the mark with diluents. The resulting solution was filtered through 0.45µ syringe filter.

Each impurity was injected at nominal concentration of 2µg/ml. The preparation was done as per standard preparation.

The diluents (blank), placebo solution, individual impurities (2µg/ml) and standard drug solution (100µg/ml) were injected in sequence for evaluation of specificity of proposed method. The chromatograms were monitored for any peak eluted at the retention time of drug.

FORCED DEGRADATION:

The forced degradation studies in acidic, hydrolysis and Oxidation condition were carried out during method development which confirmed that Nortriptyline is sensitive to oxidative conditions, potential degradation products formed at this condition. This study also confirmed that there is no co-elution of blank, placebo, known impurities or other substance with the principal peak.

PRECISION:

Precision express the measure of how close the analytical results are to each other from a set of measurements under controlled analytical conditions. Precision proves random errors of the measurement.

Precision is a measure of the degree of repeatability (Intra-day), intermediate precision and reproducibility (inter-day) of the analytical method under normal operating circumstances. Precision is usually measured as the relative standard deviation (RSD) of analytical results acquired from independently prepared quality control standards.

Method precision was evaluated by six sample preparations of same homogeneous sample of Nortriptyline tablets (25mg/tablet) test sample and calculated % recovery for Nortriptyline HCl in each sample preparation. The % RSD for set of six preparations was calculated.

The intermediate precision of the method was also evaluated using different analyst and a different instrument in the same laboratory by carrying out six sample preparations of tablets and calculated % recovery for Nortriptyline HCl in each preparation. Calculated the %RSD for 12 results. The acceptance criteria for % RSD was not more than 2%.

ACCURACY (RECOVERY):

Accuracy is extremely important in analytical method validation as it assures the closeness of agreement between a test result and the accepted reference value. Accuracy is expressed as trueness and involves a combination of random components and a common systematic error or bias component. The accuracy of the method was performed by recovery studies.

In order to evaluate the accuracy of the proposed method, a recovery test was performed by adding known amounts of standard solution to the placebo formulation sample, followed by analysis using the proposed chromatographic method.

The recovery studies were done for three different levels at 50%, 100% and 150% with three determinations of working level concentration using standard spiking method in placebo.

All the above solutions were prepared in triplicate and were analyzed using proposed chromatographic condition. The recovery at each level was calculated by using the theoretical value from exact weight taken for spiking. The % recovery was calculated with respect to amount added. The acceptance criteria for % recovery was in the range of 98 - 102%.

LINEARITY:

The linearity of an analytical method is its ability to elicit test results that are directly, or by means of well-defined mathematical transformations, proportional to the concentration of analyte in the samples within a given range. The linearity plot was constructed for Nortriptyline HCl in the concentration range of 50 to 150µg/ml. The primary stock solution of Nortriptyline HCl working standard was prepared. From the primary stock solution, appropriate dilutions were made to get concentration of 50, 60, 80, 100, 120 and 150µg/ml. The calibration curve was plotted as concentration of the respective drug solutions versus the peak area at each level. The results were statistically evaluated and correlation coefficient determination (r2), slope and y-intercept values were calculated.

ROBUSTNESS:

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. One consequence of evaluation of robustness is that, a series of system suitability parameters is established to ensure that the analytical procedure is maintained whenever used. In the present study, the working concentration 100.0µg/ml of Nortriptyline HCl was used for the determination of the robustness of the method. The following parameters were considered for the robustness of the proposed chromatographic method.

Effect of pH in the mobile phase (±0.2)

Effect of organic modifier in mobile phase composition (± 2%)

Effect of flow rate (±10%)

SOLUTION STABILITY OF SAMPLE AND STANDARD IN MOBILE PHASE:

The solution stability of sample solution in diluents was performed to understand stability which will be helpful to understand sample handling in proposed chromatographic method. Solution stability was carried out for sample solution (100.0µg/ml) in a tightly capped volumetric flask at ambient temperature for 72 hour. The sample and standard solution after preparation were injected immediately to the system considering as an initial at 0 hour as baseline.

RESULTS AND DISCUSSION:

METHOD DEVELOPMENT:

The drug and impurities were scanned on UV and the spectra of drug and individual impurity was recorded. The wavelength 220nm was selected which was permitting the detection of Nortriptyline HCl with adequate sensitivity. In this study, the chromatographic method optimization was carried out by utilizing different stationary phase containing C8 and C18 to achieve the resolution of impurities from main drug. The column of make Inertsil ODS (C18, 250 X 4.6 mm, 5µ) had offered more advantages regarding resolution of impurities.

Many different compositions of organic modifier and buffer with different pH were tried for better resolution of impurities.

Individual drug solution and known impurities was injected into column and elution pattern of the drug and its known impurities was observed. The resolution pattern was studied.

Forced degradation studies in different stress conditions like acidic, basic, oxidation and thermal were done and the peak purity of Nortriptyline HCl was monitored.

METHOD VALIDATION:

SPECIFICITY:

The specificity chromatogram known impurities (Fig. 9), sample (Fig. 10) and peak purity of sample (Fig. 11) along with diluents and placebo solution were revealed that there is no co-elution of any impurity with drug peak.

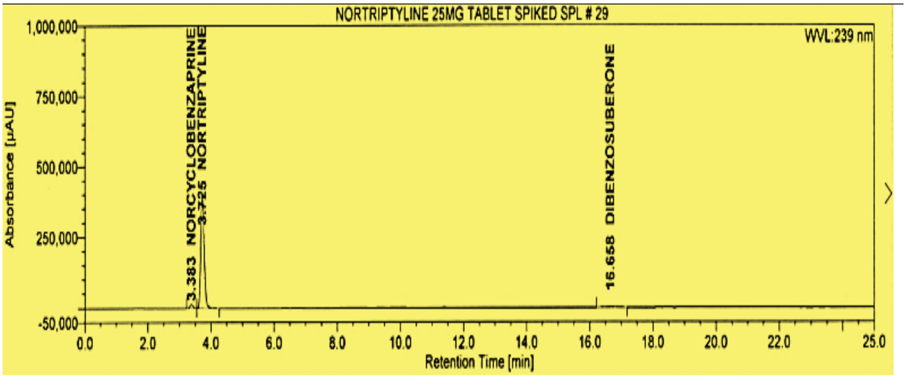

Fig. 9 Chromatogram of Known Impurities and sample in Nortriptyline assay method

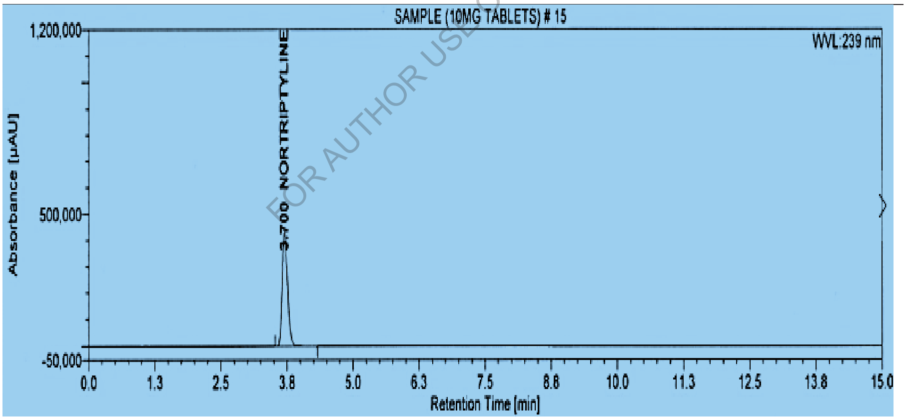

Fig. 10 Chromatogram of Sample with diluents and placebo in Nortriptyline assay method

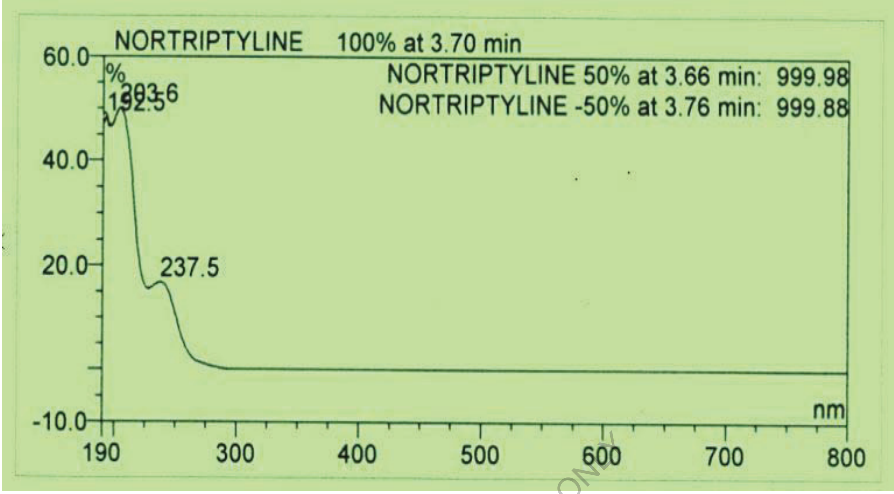

Fig. 11 Peak purity of Sample with diluents and placebo in Nortriptyline assay method

FORCED DEGRADATION:

The forced degradation study proved that the proposed method is stability indicating. All the peaks are pure. During this study, it is found that Nortriptyline HCl is sensitive to oxidative conditions, potential degradation products formed at this condition. All the unknown peaks were resolved properly. This study also confirmed that there is no co-elution of blank, placebo, known impurities or other substance with the degradants and principal peak.

Forced degradation was done in different stressed conditions like acidic -0.5M HCl (Fig. 12), basic - 0.1N NaOH (Fig. 13) and oxidation -30% Hydrogen Peroxide (Fig. 14) and the peak purity of Nortriptyline HCl were monitored. Peak purity of Nortriptyline HCl peak was found passing in all conditions (Table No. IX). The developed chromatographic method was found to be highly specific for determination of Nortriptyline HCl in Nortriptyline tablets. Reference chromatograms of forced degradation study are given below.

Table IX – Forced degradation study results of Nortriptyline tablets assay method

CONDITION	% ASSAY	% DEGRADATION	PURITY MATCH NLT 990	REMARK
Normal Conditions	100.5	Not applicable	1000	Peak purity of main peak passed
Acid degradation: 0.5N HCl solution, heating at 80°C for 2 hours	100.1	No degradation	1000	Peak purity of main peak passed
Alkali degradation:0.1N NaOH solution, at ambient for 2 hours	75.5	Nortriptyline HCl is insoluble in 0.1N NaOH, therefore no degradation	1000	Peak purity of main peak passed
Peroxide degradation: 30% H_2O_2 solution, heating at 60°C for 1 hour	90.1	9.9% degradation was observed	1000	Peak purity of main peak and degradants passed

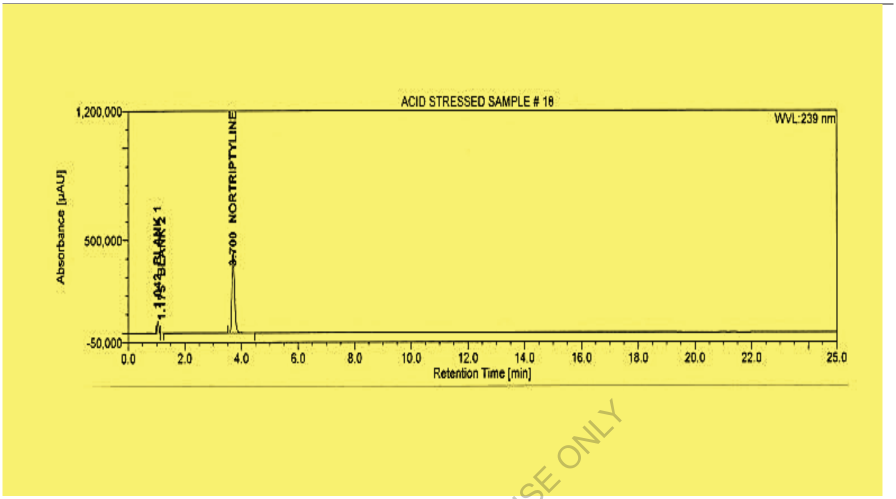

Fig. 12 Chromatogram of Forced degradation of Nortriptyline HCl in Acidic condition in assay

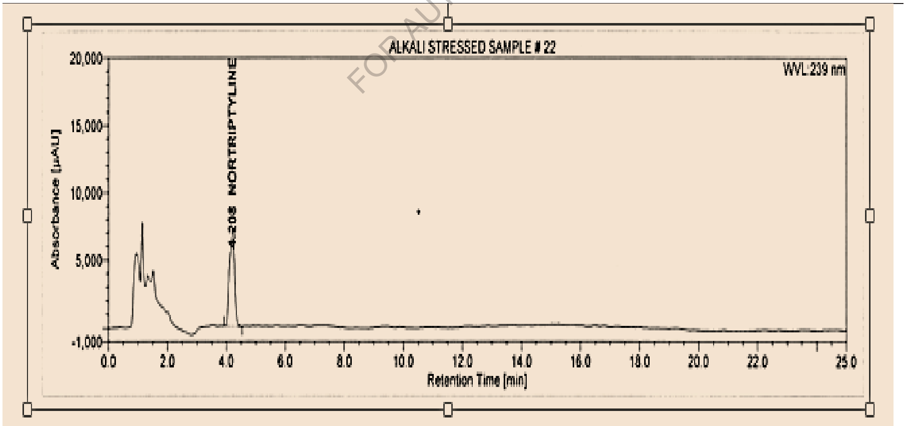

Fig. 13 Chromatogram of Forced degradation of Nortriptyline HCl in Alkali condition in assay

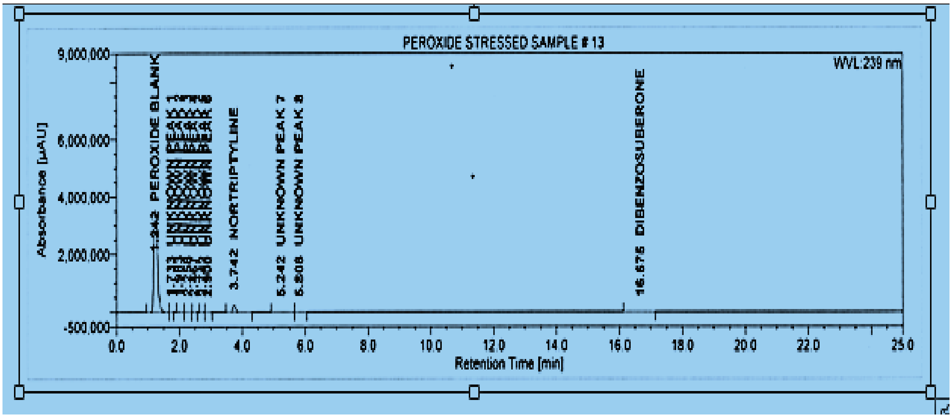

Fig. 14 Chromatogram of Forced degradation of Nortriptyline HCl in Hydrogen Peroxide in assay

Based on overall outcome of degradation studies it is concluded that proposed chromatographic method is specific and stability indicating.

PRECISION:

The method and system precision were performed at 100% working level concentration be preparing six samples of sample. The study was performed in two sets. First set analysis was done for intraday precision. The intra-day precision was evaluated by performing six (n = 6) assay determinations on same homogeneous sample of Nortriptyline tablets 25mg. Calculated %RSD for the six results and % RSD was found to be 0.41%.

The second set analysis was performed by another analyst using different instrument (HPLC) to prove the inter day precision. Six sample preparations were done on same homogeneous sample. Calculated % RSD for the six results and found to be 0.43%.

The overall % RSD of two sets (n=12) for their % assay was found to be 0.44%. The absolute difference between two sets of results for intermediate precision found 0.2% (Table No. X).

Table X – Precision results of Nortriptyline tablets assay method

Sample	% Assay	
	Intraday Precision	Inter-day (Intermediate) precision
Sample-1	100.5	99.7
Sample-2	100.1	100.0
Sample-3	99.6	99.6
Sample-4	100.8	100.5
Sample-5	100.3	100.8
Sample-6	100.5	99.9
Mean of individual sets	100.3	100.1
%RSD	0.38	0.43
Overall mean of 12 results	100.2	
Overall STDEV	0.44	
Overall % RSD	0.44	
% Difference between set I and set II results	0.2%	

ACCURACY (RECOVERY):

The recovery study was done in triplicate on three different concentrations. The range covered for this study was 50% to 150%. The three concentrations selected for this study were 50%, 100% and 150% of working level concentration. The appropriate quantity of Nortriptyline HCl drug was spiked in placebo and diluent at selected level. The recovery of drug was calculated against known spiked drug concentration. The mean % recovery of three preparations at 50%, 100% and 150% was found to be 100.2±0.4, 100.0± 0.2, and 100.3±0.2 % respectively (Table No. XI).

The overall mean recovery at all three level was found to be 100.1± 0.1%. The recovery results were found within acceptance criteria (Table No. XI). The developed method found to be accurate for determination of Nortriptyline HCl in Nortriptyline tablets.

Table XI – Recovery results of Nortriptyline tablets assay method

Accuracy conc.	Sample	% Recovery	Mean recovery	% RSD
50%	Sample-1	100.2	100.2	0.4
	Sample-2	100.6		
	Sample-3	99.9		
100%	Sample-1	99.8	100.0	0.2
	Sample-2	100.2		
	Sample-3	100.0		
150%	Sample-1	100.2	100.3	0.2
	Sample-2	100.0		
	Sample-3	100.3		
Overall Mean			100.1	0.1

LINEARITY:

The linearity for the response of Nortriptyline HCl drug was performed in the range of 50-150µg/ml and response for Nortriptyline HCl found to be linear. Total six concentration level were selected for linearity study as 50%, 60%, 80%, 100%, 120% and 150%. The linearity plot was constructed for concentration verses response. The representative regression equation was found to be y=29470.1551x +42985.8379 with lowest correlation coefficient (r2) was found to be 1.0000. The linearity was found with in acceptance criteria.

ROBUSTNESS:

The robustness study was performed to check the impact of small variables on method. During routine analysis, there can be small changes in the method parameters due to analytical variations due to analyst or and instrument. The robustness of the method was verified by making the deliberate variation in the critical method parameters like;

pH of the buffer - ±0.2

Organic modifier composition in mobile phase - ± 2%

Pump flow rate - ±10%

Even after making the small changes in the critical method parameters, the method delivered the expected results. The results are presented in Table No. XII and found within acceptance criteria.

Table XII – Robustness results of Nortriptyline tablets assay method

Method Parameter	%RSD of Standard Area	% Assay
Normal condition	0.5	99.9
Change in pH of Buffer + 0.2	0.2	99.5
Change in pH of Buffer - 0.2	0.8	100.1
Change in organic modifier + 2%	0.1	100.2
Change in organic modifier - 2%	0.8	99.6
Change in flow rate + 0.1ml/min	1.0	100.3
Change in flow rate - 0.1ml/min	0.5	100.0

SOLUTION STABILITY OF SAMPLE AND STANDARD:

The solution stability of standard and sample in selected diluent is very important during routine analysis. The analyst can plan the analysis, number of samples etc. based on solution stability of standard and sample. Hence solution stability of standard and sample was carried out up to 72 hours with testing frequency of 24 hours. The Nortriptyline HCl sample found to be stable up to 72hour in diluents at ambient temperature. The results after 72h were found to be 99.9% and 99.8% for standard and sample respectively (Table No. XIII).

Table No. XIII – Solution stability results of Nortriptyline tablets assay method

Solution stability of sample			Solution stability of standard		
Time	**%Assay**	**% difference**	**Time**	**%Assay**	**% difference**
Initial	100.5	NA	Initial	100.0	NA
24 Hour	99.8	0.7	24 Hour	100.3	-0.3
48 Hour	100.2	0.3	48 Hour	100.0	0.0
72 Hour	99.8	0.7	72 Hour	99.9	0.1

APPLICATION OF DEVELOPED METHOD:

The proposed chromatographic method was used for determination of Nortriptyline HCl in Nortriptyline tablets and the results were found within the specification.

CONCLUSION:

The proposed HPLC method is simple, precise, accurate and stability indicating rugged methodology for determination of Nortriptyline HCl in Nortriptyline tablets of different strengths. The validation of the method proven that the method is linear in the range of 50 –150 µg/ml of Nortriptyline HCl, and it proved to be precise and accurate over the range.

The validation of the method is done accordingly to ICH Q2 (R1) and USP 38. All the parameters are meeting the acceptance criteria. Since method is simple and time saving hence it can be used conveniently by laboratories to determine the Nortriptyline hydrochloride in different strengths of Nortriptyline tablets.

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CHAPTER III

3.0 THE DEVELOPMENT AND VALIDATION OF AN ANALYTICAL METHODS FOR ASSAY, RELATED SUBSTANCES AND DISSOLUTION OF URSODEOXYCHOLIC ACID TABLETS BY LIQUID CHROMATOGRAPHY

3.1 SECTION A:

DEVELOPMENT OF AN ANALYTICAL METHOD FOR DETERMINATION OF RELATED SUBSTANCES IN URSODEOXYCHOLIC ACID TABLETS

INTRODUCTION:

Ursodeoxycholic acid (UDCA) is a chemical called a bile acid (Fig. 2). It occurs naturally in bile and can be used to dissolve gallstones. The liver produces bile, which is then stored in the gall bladder. Bile is released by the gall bladder to aid the digestion of fats. UDCA the 7beta-epimer of chenodeoxycholic acid has multiple hepato protective activities. UDCA modifies the bile acid pool, decreasing levels of endogenous, hydrophobic bile acids while increasing the proportion of nontoxic hydrophilic bile acids. UDCA has a choleretic effect, increasing hepatocellular bile acid excretion, as well as cryoprotective, antiapoptotic, and immunomodulatory properties. UDCA has been shown to delay development of gastro esophageal varices and progression to cirrhosis as well as to improve long-term survival in patients with primary biliary cirrhosis. Significant improvement of abnormal liver tests may be achieved during UDCA therapy in patients with primary sclerosing cholangitis, intrahepatic cholestasis of pregnancy, cystic fibrosis-associated liver disease, nonalcoholic fatty liver disease, graft-versus-host disease of the liver, total parenteral nutrition-induced cholestasis, and in some pediatric cholestatic liver diseases. However, unlike the effects of UDCA in primary biliary cirrhosis, the long-term effects of UDCA in disease progression and survival in these other conditions remain to be established [1-3].

This research was focused on development of a simple, precise, accurate, specific and robust stability indicating reverse phase chromatographic method for quantification of relates substances of Ursodeoxycholic Acid in its pharmaceutical dosage forms [4-5]. This method can be used in quality control of manufactured and developed dosage forms. The simplicity of proposed method is economical and can be utilized by common laboratories.

The developed method was validated as per International Conference on Harmonization (ICH) Q2(R1) guideline and United State of Pharmacopoeia (USP) 38 chapter <1225> and chapter <1092> [6-8]. The method found to be specific, precise, accurate and robust with compliance to acceptance criteria of ICH and USP 38 [9].

MATERIALS

HPLC grade methanol, acetonitrile was purchased from Merck Chemicals, Mumbai, India.

Ultrapure water was generated from Milli-Q water purifier.

INSTRUMENTATION AND METHODS

INSTRUMENT:

Thermo dionex –Ultimate 3000 High performance liquid chromatography (HPLC) system with RI Detector.

CHROMATOGRAPHIC PARAMETERS:

The chromatographic column used was a Luna Phenomenox, C18(2) 100 A°, 250 mm x 4.6 mm, 5µm which was maintained at 40º C. The mobile phase was prepared by mixture of buffer, Acetonitrile and methanol of 35:28:37v/v. The flow rate of the mobile phase was 1.0 ml/min. The injection volume was 100.0 µL. The column effluents were monitored by RI detector at sensitivity 256 mV. The detector temperature and column oven temperature were set at 40.0º C.

PREPARATION OF SOLUTIONS

PREPARATION OF BUFFER SOLUTION:

Dissolve 0.78g of sodium dihydrogen orthophosphate dihydrate in 1 L of water. Adjust pH to 3.00 ± 0.05 with orthophosphoric acid (10 % v/v), mix well and filter through 0.45µm nylon membrane filter paper.

PREPARATION OF MOBILE PHASE:

Mix buffer, acetonitrile and methanol in the ratio of 35:28:37v/v/v. Mix well and sonicate to degas before use.

PREPARATION OF SAMPLE DILUENT:

Use mobile phase as sample diluent.

PREPARATION OF BLANK SOLUTION:

Dilute 5 mL of methanol to 50 mL with sample diluent in a 50mL volumetric flask and mix well.

STANDARD STOCK SOLUTION:

Transfer an accurately weighed quantity of about 22.5 mg of UDCAC RS/WS into a 50mL volumetric flask. Dissolve in about 20 mL methanol and dilute to volume with same.

STANDARD SOLUTION:

Dilute 5.0 mL of calibration stock solution to 50.0 mL with sample diluent in 50 mL volumetric flask. Mix well and filter through 0.45 µm PTFE syringe filter discarding first few mL.

SAMPLE SOLUTION:

Weigh and triturate 20 tablets to fine powder. Transfer an accurately weighed quantity of powder sample equivalent to about 450 mg of UDCA into a 50mL clean and dry volumetric flask. To this add 5.0 mL of methanol, sonicate for 5 minutes with intermittent shaking, allow solution to cool to room temperature and make to volume with sample diluent and mix well. Centrifuge at 3000 rpm for 5 minutes. Collect the clear supernatant and filter through 0.45 µm PTFE syringe filter discarding first 3mL of filtrate.

METHOD VALIDATION

SYSTEM SUITABILITY:

The rationale of the system suitability assessment is to make sure that during the complete testing, system (including instrument, reagents, columns, analysts) is appropriate for the intended application.

System suitability tests (SST) are vital part of liquid chromatographic methods. They are used to verify the reproducibility of the chromatographic parameters and system is satisfactory for the analysis to be done.

SST is support on the concept that the equipment, electronics, analytical operations and samples to be analyzed comprise an integral system that can be evaluated as such. The system suitability test was performed in accordance with USP [18].

SPECIFICITY:

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities or expected to present. The specificity of the developed LC method for all impurities was carried out by injecting placebo, known impurities of UDCA. The placebo was prepared by dissolving 10mg placebo in 10 ml of volumetric flask in mobile phase and sonicated. The volume was made up to the mark with diluents. The resulting solution was filtered through 0.45µ syringe filter. Each impurity was injected at nominal concentration (limit level). Preparation was done as per standard preparation.

The diluents (blank), placebo solution, individual impurities and standard drug solution (100µg/ml) were injected in sequence for evaluation of specificity of proposed method. The chromatograms were monitored for any peak eluted at the retention time of drug.

FORCED DEGRADATION:

The forced degradation studies in acidic, hydrolysis, Oxidation and thermal condition were carried out during development which confirmed that UDCA is sensitive to oxidative conditions, potential degradation products forms at this condition. This study also confirmed that there is no co-elution of blank, placebo or other substance with the principal peak.

PRECISION:

Precision express the measure of how close the analytical results are to each other from a set of measurements under controlled analytical conditions. Precision proves random errors of the measurement.

Precision is a measure of the degree of repeatability (Intra-day), intermediate precision and reproducibility (inter-day) of the analytical method under normal operating circumstances.

Precision is usually measured as the coefficient of variation or relative standard deviation of analytical results acquired from independently prepared quality control standards.

Method precision was evaluated by six sample preparations of same homogeneous sample of UDCA tablet test sample and calculated % recovery for known impurities in each sample preparation. The %RSD for set of six preparations was calculated.

The intermediate precision of the method was also evaluated using different analyst and a different instrument in the same laboratory by carrying out six sample preparations of tablets and calculated % recovery for impurities in each preparation. Calculated the %RSD for 12 results. The acceptance criteria for % RSD is not more than 15%.

RECOVERY (ACCURACY):

Accuracy is extremely important in analytical method validation as it assures the closeness of agreement between a test result and the accepted reference value. Accuracy is expressed as trueness and involves a combination of random components and a common systematic error or bias component. The accuracy of the method was performed by recovery studies.

In order to evaluate the accuracy of the proposed methods, a recovery test was performed by adding known amounts of standard solutions to the placebo formulation sample, followed by analysis using the proposed chromatographic method.

The recovery studies were done for three different levels at LOQ (20%), 100% and 150% with three determination of working level concentration using standard spiking method in placebo.

All the above solutions were prepared in triplicate and were analyzed using proposed chromatographic condition. The recovery at each level was calculated by using the theoretical value from exact weight taken for spiking. The % recovery was calculated with respect to amount added. The acceptance criteria for % recovery was in the range of 90 - 110%.

LINEARITY:

The linearity of an analytical method is its ability to elicit test results that are directly, or by means of well-defined mathematical transformations, proportional to the concentration of analytes in the samples within a given range.

The linearity plot was constructed for known impurities in the concentration range of LOQ to 150% impurity limit level.

The primary stock solution of UDCA working standard and impurity solution was prepared. From the primary stock solutions, appropriate dilutions were made to get concentration of LOQ, 50, 80, 100, 120 and 150%. The calibration curve was plotted as concentration of the respective drug and impurity solutions versus the peak area at each level. The results were statistically evaluated and correlation coefficient determination (r2), slope and y-intercept values were calculated.

ROBUSTNESS:

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. One consequence of evaluation of robustness is that a series of system suitability parameters is established to ensure that the analytical procedure is maintained whenever used. In the present study the working concentration 9000.0µg/ml of UDCA was used for the determination of the robustness of the method. The following parameters were considered for the robustness of the proposed chromatographic method.

Effect of flow rate (±10%)

Column oven temperature (±5°C)

SOLUTION STABILITY OF SAMPLE AND STANDARD IN MOBILE PHASE:

The solution stability of sample solution in diluents was performed to understand stability which will be helpful to understand sample handling in proposed chromatographic method. Solution stability was carried out for sample solution (100.0µg/ml) in a tightly capped volumetric flask at ambient temperature for 72 hr. The sample and standard solution after preparation were injected immediately to the system considering as an initial at 0 hr as baseline.

RESULTS AND DISCUSSION

METHOD VALIDATION

SPECIFICITY:

No peak(s) due to blank and placebo were observed at the retention time of ursodeoxycholic acid, chenodeoxycholic acid (impurity A) and impurity H and cholic acid.

The RT and RRT data of known individual related substances confirmed that all known individual related substances are well separated.

The chromatogram of diluent (blank) (Fig. 15), placebo (Fig. 16), known impurities and sample solution (Fig. 17 and Fig. 18) were revealed that there is no co-elution of any impurity with drug peak. Based on overall outcome of degradation studies, it is concluded that method is specific.

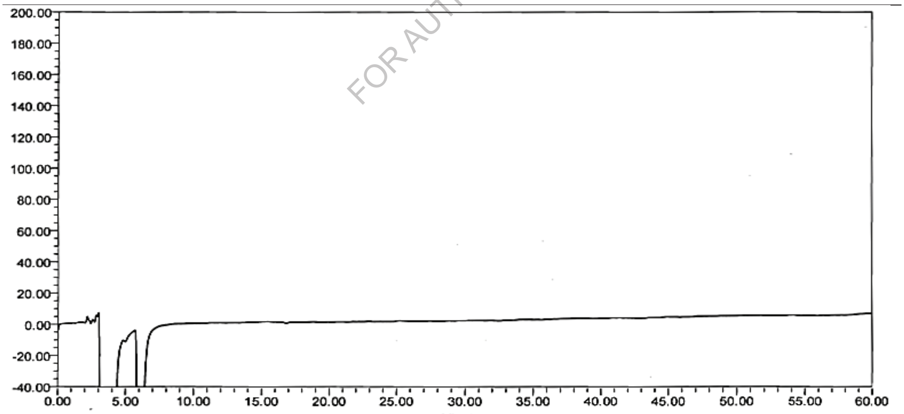

Fig. 15 Blank Chromatogram by method for related substances in Ursodeoxycholic acid tablets

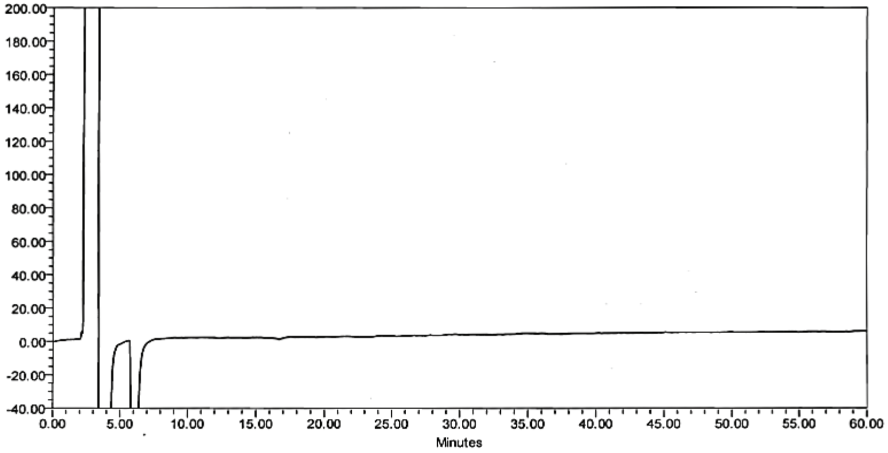

Fig. 16 Placebo Chromatogram by method for related substances in Ursodeoxycholic acid tablets

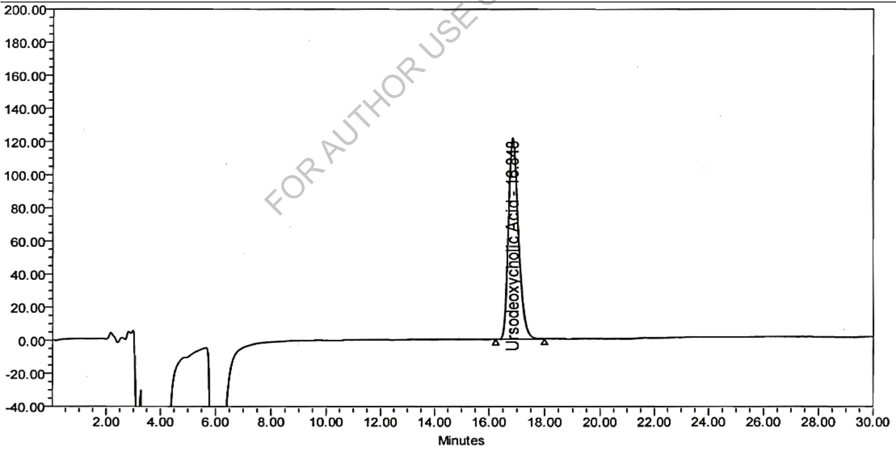

Fig. 17 Standard Chromatogram by method for related substances in Ursodeoxycholic acid tablets

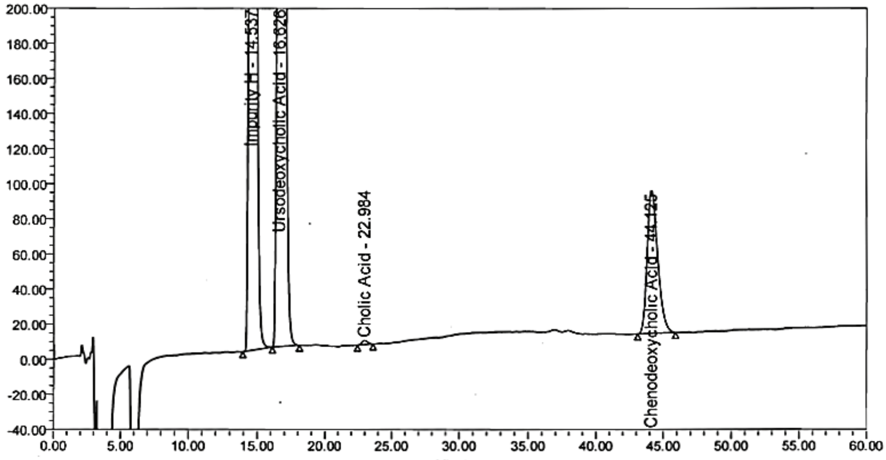

Fig. 18 Chromatogram of Impurity and Sample of method for related substances in Ursodeoxycholic acid tablets

FORCED DEGRADATION:

Forced degradation was done in different stressed conditions like acidic (0.5M HCl), basic (0.1N NaOH), oxidation (30% Hydrogen Peroxide) and thermal (was directly exposed to heat at 80°C for 48 hours) and the peak purity of UDCA was monitored. Peak purity of UDCA and impurity peak was found passing in all conditions. The developed chromatographic method was found to be highly specific for determination of impurities in UDCA tablets.

Based on overall outcome of degradation studies it is concluded that proposed chromatographic method is specific and stability indicating.

PRECISION:

The intra-day precision was evaluated by performing analysis six (n = 6) times on same homogeneous sample of UDCA tablets and the % RSD was found to be 1.6%. The % RSD for inter day precision for two sets (n=12) for their % recovery was found to be 2.2%.

ACCURACY (RECOVERY):

The % recovery at LOQ, 50%, 100% and 150% of Chenodeoxycholic acid was found to be 97.2±6.8, 100.2±2.2, 103.0± 3.2, and 101.8±0.7% respectively (Table XIV). The overall mean recovery was found to be 100.6± 4%. The recovery results were found within acceptance criteria (Table XIV). The developed method found to be accurate for determination of impurities in UDCA tablets.

Table XIV: Accuracy Results of Chenodeoxycholic Acid with Respect to Ursodeoxycholic Acid

Accuracy Level	% Recovery	Mean % recovery	% RSD
LOQ	99.1	97.2	6.8
	102.7		
	89.9		
50%	98.1	100.2	2.2
	99.9		
	102.5		
100%	99.9	103.0	3.2
	106.5		
	102.5		
150%	102.6	101.8	0.7
	101.5		
	101.4		
Overall Mean		100.6	4.0

LINEARITY:

The linearity of the response of UDCA drug and chenodeoxycholic acid [impurity A] was performed in the range of LOQ-150% of impurity specification level and response for both found to be linear. The graphs were plotted for UDCA and chenodeoxycholic acid [impurity A] related substances with concentration on X-axis and area response on Y-axis. Correlation coefficient (C.C) ‘r’ and % Y intercept were calculated. The representative regression equation for UDCA was found to be y = 1.351x - 0.055 and correlation coefficient (r2) was found to be 0.999. The representative regression equation for chenodeoxycholic acid [impurity A] was found to be y = 1.306x - 0.498 and correlation coefficient (r2) was found to be 1.000. The linearity was found with in acceptance criteria.

LIMIT OF DETECTION [LOD] AND LIMIT OF QUANTITATION [LOQ]:

The limit of detection and limit of quantification for chenodeoxycholic acid (impurity A) and UDCA was determined by signal to noise ratio method determined from linearity study (refer section 6). % LOD and LOQ values were reported with respect to nominal sample concentration of UDCA[9mg/mL].

The results of LOD and LOQ are tabulated in Table XV.

Table XV: Summary of LOD and LOQ values of analyte by S/N ratio

Analyte	**Concentration [µg/mL]**		**Signal to Noise ratio**		**% w.r.t UDCA [9mg/mL]**	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
Ursodeoxycholic acid	1.4842	4.9473	4	15	0.016%	0.055%
Chenodeoxycholic acid	5.5979	13.9948	6	14	0.062%	0.155%

ROBUSTNESS:

The robustness of the method was verified by making the deliberate variation in the critical method parameters like;

Pump flow rate - ±10%)

Column oven temperature - ±5°C

The results are presented in table XVI and table XVII, and found within acceptance criteria.

Table – XVI: Impact of change in flow rate of mobile phase on related substances data

% Impurity	**Flow rate 0.8mL/min [Normal conditions]**		**Flow rate 0.7mL/min**		**Flow rate 0.9mL/min**	
	RRT	**%Imp**	**RRT**	**%Imp**	**RRT**	**%Imp**
Chenodeoxycholic acid (impurity A)	2.870	1.525	2.801	1.605	2.818	1.549
Any other impurity (impurity H)	0.901	0.073	0.909	0.081	0.907	0.074
Sum of total impurities (including chenodeoxycholic acid)	-	1.598	-	1.686	-	1.623

Table XVII: Impact of change in column oven temperature on related substances

% Impurity	Column oven temperature 40°C [Normal conditions]		Column oven temperature 35°C		Column oven temperature 45°C	
	RRT	%Imp	RRT	%Imp	RRT	%Imp
Chenodeoxycholic acid (impurity A)	2.870	1.525	3.009	1.523	2.752	1.616
Any other impurity (impurity H)	0.901	0.073	0.894	0.079	0.907	0.079
Sum of total impurities (including chenodeoxycholic acid)	-	1.598	-	1.602	-	1.695

SOLUTION STABILITY OF SAMPLE AND STANDARD:

The UDCA sample found to be not stable more than 2hour in diluents at ambient temperature. Based on the data, it is recommended and concluded that the standard solution can be used up to 5 days from the date of preparation when stored at room temperature [25°C] or at 2-8°C.

APPLICATION OF DEVELOPED METHOD:

The proposed chromatographic method was used for determination of impurities in UDCA tablets and the results were found within the specification.

CONCLUSION:

The proposed HPLC method is simple, precise, accurate and stability indicating rugged methodology for determination of impurities in UDCA tablets of different strengths. The validation of the method proven that the method is linear in the range of LOQ –150 % of impurity concentration limit, and it is proved to be precise and accurate over the range. The validation of the method is done accordingly to ICH Q2 (R1) and USP 38. All the parameters are meeting the acceptance criteria. Since method is simple and stability indicating hence it can be used conveniently by laboratories to determine the impurities in different formulations of Ursodeoxycholic Acid.

PUBLICATION:

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3.2 SECTION B:

CHROMATOGRAPHIC METHOD DEVELOPMENT AND VALIDATION FOR QUANTITATIVE DETERMINATION (ASSAY) OF URSODEOXYCHOLIC ACID IN URSODEOXYCHOLIC ACID TABLETS

INTRODUCTION

Ursodeoxycholic acid (UDCA), is generally used in the treatment of liver diseases. UDCA is a naturally occurring bile acid with multiple hepatoprotective activities, improves liver condition in patients with a wide range of chronic liver diseases. UCDA is increasingly used for the treatment of cholestatic liver diseases. Studies done on UDCA suggests different mechanisms of action like: (1) protection of cholangiocytes against cytotoxicity of hydrophobic bile acids, resulting from modulation of the composition of mixed phospholipid-rich micelles, reduction of bile acid cytotoxicity of bile and, possibly, decrease of the concentration of hydrophobic bile acids in the cholangiocytes; (2) stimulation of hepatobiliary secretion, putatively via Ca2+- and protein kinase C-a-dependent mechanisms and/or activation of p38MAPK and extracellular signal-regulated kinases (Erk) resulting in insertion of transporter molecules (e.g., bile salt export pump, BSPE, and conjugate export pump, MRP2) into the canalicular membrane of the hepatocyte and, possibly, activation of inserted carriers; (3) protection of hepatocytes against bile acid-induced apoptosis, involving inhibition of mitochondrial membrane permeability transition (IMPT), and possibly, stimulation of a survival pathway (1,2).

There are few methods published currently for the estimation of UDCA in Ursodeoxycholic acid tablets [3-5]. This research work is done to develop stability indicating, precise, accurate, specific and robust chromatographic methods for quantification of UDCA in Ursodeoxycholic acid tablets in its pharmaceutical dosage forms. This method can be used in the quality control or in research laboratories of pharmaceutical companies for assay determination of Ursodeoxycholic acid tablets.

The developed method is validated as per International Conference on Harmonization (ICH) Q2 (R1) and United State of Pharmacopoeia (USP) chapter <1225> and chapter <1092> [6-10]. The method found to be specific, precise, accurate, robust and stability indicating. The method validation is compliant with validation criteria of ICH Q2 (R1) and USP guidelines.

EXPERIMENTAL

MATERIALS AND METHODS

Sodium dihydrogen orthophosphate dihydrate, Disodium hydrogen orthophosphate dihydrate, Tetrabutylammonium hydroxide 40% - HPLC grade, Tetrabutylammonium hydrogen sulphate, Acetonitrile and ultrapure water was generated from milli- Q water purifier.

INSTRUMENTS

Waters High Performance Liquid Chromatography (HPLC) system with PDA detector.

CHROMATOGRAPHIC PARAMETRS

The chromatographic column used was ODS (C18), 250mm X 4.6mm, 5µm (Devlosil) which was maintained at 450C. The mobile phase was prepared by mixture of buffer and Acetonitrile in the ratio of 50:50 v/v. The flow of the mobile phase was 1.5 ml/min. The injection volume was 10.0 µL. The column effluents were monitored by UV detector at 210nm. The column oven temperature and sample oven temperature was at ambient.

PREARATION OS SOLUTIONS:

BUFFER SOLUTION:

Dissolved about 12 gm of sodium dihydrogen orthophosphate dihydrate, 4 gm of disodium hydrogen orthophosphate dihydrate and 11 gm of tetra butyl ammonium hydrogen sulphate in 1000 ml of HPLC grade water. Sonicated for degassing and filtered through 0.45 µm membrane filter.

DILUENT:

Dissolved 6 gm of sodium dihydrogen orthophosphate dihydrate, 2gm of disodium hydrogen orthophosphate dihydrate in 535 ml of water and added 65 ml of tetrabutyl ammonium hydroxide 40 % to it. Mixed this solution with 400 ml of acetonitrile.

STANDARDPREPARATION:

Weighed accurately about 300mg of UDCA working standard and transferred to 20 ml volumetric flask. The content of the flask was dissolved in diluent with sonication and intermittent shaking and volume was made up to the mark with diluent to nominal concentration about 6000 µg/ml.

SAMPLEPREPARATION:

Powdered 20 tablets and weighed powder equivalent to 300 mg of UDCA in 20ml volumetric flask and dissolved in diluent with sonication and intermittent shaking (15000µl). Filtered through 0.45 µm nylon syringe filter.

METHOD VALIDATION

Developed analytical method is validated for parameters as suggested by ICH Q2 (R1) and USP.

SYSTEMSUITABILITY:

System suitability test (SST) are vital part of liquid chromatographic methods. They are used to verify the reproducibility of the chromatographic parameters and system is satisfactory for the analysis to be done. SST is support on the concept that the equipment, electronics, analytical operations and samples to be analyzed comprised and integral system that can be evaluated as such. The system suitability test was performed in accordance with USP [18].

SPECIFICITY:

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities or expected to be present. The specifity of the developed LC method for all impurities was carried out by injecting placebo, known impurities of UDCA (Chenodeoxycholic acid, Cholic acid and Lithocholic acid).

The diluent (blank), placebo solution, individual impurities and standard drug solution (15000 µg/ml) were injected in sequence for evaluation of specificity of proposed method. The chromatograms were monitored for any peak eluted at the retention time of drug.

FORCEDDEGRADATION:

The forced degradation studies in acidic, alkali, oxidation and thermal condition were carried out during development. No degradation was observed which confirmed that UDCA is stable at all conditions. This study also confirmed that there is no co-elution of blank, placebo or other substance with the principle peak.

PRECISION:

Precision is a major of the degree of repeatability (intra-day), intermediate precision and reproducibility (Inter-day) of the analytical method under normal operating circumstances.

Precision is usually measured as the coefficient of variation or relative standard deviation of analytical results acquired from independently prepared quality control standards.

Method precision was evaluated by six sample preparations as per above mentioned procedure for sample preparation of same homogeneous powdered sample of UDCA tablets and calculated % recovery for UDCA in each sample preparation. The %RSD for set of six preparations was calculated.

The intermediate precision of the method was also evaluated using different analyst and a different instrument in the same laboratory by carrying out six sample preparations of tablets and calculated % recovery for UDCA in each preparation. Calculated the %RSD for 12 results. The acceptance criteria for % RSD was not more than 2% and the absolute difference between results by two analysts was not more than 2%.

RECOVERY (ACCURACY):

Accuracy is extremely important in analytical method validation as it assures the closeness of agreement between test results and the accepted reference value. Accuracy is expressed as trueness and involves the combination of random components and common systematic errors or bias component. The accuracy of the method was performed by recovery studies.

In order to evaluate the accuracy of the proposed method, recovery test was performed by adding known amount of standard solution to the placebo formulation sample, followed by analysis using the proposed chromatographic method.

The recovery studies were done at three different levels at 50%, 100% and 150% with three determinations of working level concentration using standard spiking method in placebo.

All the above solutions were prepared in triplicate and were analyzed using proposed chromatographic condition. The recovery at each level was calculated by using the theoretical value from exact weight taken for spiking. The % recovery was calculated with respect to amount added. The acceptance criteria for % recovery was – the recovery at each level should be in the range of 98% to 102% and overall %RSD of nine results should be less than 2%.

LINEARITY:

The linearity of an analytical method is its ability to elicit test results that are directly or by means of well-defined mathematical transformations, proportional to the concentration of analytes in the samples within a given range.

The linearity plot was constructed for UDCA in the concentration range of 50% to 150% of the sample concentration (15000 µg/ml). The primary stock solution of UDCA working standard was prepared. From the primary stock solution, appropriate dilutions were made to get concentration 0f 7500, 12000, 15000, 18000 and 22500 µg/ml. The calibration curve was plotted as concentration of the respective drug solutions verses the peak area at each level. The results were statistically evaluated and correlation coefficient determination, slope and y-intercept values were calculated.

ROBUSTNESS:

Robustness is a capacity of a method to remain unaffected by small deliberate changes in method parameters.

For robustness study, the sample concentration 15000 µg/ml of UDCA was used. Following chromatographic parameters were considered for the robustness study.

Effect of mobile phase composition (±2%)

Effect of flow rate (±0.1ml/min)

Column oven temperature (±20C)

SOLUTION STABILITY OF SAMPLE AND STANDARD IN DILUENT:

The solution stability of sample and standard solution in diluent was performed to understand the stability which will be helpful for sample handling during routine analysis by using the proposed method.

Solution stability was carried out for sample solution (15000 g/ml) in a tightly capped volumetric flask at ambient temperature for 72 hr. The sample and standard solution were injected immediately after preparation in the HPLC system considering it as an initial (0 hr.) as baseline.

RESULTS AND DISCUSSION

METHOD DEVELOPMENT:

Literature survey revealed that UDCA is having molecular weight 392.58 g/mol. The solubility of UDCA in water was found as 20mg/liter. The drug solution was scanned on PDA and the spectra of UDCA was recorded.

The wavelength 210nm was selected which was permitting the detection of UDCA with adequate sensitivity.

In this study, the chromatographic method optimization was carried out by utilizing different stationary phase (C8 and C18) to achieve the resolution of potential impurities from main drug. The column of make Devlosil (C18, 250 X 4.6 mm, 5µ) had offered more advantages regarding the resolution of impurities and UDCA.

Many different compositions of organic modifier with buffer were tried for better resolution of impurities at different pH mobile phase. Individual drug solution and known impurities was injected into column and elution pattern of the UDCA and its impurities was injected into column and elution pattern was studied. Forced degradation studies in different stress conditions like acidic, basic, oxidation and thermal were performed and the peak purity of UDCA was monitored.

METHOD VALIDATION

SPECIFICITY:

The chromatograms during specificity study (fig. 19,20,21 and 22) of diluent, placebo, standard and sample solution shows that there is no co elution of any peak with UDCA.

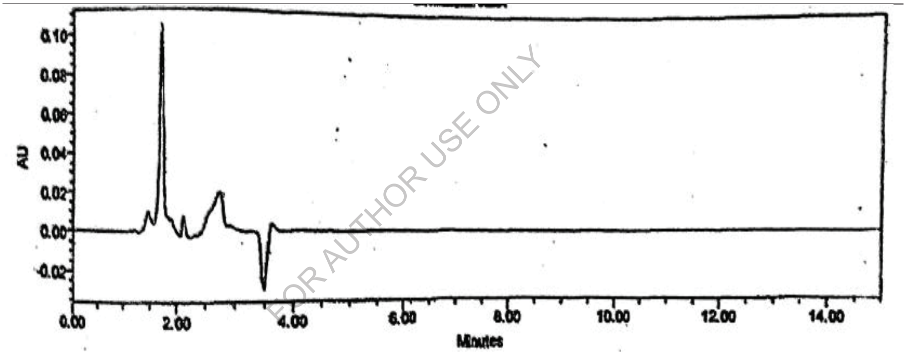

Fig. 19 – Blank Chromatogram of UDCA assay method

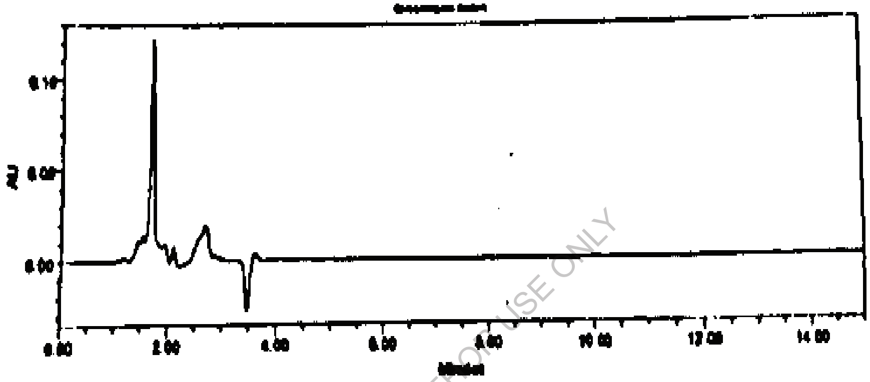

Fig. 20 – Placebo Chromatogram of UDCA assay method

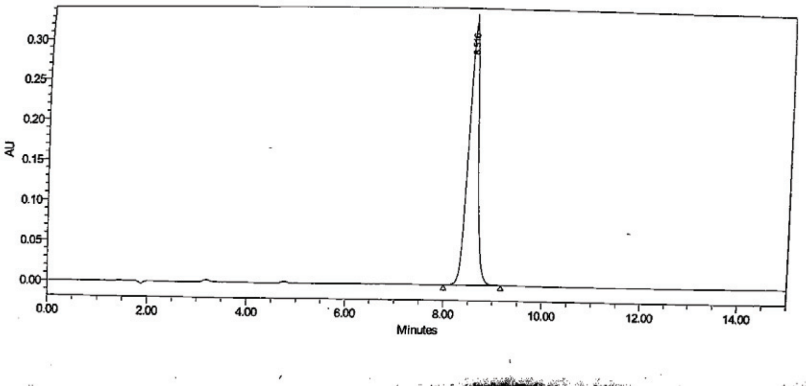

Fig. 21- Standard Chromatogram of UDCA assay method

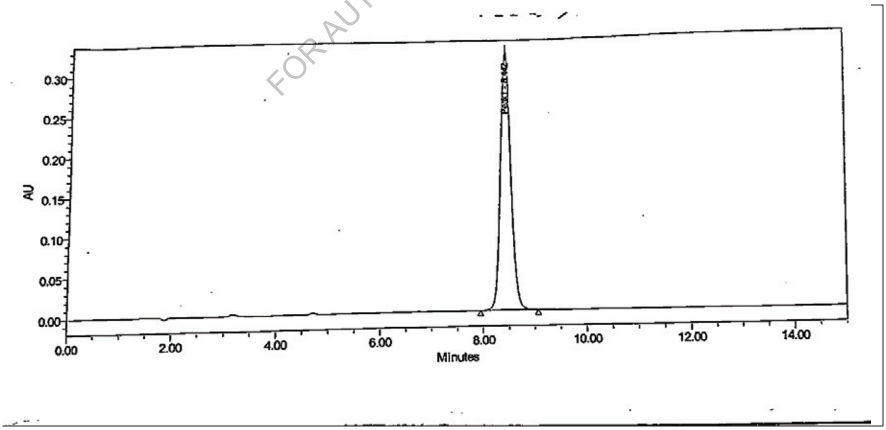

Fig. 22 – Sample Chromatogram of UDCA assay method

FORCED DEGRADATION:

During method development, forced degradation study was done to check the co-elution of any degradants along with drug peak. The study was done in different stressed conditions like acidic (0.1M HCl), basic (0.1N NaOH), Oxidation (3% H2O2), and thermal (was directly exposed to heat at 800C for 48hrs.) and the peak purity of UDCA was monitored. Peak purity of UDCA peak was found passing in all conditions (Table XVIII). The developed chromatographic method was found to be highly specific and stability indicating for quantitative determination of UDCA in Ursodeoxycholic Acid tablets.

Table XVIII – Forced degradation study results of UDCA assay method.

Condition	%Assay	%Degradation	Peak Purity
Normal conditions	98.5	Not applicable	Pass
Acid degradation: 5ml of 0.1N HCl solution, heating at 700C for 2 hours on water bath	97.0	No degradation	Pass
Alkali Degradation: 5ml of 0.1N NaOH solution heating at 700C for 2 hours on water bath	98.5	No degradation	Pass
Peroxide degradation: 2ml 3% H2O2 solution, heating at 600C for 1 hours on water bath	98.1	No degradation	Pass
Thermal Degradation: heating at 800C for 24 hours	98.0	No degradation	Pass

PRECISION:

The intraday precision was evaluated by performing six (n=6) assay determinations on same homogeneous sample of Ursodeoxycholic acid tablets and the %RSD was found to be 0.44%. The %RSD for inter-day precision for two sets (n=12) for their %recovery was found to be 0.46%. The absolute difference between results for intermediate precision was found 0.5% (table XIX).

Table XIX: Precision results of UDCA assay method

Sample No.	% Assay (Precision)	% Assay (Intermediate Precision)
1	98.9	99.9
2	99.3	100.2
3	99.2	100.1
4	99.8	99.5
5	100.1	100.5
6	99.6	99.6
Mean	99.5	100.0
%RSD	0.44	0.38
Overall mean		99.7
Over all %RSD		0.46
Absolute difference of assay results		0.5%

ACCURACY (RECOVERY):

The % recovery at 50%, 100% and 150% was found to be 99.8±0.3, 99.6± 0.45 and 99.8± 0.45% respectively (table 3). The overall mean recovery was found to be 99.7± 0.4%. The recovery results were found within acceptance criteria (table XX). The developed method found to be accurate for determination of UDCA in Ursodeoxycholic Acid tablets.

Table XX: Accuracy results of UDCA assay method

Level	Sample no.	% Recovery	Mean	%RSD
50%	1	99.5	99.8	0.3
	2	99.8		
	3	100.1		
100%	1	99.1	99.6	0.5
	2	100.0		
	3	99.6		
150%	1	99.3	99.8	0.5
	2	99.8		
	3	100.2		
Over all mean			**99.7**	**0.4**

LINEARITY:

Linearity of the response of UDCA drug against concentration at five different level was performed in the range of 50 – 150 µg/ml and response for UDCA found to be linear. The representative regression equation was found to be y=29470.1551x + 42985.8379 with lowest correlation coefficient (r) was found to be 1.0000. The linearity was found with in acceptance criteria.

ROBUSTNESS:

The robustness of the method was verified by making the deliberate changes in the critical chromatographic parameters like;

Organic modifier composition

Pump flow rate

Column oven temperature

The results are presented in table - XXI and found within the acceptance criteria.

Table XXI- Results of Robustness study of UDCA Assay method

HPLC Parameter		**% Results**
Initial Assay Results	As per actual method	98.9
Organic modifier (Acetonitrile) composition in mobile phase (± 2%)	+2% (52%)	99.2
	-2% (48%)	99.5
Pump flow rate ± 0.1 ml/min	1.6 ml/min	98.9
	1.4ml/min	99.0
Column oven temperature ± 20C	430C	99.1
	470C	99.0

SOLUTION STABILITY OF SAMPLE AND STANDARD:

The Ursodeoxycholic Acid sample found to be stable up to 72 hours in diluents at ambient temperature. The results after 72 hours were found to be 99.8% and 99.9% for standard and sample respectively.

APPLICATION OF DEVELOPED METHOD:

The proposed chromatographic method as used for determination of Ursodeoxycholic Acid tablets were found within the specification.

CONCLUSION:

The proposed HPLC method is accurate, linear, precise, stability indicating and rugged methodology for quantitative determination (Assay) of Ursodeoxycholic Acid in Ursodeoxycholic Acid tablets of different strengths. The results of the method validation study show that the method is accurate and linear in the range of 7500-22500 µg/ml of Ursodeoxycholic Acid. The analytical method validation was done as per ICH Q2(R1) and USP guidelines. This method can be used by quality control or research laboratories for quantitative determination of the content (assay) of Ursodeoxycholic Acid tablets of different strength.

CONFERENCE:

This paper is presented in **3rdInternational Conference on Global Trends in Pure and Applied Chemical Sciences on 8th and 9th December 2017 at SRM university, Delhi-NCR Campus, Ghaziabad (U.P.).**

PUBLICATION:

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9. United States of Pharmacopoeia 38 NF 33 (2015). United States Pharmacopeial Convention General chapter <1225>, Validation of Compendial Procedures, pp. 1445-1450.
10. United States of Pharmacopoeia 38 NF 33 (2015), United States Pharmacopeial Convention General chapter <621>, Chromatography, pp. 424-434.

3.3 SECTION C:

METHOD DEVELOPMENT AND VALIDATION FOR DETERMINATION OF DRUG RELEASE (DISSOLUTION) OF URSODEOXYCHOLIC ACID IN URSODEOXYCHOLIC ACID TABLETS

INTRODUCTION:

Ursodeoxycholic acid (UDCA), is a bile acid (fig. 2) and is generally used for the treatment of liver diseases. UDCA is a naturally occurring bile acid and used to dissolve gallstones in the gall bladder [1-4].

It is very important to measure the drug release of UDCA for better quality of products so that the disease can be cured very fast. Hence this research was focused to develop dissolution method and measurement of drug release accurately in dissolution samples. The measurement method is chromatographic method by HPLC. There are few methods published currently for the estimation of UDCA in Ursodeoxycholic acid tablets [5-9]. This method can be used in the quality control or in research laboratories of pharmaceutical companies for assay determination of Ursodeoxycholic acid tablets. The dissolution method along with chromatographic method which is used for determination of drug is validated as per International Conference on Harmonization (ICH) Q2 (R1) and United State of Pharmacopoeia (USP) chapter <1225> and chapter <1092> [10-12]. The method found to be precise, accurate, linear and robust.

EXPERIMENTAL:

MATERIALS:

Potassium hydrogen orthophosphate, Sodium hydroxide, Acetonitrile and HPLC grade water.

METHOD AND INSTRUMENTS:

Dissolution apparatus of Lab India, Waters High Performance Liquid Chromatography (HPLC) system with UV detector.

DISSOLUTION METHOD:

Dissolution Medium: 0.05M Phosphate buffer -pH-7.5 (900 ml)

Apparatus: Paddle (USP II)

Rotation per minute (RPM): 75 RPM

Time: 60 minutes

CHROMATOGRAPHIC PARAMETRS:

HPLC column: Hypersil - C18, 250 X 4.6 mm, 5µm

Pump Flow: 1.0 ml/min

Injection volume: 50.0 µL

Mobile Phase: Mixture of buffer and Acetonitrile in the ratio of 50:50 v/v.

Wavelength: UV detector at 210nm.

The column oven temperature: Ambient.

PREARATION OF SOLUTIONS:

BUFFER SOLUTION FOR MOBILE PHASE:

Dissolved about 6.8 gm of potassium hydrogen orthophosphate in 1000 ml of HPLC grade water and adjusted the pH to 6.5 with dilute sodium hydroxide. Sonicated for degassing and filtered through 0.45 µm membrane filter.

STANDARD PREPARATION:

Weighed accurately about 40mg of UDCA working standard and transferred to 100 ml volumetric flask. The UDCA was dissolved in 5 ml methanol with sonication and intermittent shaking and diluted to volume with dissolution medium.

DISSOLUTION MEDIUM:

Dissolved 68 gm of potassium dihydrogen orthophosphate in 10000 ml of purified water, adjusted pH to 7.5 with dilute sodium hydroxide solution. Degassed the solution with means of sonication.

SAMPLEPREPARATION:

Placed one tablet in each individual jar (six tablets in six individual jars) which was contained 900 ml of dissolution medium maintained at 37.0. The paddle was rotated at speed of 75 rpm. Aliquot was withdrawn after 60 minutes. Filtered through 0.45µ filter and injected in chromatographic system.

PROCEDURE:

Injected blank (dissolution medium), standard solution and sample solution in HPLC system using the newly developed chromatographic method. The concentration of drug release was calculated using sample area against standard area.

METHODVALIDATION

Developed analytical method is validated for parameters as suggested by ICH Q2 (R1) and USP.

SYSTEMSUITABILITY:

System suitability test (SST) is very important in liquid chromatographic method to prove that the system is working accurately and all the chromatographic conditions are fine. They are used to verify the reproducibility of the chromatographic parameters and system is satisfactory for the analysis to be done. The system suitability test was performed in accordance with USP [18].

SPECIFICITY:

Specificity is the ability of the method to measure the analyte response in the presence of other substances or expected to be present. The specificity of the developed LC method was carried out by injecting dissolution medium and placebo.

The diluent (blank), placebo solution was injected in sequence for evaluation of specificity of proposed method. The chromatograms were monitored for any peak eluted at the retention time of drug.

PRECISION:

Precision is a major of the degree of repeatability (intra-day), intermediate precision and reproducibility (Inter-day) of the analytical method under normal operating circumstances.

Precision is usually measured as the coefficient of variation or relative standard deviation of analytical results acquired from independently prepared samples (six tablets in case of dissolution).

Method precision was evaluated by performing the dissolution using proposed method (dissolution parameters and chromatographic method) on six tablets of UDCA tablets and calculated % release of UDCA in each sample. The %RSD for set of six tablets was calculated.

The intermediate precision of the method was also evaluated using different analyst and a different instrument in the same laboratory by carrying out dissolution on six more tablets using proposed method and calculated % release of UDCA in each sample. Calculated the %RSD for 12 results. The acceptance criteria for %RSD was not more than 5% and the absolute difference between results by two analysts was not more than 5%.

RECOVERY (ACCURACY):

Accuracy is the closeness of agreement between test results and the accepted reference value. The accuracy of the method was performed by recovery studies.

In order to evaluate the accuracy of the proposed method, recovery test was performed by adding known amount of standard solution to the placebo formulation sample, followed by analysis using the proposed dissolution parameters and chromatographic method. The recovery studies were done at three different levels at 50%, 100% and 150% with three determinations of working level concentration using standard spiking method in placebo.

All the above solutions were prepared in duplicate, the sample aliquots were withdrawn after 60 minutes and were analyzed using proposed chromatographic condition. The recovery at each level was calculated by using the theoretical value from exact weight taken for spiking. The % recovery was calculated with respect to amount added. The acceptance criteria for % recovery was – the recovery at each level should be in the range of 95% to 105% and overall %RSD of nine results should be less than 5%.

LINEARITY:

The linearity of an analytical method is its ability to elicit test results that are directly or by means of well-defined mathematical transformations, proportional to the concentration of analytes in the samples within a given range.

The linearity plot was constructed for UDCA in the concentration range of 50% to 150% of the sample concentration (390 µg/ml). The primary stock solution of UDCA working standard was prepared. From the primary stock solution, appropriate dilutions were made to get concentration of 190, 290, 390, 490 and 590 µg/ml. The calibration curve was plotted as concentration of the respective drug solutions verses the peak area at each level. The results were statistically evaluated and correlation coefficient determination, slope and y-intercept values were calculated.

ROBUSTNESS:

Robustness is a capacity of a method to remain unaffected by small deliberate changes in method parameters.

For robustness study, the standard concentration 550 µg/ml of UDCA was used. Following chromatographic parameters were considered for the robustness study.

- Effect of acetonitrile in mobile phase composition (±2%)
- Effect of flow rate (±0.1ml/min)
- Effect of wavelength (±2nm)

The robustness of dissolution method was also evaluated by making deliberate changes in the dissolution parameters and analysis was performed using the proposed chromatographic method. The below dissolution parameters were changed during this study;

- Change in pH of dissolution medium ± 0.1
- Change in RPM ± 3 RPM

SOLUTION STABILITY OF SAMPLE AND STANDARD IN DISSOLUTION MEDIUM:

The solution stability of sample and standard solution in dissolution medium was performed to understand the stability which will be helpful for sample handling during routine analysis in laboratory by using the proposed method.

Solution stability was carried out for sample solution at 2-8 and ambient temperature for 24 hr. The sample and standard solution were injected immediately after preparation in the HPLC system considering it as an initial (0 hr.) as baseline.

RESULTS AND DISCUSSION

METHOD DEVELOPMENT:

Literature survey revealed that UDCA is having molecular weight 392.58 g/mol. The UDCA solution of 400 µg/ml concentration prepared in dissolution medium was scanned on PDA and the spectra of UDCA was recorded. It was observed that UDCA doesn’t have good absorption after 210nm. Hence to detect the drug adequately the wavelength of 210nm was selected as detection wavelength.

The blank (dissolution medium), placebo solution and sample solution was injected in HPLC system by using stationary phase C18 to check the interference due to diluent or placebo in main drug. The column of make Hypersil (C18, 250 X 4.6 mm, 5µ) was found suitable.

To achieve the optimum run time and capacity factor different compositions of organic modifier with buffer were tried at different pH buffer. The elution pattern of the UDCA was studied.

METHOD VALIDATION:

SPECIFICITY:

The chromatograms during specificity study (fig. 23, 24 and 25) of diluent, placebo and sample solution shows that there is no co elution of any peak with UDCA due to blank and placebo.

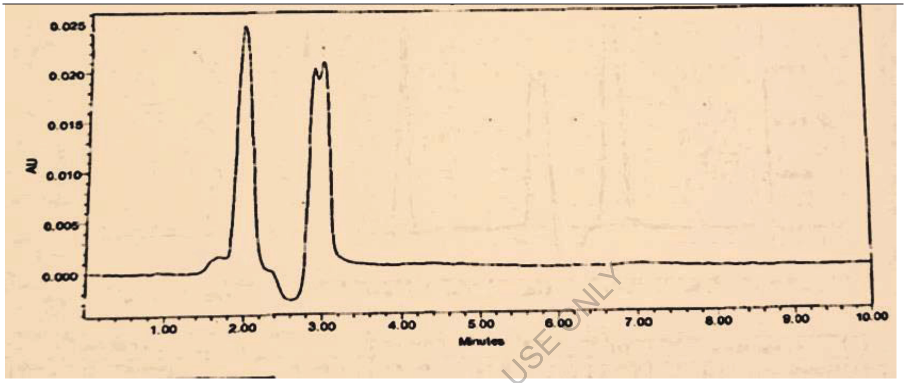

Fig. 23 – Blank Chromatogram of UDCA tablets dissolution method

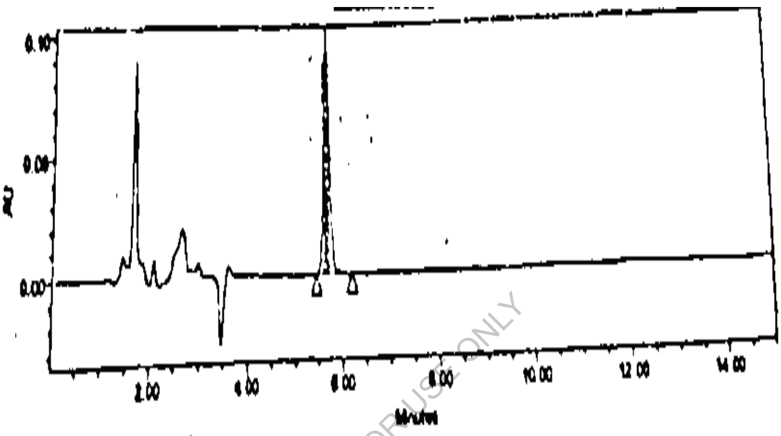

Fig. 24- Standard Chromatogram of UDCA tablets dissolution method

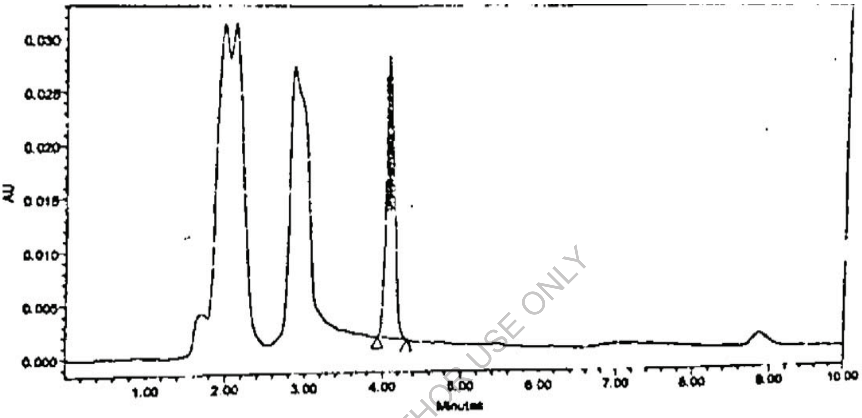

Fig. 25- Standard Chromatogram of UDCA tablets dissolution method

PRECISION:

SYSTEM PRECISION:

The system precision was evaluated by injecting six standard solutions in HPLC by using the proposed chromatographic conditions, and the % RSD of Area and retention time was calculated. The %RSD of area was found 0.85%, and %RSD of retention time was found 0.43%.

METHOD PRECISION:

Method precision was divided in two parts as below;

Intraday precision: Intraday precision was evaluated by performing dissolution of Ursodeoxycholic acid 300 mg tablets on six tablets (n=6) using the proposed dissolution and chromatographic parameters, % drug release was calculated. The %RSD for six results (of six tablets) was calculated and found 1.6%.

INTER-DAY PRECISION (RUGGEDNESS):

The ruggedness of the method was evaluated by performing dissolution of Ursodeoxycholic acid 300 mg tablets on six tablets (n=6) using the proposed dissolution and chromatographic parameters on different day and on different instrument. The % drug release was calculated and %RSD of six results was found 1.5%. The inter-day precision for two sets (n=12) for their %recovery was found to be 1.5%. The absolute difference between results for intermediate precision was found 2% (table XXII).

Table XXII: Method Precision results of UDCA tablets dissolution method

Sample No.	% Dissolution (Precision)	% Dissolution (Intermediate Precision)
1	98	95
2	96	96
3	99	99
4	98	95
5	100	96

6	96	96
Mean	98	96
%RSD	1.6	1.5
Overall mean		97
Over all %RSD		1.5
Absolute difference of assay results		2%

ACCURACY (RECOVERY):

The % recovery at 50%, 100% and 150% was found to be 99.4±0.38, 99.6± 0.36 and 99.8± 0.35% respectively (table XXIII). The overall mean recovery was found to be 99.6± 0.38%. The recovery results were found within acceptance criteria (table XXIII). The developed method found to be accurate for determination of drug release of UDCA in dissolution samples of Ursodeoxycholic Acid tablets.

Table XXIII: Accuracy results of UDCA tablets dissolution method

Level	Sample no.	% Recovery	Mean	%RSD
50%	1	99.2	99.4	0.38
	2	99.8		
	3	99.1		
100%	1	99.5	99.6	0.36
	2	100.0		
	3	99.3		
150%	1	99.5	99.8	0.35
	2	99.8		
	3	100.2		
Over all mean and % RSD			99.6	0.38

LINEARITY:

Linearity was evaluated at five different (50% to 150%) level considering the working concentration level as 100%. The area response of UDCA drug against concentration at five different level was performed in the range of 50% – 150% and response for UDCA found to be linear. The correlation coefficient (r) was found to be 0.999. The linearity was found with in acceptance criteria.

ROBUSTNESS:

The robustness of the method was verified by making the deliberate changes in the critical chromatographic parameters and the system suitability was checked. Below chromatographic parameters (one parameter at a time) were changed.

- Organic modifier composition
- Pump flow rate
- Column oven temperature

The results are presented in table - XXIV and found within the acceptance criteria.

Table - XXIV Robustness Results for chromatographic parameter of UDCA tablets dissolution method

HPLC Parameter		% RSD of standard
Initial	As per actual method	0.62
Acetonitrile composition in mobile phase (± 2%)	+2% (52%)	0.45
	-2% (48%)	0.52
Pump flow rate ± 0.1 ml/min	1.1 ml/min	0.44
	0.9 ml/min	0.21
Wavelength ± 2nm	208 nm	0.15
	212 nm	0.24

The robustness of the dissolution method was evaluated by making the changes in proposed dissolution parameters.

The results are presented in table - XXV and found within the acceptance criteria.

Table XXV – Robustness results for dissolution parameter of UDCA tablets dissolution method

Dissolution Parameter		% Average Drug Release	%RSD of six tablets
Initial	As per actual method	95	2.8
pH of Dissolution Medium (± 0.1)	+0.1 (7.6)	97	3.2
	-0.1 (7.4)	92	2.9
Rotation of paddle per minute ± 0.1 ml/min	+3 (78 RPM)	93	1.8
	- 3 (72 RPM)	95	3.0

SOLUTION STABILITY OF SAMPLE AND STANDARD:

The Ursodeoxycholic Acid sample found to be stable up to 24 hours in dissolution medium at ambient temperature. The average % results after 24 hours were found to be 95%.

APPLICATION OF DEVELOPED METHOD:

The proposed dissolution method with chromatographic method was used for measurement of % drug release of Ursodeoxycholic Acid tablets 500mg, the results were found within the specification. The proposed method can be used in laboratory for dissolution of Ursodeoxycholic Acid tablets.

CONCLUSION:

The proposed HPLC method is accurate, linear, precise and rugged methodology for quantitative determination (Assay) of Ursodeoxycholic Acid in Ursodeoxycholic Acid tablets of different strengths. The results of the method validation study show that the method is accurate and linear in the concentration range of 50%-150 % of Ursodeoxycholic Acid. The analytical method validation was done as per ICH Q2(R1) and USP guidelines. This method can be used by quality control or research laboratories for quantitative determination of the content of Ursodeoxycholic Acid in dissolution sample of UDCA tablets of different strength.

PUBLICATION:

This research paper is in UGC approved international journal "Journal of Drug Delivery and Therapeutics" (JDDT), (ISSN 2250-1177) Dec; 2017.

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12. United States of Pharmacopoeia 38 NF 33 (2015), United States Pharmacopeial Convention General chapter <621>, Chromatography, pp. 424-434.

CHAPTER IV

4.0 SUMMARY AND CONCLUSION

SUMMARY :

4.1 SECTION A: ALZHEIMER’S DISEASE (MEMANTINE HCl)

Alzheimer’s disease (AD) is the most common type of dementia. Memantine HCl is one of the common drug used in the treatment of Alzheimer’s disease. Memantine HCl lacks the chromophores. Due to this limitation it was difficult to develop specific and accurate chromatographic analytical method for its quantification in drug formulations. All the currently published methods require derivatization of Memantine HCl which is tedious, time consuming and hence can lacks the accuracy. Hence this research was focussed to develop simple, specific and accurate methods to determine the drug quantity in its dissolution samples and to determine its assay in drug formulations.

Two chromatographic analytical methods, one for quantification of memantine hydrochloride in dissolution samples and second for determination of quantity of memantine hydrochloride in its formulations are developed using refractive index (RI) detector. Memantine HCl lacks the chromophores, hence conventional UV/PDA detectors can’t be used in detection of samples. To use conventional detectors, derivatization of Memantine HCl is required which is time consuming and tedious. Lot of manpower and chemicals also required for derivatization. Hence current methods are developed by using refractive index detector. Simplicity of these methods is no derivatization is required for detection of Memantine HCl. Both methods are validated as per ICH guidelines and the results shown that the methods are specific, accurate, precise and robust.

SUMMARY OF DISSOLUTION METHOD:

The present study was aimed to develop an analytical method for quantification of memantine hydrochloride (MEM) in dissolution samples using high performance liquid chromatography with refractive index (RI) detector. The chromatographic separation was achieved on C18 (250 × 4.5 mm, 5µ) column using isocratic mobile phase comprises of buffer (pH-5.2): methanol (40:60 v/v) pumped at a flow rate of 1.0 mL/min. The column effluents were monitored using RI detector. The retention time of MEM was found to be about 6.5±0.3 min. The developed chromatographic method was validated and found to be linear over the concentration range of 5.0 - 45.0 µg/mL for MEM. Mean recovery of MEM was found to be 99.2±0.5%w/w. The method was found to be simple, fast, precise and accurate which can be utilized for the quantification of MEM in dissolution samples.

SUMMARY OF ASSAY METHOD:

The chromatographic separation was achieved on C18 (250 × 4.5 mm, 5µ) column using isocratic mobile phase comprises of buffer (pH-6.0): Methanol (45:55 v/v) pumped at a flow rate of 1.0 ml/min. The detection of effluent was monitored using RI detector.
The retention time of MEM was found about 6.9±0.2 minutes. The method was validated and found to be accurate, precise and linear over the range of 50-150µg/ml. The mean recovery of MEM in this range was found 100.0±0.2%.

These methods can be used by common laboratories for analysis of Memantine HCl drug formulations. These methods will save time, chemicals and reagents of the laboratories, hence very economical. It will reduce the pollution of environment due to use of less chemicals and reagents.

4.2 SECTION B: DIPRESSION DISEASE

(NORTRIPTYLINE HCl)

Nortriptyline hydrochloride, the N-demethylated active metabolite of amitriptyline, is a dibenzocycloheptene-derivative. Nortriptyline hydrochloride is a tricyclic antidepressant agent used for short-term treatment of various forms of depression.

The Development and Validation of Stability Indicating Analytical Method for Determination of Nortriptyline In Nortriptyline HCl Tablets by Liquid Chromatography

Summary: A rapid, rugged, precise and an accurate stability indicating chromatographic analytical method is developed and validated for determination of Nortriptyline HCl in Nortriptyline tablets. The separation of impurities and Nortriptyline HCl drug was achieved by an isocratic liquid chromatographic method using Inertsil, C18, 250mm x 4.6mm, 5µm column at 45^0C. The mobile phase consists of 70% Methanol and 30% phosphate buffer of pH-7.5 pumped at a flow rate of 1.0 ml/min. The detection was carried out at a wavelength 220 nm. The retention time of Nortriptyline HCl is about 3.8 minutes.

The developed chromatographic method was validated and found to be linear over the concentration range of 50 - 150.0 µg/ml. Mean recovery of Nortriptyline HCl was found to be 100.1±0.1%w/w.

All the validation parameters are meeting the acceptance criteria.

Developed analytical method is stability indicating, simple, accurate. It will save time of analysis and hence it is more economical than the published methods.

4.3 SECTION C: LIVER DISEASE (URSODEOXYCHOLIC ACID)

Ursodeoxycholic acid (UDCA), is generally used in the treatment of liver diseases. UDCA is a naturally occurring bile acid with multiple hepato protective activities, improves liver condition in patients with a wide range of chronic liver diseases. Generally during analysis of UDCA tablets, assay, dissolution and related substances tests are performed to determine the drug quantity and impurities in its formulations. Hence simple, precise, accurate, specific, robust and stability indicating reverse phase chromatographic methods for these tests are developed individually.

Determination of Ursodeoxycholic acid in dissolution samples of Ursodeoxycholic acid tablets:

Summary: An HPLC assay method is developed for quantitative determination of Ursodeoxycholic Acid in Ursodeoxycholic Acid tablets. The developed chromatographic is a simple, accurate, rugged, precise and stability indicating chromatographic method.

The developed chromatographic method is validated. The method is accurate, precise and linear over the concentration range of 50 - 150.0 µg/ml. Mean recovery of Ursodeoxycholic Acid was found to be 99.7±0.4%w/w.

The method is simple and stability indicating and can be used in common laboratories for the determination of assay of Ursodeoxycholic Acid in Ursodeoxycholic Acid tablets.

Determination of Ursodeoxycholic acid drug quantity (assay) in Ursodeoxycholic acid tablets:

Summary : An HPLC assay method is developed for quantitative determination of Ursodeoxycholic Acid in Ursodeoxycholic Acid tablets. The developed method is a simple, accurate, rugged, precise and stability indicating chromatographic method. The stationary phase used in separation of known impurities and Ursodeoxycholic Acid drug was C18, 250mm

X4.6mm, 5µm. The mobile phase was prepared by mixing of buffer and acetonitrile in the ratio of 50:50v/v pumped at a flow rate of 1.5ml/minute.

The developed chromatographic method is validated. The method is accurate, precise and linear over the concentration range of 50 – 150%. Mean recovery of Ursodeoxycholic Acid tablets is found 99.7 ± 0.4.

The method is simple and stability indicating and hence can be used by common laboratories for the determination of Ursodeoxycholic Acid (assay) in Ursodeoxycholic Acid tablets.

Determination of related substances (impurity) of Ursodeoxycholic acid in Ursodeoxycholic acid tablets:

Summary : To develop rapid, rugged, precise and an accurate stability indicating analytical method forestimation of related substances in Ursodeoxycholic Acid tablets. The separation of impurities and Ursodeoxycholic Acid drug is achieved by an isocratic chromatographic method on C18, 250 mmx 4.6 mm, 5µm column. The mobile phase consists of buffer, acetonitrile and methanol in the ratio of 35:28:37 v/v/v pumped at a flow rate of 1.0 ml/minute 35:37:28v/v/v pumped at a flow rate of1.0 ml/minute. The detection was carried out by using refractive index (RI) detector. The proposed chromatographic method was validated and found to be linear over the concentration range from LOQ to 150.0% of impurity limit level. Overall mean recovery of Chenodeoxycholic acidimpurity was found to be 100.6±4.0%w/w. The method was found to be simple, stability indicating, precise, accurate and robust which can be utilized for estimation of related substances in Ursodeoxycholic Acid tablets.

4.4 SECTION D: CONCLUSION

The chromatographic technique like HPLC is a more accurate technique to determine the drug quantities, percentage of drug release and determination of impurities/related substances and or degradation products. Delivering the high quality standard drug formulations to the patient will become easier with the help of simple, accurate and robust analytical technique. This will also meet the regulatory expectations. Hence during this research work an attempt is being made to develop few new chromatographic methods which will certainly help the pharmaceutical industry.

Six different methods are developed and validated for analysis of Memantine HCl tablets (Assay, Dissolution), Nortriptyline tablets (Related substances) and Ursodeoxycholic acid tablets (Assay, Dissolution and Impurities). The outcome of method validation proved that the developed methods are accurate, precise, stability indicating and robust.

These methods are published in international publications. Applications of these methods proved that methods can be used as such for analysis of drug formulations.

This research will help to pharmaceutical industry to improve drug quality and to meet compliance of regulatory guidelines. This work will also help to reduce economic burden on industry.

LIST OF PUBLICATIONS:

1) ***Tukaram B Sawant, Vikas S Wakchaure, Udaykumar K Rakibe, Prashant B Musmade, Bhata R Chaudhari, Dhananjay V Mane. The development and validation of novel, simple high-performance liquid chromatographic method with refractive index detector for quantification of memantine hydrochloride in dissolution samples.Journal of chromatographic sciences" (JCS), Volume 55, Issue 6, 1 July 2017, Pages 603–609 – An Oxford University Journal (USA).***

2) ***Tukaram B Sawant, Dhananjay V Mane.To Develop HPLC Method for The Assay of Memantine Hydrochloride Tablets Using Refractive Index (RI) Detector.Indo American Journal of Pharmaceutical Sciences (IAJPS) ISSN 2349-7750, 4(11), Nov; 2017, Pages 4391–4397.***

3) ***Sawant TB, Mane D. V. The Development and Validation of Stability Indicating Analytical Method for Determination of Nortriptyline in Nortriptyline HCl Tablets by Liquid Chromatography. 2017; 51(4S): S761-S8.***

4) ***Sawant TB, Mane D. V. Method Development and Validation for Determination of Drug Release (Dissolution) of Ursodeoxycholic Acid in Ursodeoxycholic Acid Tablets.Journal of Drug Delivery and Therapeutics (JDDT), (ISSN 2250-1177).***

5) ***Sawant Tukaram B., Wakchaure Vikas S., Mane Dhananjay V. The Development and Validation of an Analytical Methodfor Determination of Related Substances in Ursodeoxycholic Acid Tablets by Liquid Chromatography.American journal of Pharma Tech Research (AJPTR), 7(4), August 2017 Edition (ISSN 2249-3387).***

6) ***Sawant TB, Mane D. V. Chromatographic Method Development and Validation for Quantitative Determination (Assay) Of Ursodeoxycholic Acid in Ursodeoxycholic Acid Tablets. Asian journal of Chemistry (AJC), ISSN 0970-7077.***


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