METHOD DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF OMEPRAZOLE AND CINITAPRIDE IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC

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In partial Fulfillment for the award of degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

Submitted by

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NOVEMBER 2012

CERTIFICATE

This is to certify that the dissertation work entitled **METHOD DEVELOPMENT AND VALIDATION OF SIMULTANEOUS ESTIMATION OF OMEPRAZOLE AND CINITAPRIDE IN PHARMACEUTICAL DOSAGE FORMS BY RP-HPLC** is a bonafied work of **Ms.M.Deepthi** carried out in RAINBOW PHARMA TRAINING LAB, HYDERDAD under my guidance and under supervision of Mr. T. SRINIVAS REDDY,Director ,Rainbow Pharma Training lab and has completed to my fullest satisfaction for partial fulfillment of the award of degree of **Master of Pharmacy in Pharmaceutical Analysis**, RVS college of Pharmaceutical Sciences, Sulur, Coimbatore, which is affiliated to The Tamilnadu Dr. M.G.R Medical University, Chennai. It is to certify that the part or whole of the work has not been submitted either to this university or any other university. This work is original and confidential.

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INTERNAL EXAMINER

Place:

Date:

EXTERNAL EXAMINER

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. ***M.Deepthi***

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LIST OF CHROMATOGRAMS

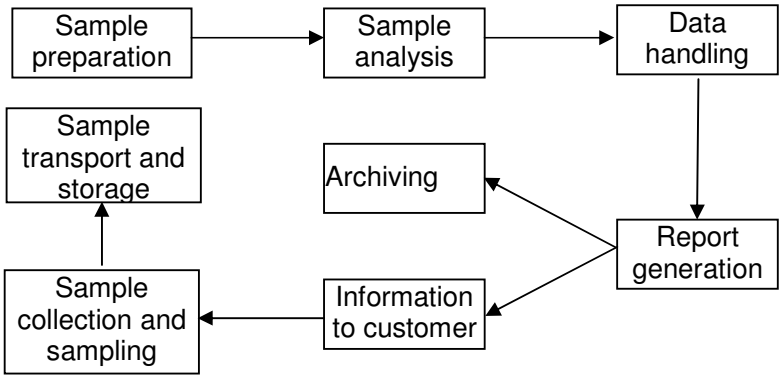
INTRODUCTION

Analytical chemistry is the science that seeks ever improved means of measuring the chemical composition of natural and artificial materials. Chemical composition is the entire picture (composition) of the material at the chemical scale and includes geometric features such as molecular morphologies and distributions of species within a sample as well as single dimensional features such as percent composition and species identity.[1]

To be effective and efficient, analyzing samples requires expertise in

1. The chemistry that can occur in a sample.
2. Analysis and sample handling methods for a wide variety of problems (the tools-of-the-trade).
3. Accuracy and precision of the method.
4. Proper data analysis and record keeping.

The major stages of an analytical process are described as follows:



Steps in analytical cycle

The pharmaceutical analysis comprises the procedures necessary to determine the "identity, strength, quality and purity" of such compounds. It also includes the analysis of raw material and intermediates during manufacturing process of drugs.

Types

Qualitative analysis

- Qualitative inorganic analysis seeks to establish the presence of a given element or inorganic compound in a sample.
- Qualitative organic analysis seeks to establish the presence of a given functional group or organic compound in a sample.

Quantitative analysis[2]

- Quantitative analysis seeks to establish the amount of a given element or compound in a sample.

Methods of detecting analytes

1. Physical means
   - Mass
   - Color
   - Refractive index
   - Thermal conductivity
2. With electromagnetic radiation (Spectroscopy)
   - Absorption
   - Emission
   - Scattering
3. By an electric charge
   - Electrochemistry
   - Mass spectrometry

Traditional analytical techniques

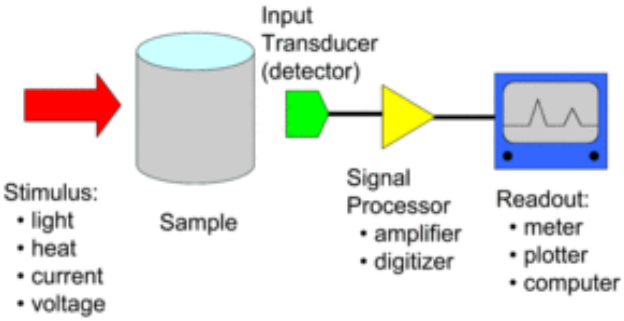
Titration

Titration involves the addition of a reactant to a solution being analyzed until some equivalence point is reached. Often the amount of material in the solution being analyzed may be determined.

Gravimetry[3]

Gravimetric analysis involves determining the amount of material present by weighing the sample before and/or after some transformation. A common example used in the determination of the amount of water in a hydrate by heating the sample to remove the water such that the difference in weight is due to the water lost.

Instrumental Analysis[4]



Block diagram of an analytical instrument showing the stimulus and measurement of response

1. **Separation Techniques**

Separation processes are used to decrease the complexity of material mixtures. Chromatography and electrophoresis is representative of this field.

Chromatographic methods

Chromatography is a technique by which the components in a sample, carried by the liquid or gaseous phase, are resolved by sorption-desorption steps on the stationary phase.

There are various advanced chromatographic techniques, which are most reliable and widely used for the estimation of multicomponent drugs in their formulation namely,

a) Gas chromatography (GC)

b) High Performance Thin Layer Chromatography (HPTLC)

c) High Performance Liquid Chromatography (HPLC)

High performance liquid chromatographic separation is based on interaction and differential partition of the sample between the mobile phase and stationary phase. The commonly used chromatographic methods can be roughly divided into the following groups,

I. Chiral
II. Ion-exchange
III. Ion pair/affinity
IV. Normal phase
V. Reverse phase
VI. Size exclusion

When compared to classical column chromatography, this technique is preferred because of its improved performance in terms of rapidity, specificity, sensitivity, accuracy, convenience, ease of automation and the cost of analysis.

Advance in column technology, high pressure pumping system and sensitive detectors have transformed liquid column chromatography into a high speed, efficient, accurate and highly resolved method of separation.

2. Hybrid Techniques

Combination of the above techniques produces 'hybrid' or 'hyphenated' techniques. Several examples are in popular use today and new hybrid techniques are under development. For example

a) GC-MS
b) LC-MS
c) HPLC/ ESI-MS
d) LC-DAD
e) CE-MS
f) CE-UV

3. Microscopy

The visualization of single molecules, single cells, biological tissues and nano-micro materials is very important and attractive approach in analytical science. Also, hybridization with other traditional analytical tools is revolutionizing analytical science.

Microscopy can be categorized into three different fields: optical microscopy, electron microscopy, and scanning probe microscopy. Recently, this field is rapidly progressing because of the rapid development of computer and camera industries.

4. Lab-on-a-chip

Miniaturized analytical instrumentation, which is also called as micro fluidics or micro total analysis system (μTAS). The beauty of lab-on-a-chip system is that a whole device can be visualized under a microscope.

Method of data analysis[6]

a. Standard Curve

A standard method for analysis of concentration involves the creation of a calibration curve. This allows for determination of the amount of a chemical in a material by comparing the results of unknown sample to those of a series known standards.

5. Internal Standard

Sometimes an internal standard is added at a known concentration directly to an analytical sample to aid in quantitation. The amount of analyte present is then determined relative to the internal standard as a calibrant.

Quality assurance plays a central role in determining the safety and efficacy of medicines. Highly specific and sensitive analytical technique holds the key to design, development, standardization and quality control of medicinal products.

The efficacy and safety of a medicinal product can be assured by analytical monitoring of its quality. It is important that analytical procedure proposed of a particular active ingredient or its dosage form should be systematically sound under the condition in which it is to be applied.

New Drug Discovery

New drugs have been discovered from two major sources

- Synthetic chemicals
- Natural products including plants, animal and microbes.

The number of drugs introduced into the market has been increasing at an alarming rate. Newer analytical methods are developed for these drugs or drug combinations because of the following reasons

i) The drug or combination may not be official in any pharmacopoeia.

ii) A literature search may not reveal an analytical procedure for the drug or its combination.

iii) Analytical methods may not be available for the drug combination due to the interference caused by excipients.

iv) Analytical methods for the quantification of drug or drug combination with other drugs may not be available.

On the other hand, the existing procedure may

- Require expensive instruments, reagents, solvents etc.
- Involve any tedious extraction or separation steps which may be quite time consuming.
- Not be rapid, reliable or sensitive.
- The newly developed analytical methods find their importance in various fields such as
- Research institutions
- Quality control department in industries
- Approved testing laboratories
- Bio-pharmaceutical and bio-equivalence studies
- Clinical pharmacokinetic studies

3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography is a very sensitive analytical technique most widely used for quantitative and qualitative analysis of pharmaceuticals. The principle advantage of HPLC compared to classical column chromatography is improved resolution of the separated substance, faster separation times and the increased accuracy, precision and sensitivity[8].

Principle of Separation and its type

There are four types of chromatography in which the mobile phase is a liquid. The mobile phase is pumped through the packed column, under high pressure.

a. Partition chromatography
    i. Normal phase chromatography
    ii. Reverse phase chromatography
b. Adsorption or liquid solid chromatography
c. Ion exchange chromatography
d. Size exclusion or gel permeation chromatography

NORMAL PHASE CHROMATOGRAPHY

In normal phase mode, the stationary phase (e.g. silica gel) is polar in nature and the mobile phase is non- polar in this technique, non-polar compounds travel faster and are eluted first. This is because less affinity between solute and stationary phase. Polar compounds are retained for longer time in the column because more affinity towards stationary phase and takes more time to be eluted from the column. This is not advantageous in pharmaceutical applications since most of the drug molecules polar in nature and takes longer time to be eluted and detected. Hence this technique is not widely used in pharmacy.

SOLUTE POLARITY:-

Adsorption strengths increased with increasing solute polarity and this is favorable interaction between the polar solutes and the polar stationary phase increases the elution time (note:-the interaction strength not only depends on the functional groups in the enlight molecule, but also stearic factors).

Reverse phase chromatography

In RP-HPLC the stationary phase is non-polar often a hydrocarbon and the mobile phase is relatively polar such as water, methanol or Acetonitrile. In RPC the solutes are eluted in the order of their decreasing polarities. These are prepared by treating the

surface of silanol group with an organochlorosilane reagent.

Reverse phase mode

Non-polar stationary phase and polar mobile phase is used here.

Mechanism

Retention by interaction of the stationary phase non-polar hydrocarbon chain with non-polar parts and sample molecules.

ADSORPTION OR LIQUID SOLID CHROMATOGRAPHY

The principle of separation is adsorption, separation of components takes place because of difference in affinity of compounds towards stationary phase. This principle is seen in normal phase as well as reverse phase mode, where adsorption will takes place .

ION-EXCHANGE CHROMATOGRAPHY

In ion exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to stationary phase. Ions of the same charge are excluded. Some types of ion Exchangers include :(1) polystyrene resins-allows cross linkage which increases the stability of the chain. Higher cross linkage reduces swerving, which increases the equilibration time and ultimately improves selectivity. (2)Cellulose and dextran ion-exchangers (gels) these possess larger pore sizes and low charge densities making them suitable for protein separation. (3) controlled-pore glass or porous silica

In general, ion-exchangers favor the binding of ions of higher charge and smaller radius. An increase in counter ion (with respect to the functional groups in resins) concentration reduces the retention time. An increase in ph reduces the retention time in cation exchange while a decrease in ph reduces the retention time in anion exchange

SIZE EXCLUSION CHROMATOGRAPHY

Size exclusion chromatography (SEC) also known as gel permeation chromatography or gel filtration chromatography, separates particles on the basics of size. It is generally a low resolution chromatography and thus it is often reserved for the final, "polishing" step of purification. It is also useful for determining the tertiary structure and quaternary structure of purified proteins, and is the primary technique for determining the average molecular weight of natural and synthetic polymers

METHODS OF QUANTITATIVE ANALYSIS:

The sample or solute is analyzed quantitatively in HPLC by either peak height or peak area measurements. Peak areas are proportional to the amount of the material eluting from the column as long as the solvent flows at constant rate. Peak heights are proportional to the amount of the material only when peak widths are constant and are strongly affected by the sample injection techniques. They are five principles evaluation methods for quantifying the solute[10]

(a)Calibaration by Standards:

Calibration curves for each component are prepared from pure standards, using identical injection volumes of operating conditions for standards and samples. The concentration of solute is read from its curve if the curve is linear

$$X=K \times Area$$

Where, x=concentration.

K=proportionality constant (slope of the curve).

In this evaluation method, only the area of the peaks of interest is measured. Relative response factors must be considered when converting areas to volume and when the response of the given detector differs for each molecular type of compounds.

(b)Internal Standard Method:

In this technique, a non quantity of internal standard is chromatographed and area Vs concentration is ascertained. Then a quantity of internal standard is added to the raw sample prior to any sample pretreatment or separation operations. The peak area of the standard in the sample run is compared with the peak are when the standard is run separately. This ratio serves as correction factor for variation in sample size, for losses in any preliminary pretreatment operations, or for incomplete elution of the sample. The material selected for the internal standard must be completely resolved from adjacent sample components and should not interfere with the sample components and never be present in samples

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

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

(c)Area normalization

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

(d)Standard addition method

If only few samples are to be chromatographed, it is possible to employ the method of standard edition (s).the chromatogram of the unknown is recorded, then a known amount of analyte (s) is added and the chromatogram is repeated using same

reagents, instruments and other conditions. From the increase in the peak area (or peak height), the original concentration can be computed by interpolation.

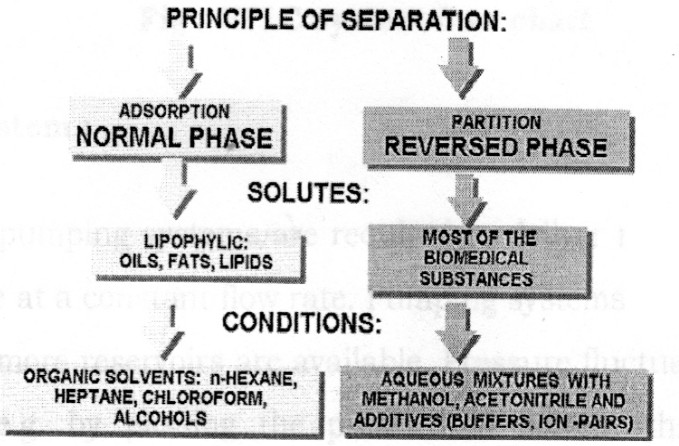
If an instrumental reading(area/height) 'Rx' is obtained, from a sample of unknown 'x' and a reading 'Rt' is obtained from the sample to which a known concentration 'a' of analyte has been added, then 'x' can be calculated from

$$\frac{x}{x+a} = \frac{Rx}{Rt}$$

A correction for dilution must be made if the amount of standard added, changes the total sample volume significantly. It is always advisable to check the result by adding at least one other standard.

Principle of separation

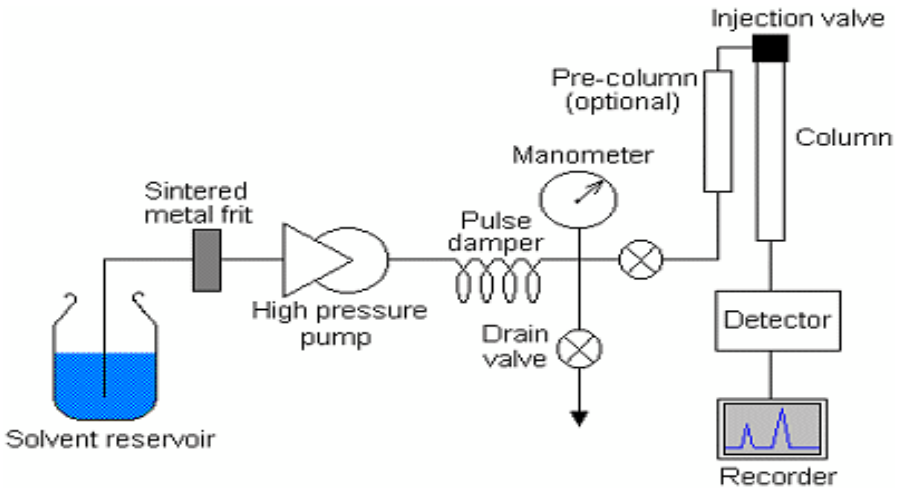
HPLC is based on the mechanism of adsorption, partition, ion exchange or size exclusion, depending on the type of stationary phase used. HPLC involves a solid stationary phase, normally packed inside a stainless- steel column, and a liquid mobile phase. Separation of the components of a solution results from difference in the relative distribution ratios of the solutes between the two phases



The majority of the HPLC separation are done with Reversed phase separation, probably over 90%.In reversed phase separation organic molecules are separated based on the degree of hydrophobicity. There is a correlation between the degree of lipophylicity

and retention in the column. This is the list of mobile phase parameters effecting retention and separation in reversed phase .Elution order in normal phase HPLC shows that the polar solutes elute later then non polar lypophilic ones.

HPLC EQUIPMENT



Schematic diagram of HPLC

The Various parts of HPLC equipment are[1]

1. Reservoir - that holds the mobile phase.
2. Pumps - A & B to pump the mobile phase.
3. Sample injection - to inject the sample.
4. Columns - that contains the stationary phase.
5. Detectors - to detect the results.
6. Recorder – to display the results.

The operation has specific procedure and it depends on various factors.

They can be described as follows:

1. **Reservoirs:**

   The solvent reservoirs are glass or stainless steel containers capable of holding uto one liter of mobile phase. The reservoir has special caps, Teflon tubing and filters.

2. **Pumps:**

   Pumps are used to pump the solvent into the column. Most of HPLC work is done under pressure between about 400 to 1500 psi.

There are two types of pumps

i) Mechanical Pumps – These pumps deliver the mobile phase at a constant flow rate. The mechanical pumps are commonly **Reciprocating Piston** types. It consists of a small motor driven piston, which moves rapidly back and forth in a hydraulic chamber that may vary from 35-400µl in volume. On the backstroke, the separation column valve is closed, and the piston pulls in solvent from the mobile phase reservoir. On the forward stroke, the pump pushes solvent out to the column from the reservoir. This type of pump system is significantly smoother because one pump is filling while the other is in the delivery cycle.

ii) Pneumatic Pumps – These pumps deliver the mobile phase at a constant pressure. The pneumatic pumps are commonly **Gas Displacements** type. It uses direct pressure from the highly compressed gas to force solvent out of tube to deliver the liquid at constant pressure. The amplification of the original gas pressure is proportional to the ratio of two ends of the piston. The pneumatic pumps having the advantage of pulse-less operation[10-11].

3. Sample Injection: -

The sample injection device is available in which sample solutions are injected through a self sealing rubber or Teflon disc using micro liter syringe. Samples are injected into the HPLC via an injection port. The injection port of an HPLC commonly consists of an injection valve and the sample loop. The sample is

typically dissolved in the suitable solvent before injection into the sample loop. The sample is then drawn into a syringe and injected into the loop via the injection valve then it injects the sample into the stream of the mobile phase. Loop volumes can range between 10µl to 1000µl.

For **syringe type and reciprocation pumps**, flow in the column can be brought to zero and rapidly resumed by diverting the mobile phase by means of a three way valve placed in front of the injector. This method can be used up to very high pressures.[10-11]

4. Analytical Columns: -

The analytical columns are heart of HPLC system which decide efficiency of separation, in which actual separation are take place. There are various columns that are secondary to the separating column or stationary phase.

I) Guard columns

They are placed anterior to the analytical column. They are having same packing material as that of analytical column but its particle size may larger. The length of columns are 2-10 cm, thus they dose not affect the separation. They are for protective purpose. They are dependable columns designed to filter or remove particles that clog the separation of the column. These are used in the following cases:

a. Compounds and ions that could ultimately cause "baseline drift", decreased resolution, decreased sensitivity and create false peaks.
b. Compounds that may cause precipitation upon contact with the stationary or mobile phase. Compounds that might co-elute and cause extraneous peaks and interfere with the detection and/or quantification.10-11

II) Analytical Columns

These columns are mainly involved in sample separation. They are made up of stainless steel / heavy glass / polyethylene / polyethylene ketone. These are also known as micro columns, capillary columns have a diameter much less than a millimeter. They allow the user to work with nanoliter sample volumes, decreased flow rate, and decreased solvent volume usage which may lead to cost effectiveness.

The stainless steel columns are commonly used because they can give good separation with high pressure. The columns are long narrow and the length varies from 8 to 10 cm and 20 to 30 cm. them having the internal diameter 2 to 5 mm. The silica gel is most frequently used for column packing. For partition chromatography it is bonded with some alkyl groups with the chain lengths of 1, 2, 8 or 18 carbons, cyanopropyl groups, phenyl groups and ion exchange[10-11].

5. Detectors

The function of the detector in HPLC is to monitor the mobile phase as it emerges from the column.

Different types of detectors can be used with HPLC.

Ultra-Violet (UV) Detectors:

The mechanism of these detectors is based upon the light absorption characteristics of the sample. They measure the ability of samples to absorb light. This can be done at single or variable wavelengths:

Fixed wavelength: Measures at single wavelength.

Variable wavelength: Measures one wavelength at a time, but can detect over a wide range of wavelengths.

Photodiode Array Detector:

These detectors can scan the entire UV spectrum repeatedly during the elution of a peak to determine if more than one substance is coeluting.

Fluorescence Detector:

These detectors are used for those compounds that fluoresces naturally or it can be made to do so by derivatization.

Differential Refractometer Detector:

It is capable of measuring refractive index changes from 10^{-4} to 10^{-5} RI units. They measure the ability sample molecules to bend or refract light. This property is called refractive index. Detection occurs when light is bent due to samples eluting from the column and this is read as a disparity between the two channels.

Radio Chemical Detectors:

Involves use of radio labeled material usually tritium (^{3}H) or carbon-14 (^{14}C). It operates by detection of fluorescence along with beta-particle ionization.

Electrochemical Detectors:

Used in analysis of compounds that undergoes oxidation or reduction reactions. They measure the difference in electrical potential when the sample passes between the electrodes. It includes amperometry, coulometry, polarography, photoconductivity principles.[10-11]

7. Recorder

Recorders are used to record the responses obtained from detector after amplification. They record the base line and all the peaks obtained with respect to time[10-11]

ANALYTICAL METHOD DEVELOPMENT[12-13]

Selecting an accurate assay procedure for each ingredient present in pharmaceutical dosage forms, either individually or complex dosage formulation containing several therapeutically and chemically compatible drugs with very similar chemical nature is a monumental undertaking.

Separation, identification and estimation of each ingredient in such complex formulation are a challenging task. The presence of excipients, additives and decomposition products further complicates the analysis. Therefore analytical development is done for new drug where no methods are available. Or, alternate method development for existing (non pharmacopoeia) products to reduce cost and time of analysis.

There are different kinds of reasons for developing new methods for analysis: -

- There is no any suitable method for a particular compound or combination of that compound.
- The existing method may be too error, contamination-prone or may be not well suitable.
- The existing method may be too expensive, time consuming or may not be easily automated.
- The existing method may be having less accuracy or precision.

- The existing method may not provide adequate sensitivity or analyte selectivity in the sample.
- There may be need of alternative method to confirm a legal or scientific reasons, analytical data originally or turned by existing method.

STEPS TO BE FOLLOWED IN METHOD DEVELOPMENT

Method development[9] starts with the documentation of the developed studies. All data related to these studies must be established and they must be recorded in laboratory notebook or an electronic data base.

1. Standard Analyte Characterization:-

- Collection of information about the analyte or drug should be collected starting from the structure, physical, chemical properties toxicity, purity, hygroscopic nature, solubility and stability.
- Reference standard for the sample should be obtained. In case of multiple components for the analysis to be analyzed in the sample, number of components should be noted, data is assembled and the availability of standards for each one is determined.
- Proper storage are set for the reference standards (refrigerators, dessicator, freezer).
- A suitable method for the sample is considered.

2. Method Requirements:-

The aim or requirement of the analytical method that need to be developed all considered and the analytical figures of merit are defined. The required detection limits, selectivity, linearity, range, accuracy and precision are defined.

3. Literature Search and prior Methodology:-

The literature for all types of information related to the analyte surveyed. Literature is done for synthesis, physical, chemical properties, solubility and related analytical method. Information can be obtained from official standard books such as USP/NF, AOAC standard books, periodicals, chemical manufacturers along with chemical abstract services and computer data banks.

4. Choosing of Method:-

- Adaptation is more efficient than "reinventing the wheel". If any of the reported methods from the literature are adaptable to the current laboratory setting and future needs, it is determined.
- From the various source a methodology is adopted. The methods are modified.
- If there are no reported details for the drug or the chemical then the drugs are investigated and are worked out.

5. Instrumental Setup and Initial Studies:-

- By using the data made for the analyte a suitable instrument method is selected. The instrument is set up by using standard operation procedure.
- Analyte reference standard is prepared in suitable concentration by using various combination of solvent system. It is important to start with reference standard substance rather then complex sample components.
- The analysis is made for the various conditions described in the existing literature.

6. Optimization:

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

7. Documentation of analytical figures:

The originally determined analytical figures of merit, Limit of quantitation [LOQ] and Limit of the detection [LOD], linearity, time per analysis cost, sample preparation etc are documented.

8. Evaluation of Method Development with actual Sample:

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

9. Determination of Percent Recovery of Actual Sample and Demonstration of Quantitative Sample Analysis:

Percent recovery for the standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery from sample to sample and whether the recovery has been optimized has been shown. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with high degree certainty.

The validity of analytical method can be verified only by laboratory studies. Therefore documentation of the successful completion of the studies is the basic requirement for determining whether a method is suitable for its intended applications.

Validation is an approach to form a basis for written procedures for production and process control which are designed to assure that the drug products have the identity strength, quality, and purity they purport or are represented to possess.

Analytical Method Validation

Definitions of validation:

World health organization (WHO):

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

Food and drug administration (FDA US):

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

European committee(EC):

Action of providing in accordance with the principle of good manufacturing practice, that any procedure process equipment material, activity or system actually read to the expected result.

Types of validation:[14]

Prospective Validation:

At least three successive production size (US Via) batches, all batches made tested and report approved before distribution facilities and equipment qualified.

Concurrent Validation:

Generation of validation data concurrent or simultaneously with normal production schedules used in exceptional cases (low volume products); interim reports required.

Retrospective Validation:

This is establishing documented evidence that the process is performed satisfactorily and consistently over time, based on review and analysis of historical data. The source of such data is production and QA/QC records. The issues to be addressed here are charged to equipment, process, specification and other relevant changes in the past.

Purpose of Validation

The principal purpose of analytical method validation is to ensure that test methods, which are used for assessing compliance of pharmaceutical products with established specifications, will give accurate, reliable and reproducible results.

The real goal of the method validation process is to challenge the method and determine limits of allowed variability for the conditions needed to run the method. It is important to have a well-conceived validation plan for testing the method and acceptance criteria before starting the validation process. Included in this plan should be detailed procedure describing the entire method (including calibration standard and sample preparation, separation, data handling and calculations) that can conveniently be executed by others.

Parameters for Validation of HPLC Methods[14-15]

The validation parameters as per ICH guidelines and USP are:-

Table: 1. validation parameters as per ICH guidelines and USP

1.System Suitability	6. Limit of Quantitation
2. Accuracy	7. Limit of Detection
3. Precision	8. Ruggedness
4. Linearity & Range	9. Robustness
5. Specificity	10. Stability

A. **ACCURACY:**

Accuracy is the closeness of test results obtained by that method to the true value.

Determination of accuracy

In case of assay of a drug substance accuracy may be determined by application of the analytical method to an analyte of known purity (e.g. reference standard) or by comparison of the results of the method with those of a second well characterized method, the accuracy of which has been stated or defined. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals.

The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentrations levels, covering the specified range (i.e., three concentrations and three replicates of each concentration).

B. PRECISION:

Precision is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample. Precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements.

Precision may be measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating conditions.

Determination of precision

Precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation (coefficient of variation). The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure.

Bias (Systematic error)

Indicative of tendency of the method to measure something other than what it is intended to measure.

Repeatability

The precision of the analytical method when repeated by the same analyst under set of laboratory conditions, the only difference being the sample.

Determination of repeatability

The repeatability of a test procedure is assessed by carrying out complete separate determinations on the separate samples of the same homogeneous batch of the material and this will provide a measure of the precision of the procedure under normal laboratory operating conditions.

Reproducibility

When the procedure is carried out by different analysts in different laboratories, using different equipments, reagents and laboratory setting.

Determination of reproducibility

The reproducibility of a test procedure is determined by evaluating the samples from the same homogeneous batch, the analytical data will provide information about the reproducibility of the test procedure under validation.

C. **SPECIFICITY:**

ICH documents defines specificity as the ability to assess unequivocally the analyte in the presence of compounds that may be expected to present, such as impurities, degradation products and matrix components.

Identification tests

Ensure the identity of an analyte.

Purity test

Ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte. (e.g., related substances test, heavy metals limit, organic volatile impurity test).

Assay

Provide an exact result, which allows as accurate statement on the content or potency of the analyte in a sample.

Determination of specificity

ICH documents state that when chromatographic procedures are used, representative chromatograms should be presented to demonstrate the degree of selectivity and peaks should be appropriately labeled. Peak purity tests (e.g., Using diode array or mass spectrometry) may be useful to show that the analyte chromatographic peak is not attributable to more than one component.

D. **DETECTION LIMIT(LOD):**

Lowest amount of analyte in a sample that can be detected, but not necessarily quantities as an exact value, under the stated experimental conditions. The detection limit is usually expressed as the concentration of analyte (e.g., percentage parts per million) in the sample.

$$D L = 3.3 \sigma / S$$

Where;

σ = the standard deviation of the response,

S = the slope of the calibration curve,

DL = Detection Limit.

The slope S may be estimated from the calibration curve of the analyte.

Determination of detection limit

For instrumental and non-instrumental methods detection limit is generally determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be reliably detected.

E. QUANTITATION LIMIT(LOQ):

It is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Quantitation limit is expressed as the concentration of analyte (e.g. percentage, parts per billion) in the sample.

Determination of quantitation limit

For instrumental and non-instrumental methods, the quantitation limit is generally determined by the analysis of samples with known concentration of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision.

$$QL=10\ s/\ S$$

Where'

s = the standard deviation of the response,

S = the slope of the calibration curve,

QL = Quantitation limit.

The slope S may be estimated from the calibration curve of the analyte.

F. LINEARITY AND RANGE:

Linearity of an analytical method is its ability to produce results that are directly proportional to the concentration of analyte in samples. The range of the procedure is an expression of the lowest and highest levels of analyte that have been demonstrated to be determinable with acceptable precision, accuracy and linearity.

Determination of linearity and range

These characteristics are determined by application of the procedure to a series of samples having analyte concentration spanning the claimed range of the procedure. When the relationship between response and concentration is not linear, standardization may be provide by means of a calibration curve. ICH recommends that for the establishment of linearity a minimum of 5 concentrations normally used.

G. SENSITIVITY:

Sensitivity is the capacity of the test procedure to record small variations in concentration. It is the slope of the calibration curve. A more general use of the term to encompass limit of detection and or / limit of quantitation should be avoided.

H. RUGGEDNESS:

Degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, different analysts, different instruments etc., normally expressed as the lack of influence on test results of operational and environmental variables of the analytical method. Ruggedness is a measure of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst.

Determination of ruggedness

By analysis of aliquots from homogenous lots in different laboratories, by different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay. Degree of reproducibility of test results is then determined as a function of the assay variables.

I. ROBUSTNESS:

Robustness of an analytical method is measure of its capacity to remain unaffectedly small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

J. SYSTEM SUITABILITY:

According to USP system suitability are an integral part of chromatographic methods. These tests verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. One consequence of the evaluation robustness and ruggedness should be that a series of system suitability parameters is established to ensure that the validity of the analytical method is maintained whenever used. System suitability tests are based on the concept that the equipment, electronics, analytical operations and samples constitute an integral system that can be evaluated as a whole.

SYSTEM SUITABILITY PARAMETERS FOR HPLC[16]

Retention time (RT)

Retention time is time of elution of peak maximum after injection of sample.

Column Efficiency (N)

Solutes are placed on an HPLC column in a narrow band

- Each solute band spreads as it moves through the column due to diffusion and mass transfer affects
- The later eluting bands will spread more
- Peak shape follow a Gaussian distribution
- The sharpness of a chromatographic peak is an indication of the quality of the chromatographic column.
- Peak sharpness is determined by measurement of the peak width
- Peak width is dependent on flow rate so measurement of the width alone is not enough
- A good measure of column efficiency is $\frac{tR}{Wb}$.

$$\mathbf{n = 16 \left(\frac{t}{w}\right) 2 \text{ or } n = 5.54 \left(\frac{t}{w1/2}\right) 2}$$

w = width of the peak at its base, obtained by extrapolating the relatively straight sides of the peak to the baseline.

w1/2 = width of the peak at half height, obtained directly by electronic integrators.

The value of 'N' depends upon the substance being chromatographed as well as the operating conditions such as mobile phase, temperature etc

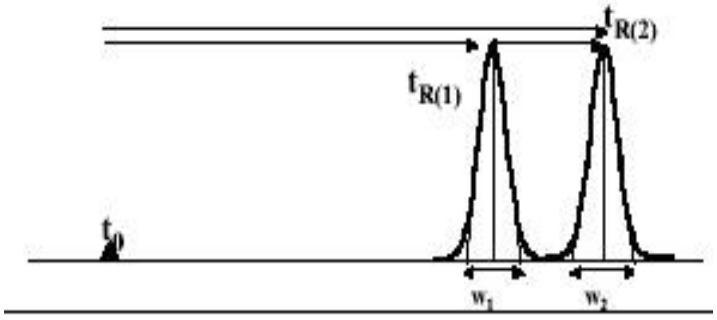
Resolution (RS)

It is function of column efficiency, and is specified to ensure that closely eluting compounds are resolved from each other to establish the general resolving power of system.

The separation of two components in a mixture the resolution is determined by the equation,

$$Rs = \frac{t_{R(2)} - t_{R(1)}}{\frac{1}{2}(W1 + W2)}$$

+



Where, tR(2) and tR(1) are the retention time of second and first compound respectively, where as W2 and W1 are the corresponding widths at the base of peaks obtained by extrapolating straight side of peaks to the base lines.

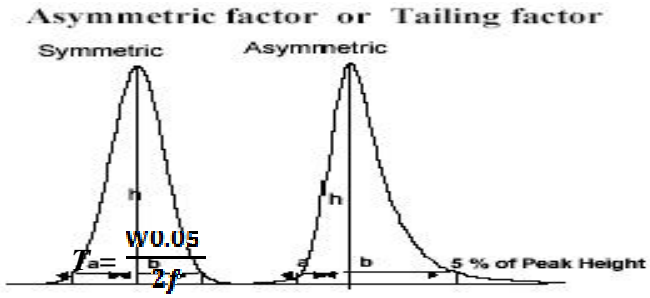
Where, electronic integrator is used, it may be convenient to determine the resolution by equation

$$RS = \frac{2(t2 - t1)}{1.7(W1\ h/2 + W2\ h/2)}$$

Where, W h/2 is peak at the half height, obtained directly by electronic integrator.

Peak Asymmetric factor or Tailing (As)

A properly packed HPLC column will give symmetrical or gaussian peak shapes. Changes in either the physical or chemical integrity of the column bed can lead to peak tailing.



Where W0.05 is the width of peak at 5% height and f is the distance from the peak maximum to the leading edge of the peak height from the baseline.

Tailing can be caused by:

- Column voids, channels, or extra-column dead volume (affects early eluting peaks most)
- Stripping of the bonded phase (affects late eluting peaks most)

Separation Factor (α)

- The separation factor, also referred to as column selectivity, is affected by changes in the chemistry of the chromatographic method such as:
- A change in the choice of solvents for the mobile phase
- A change in the packing material in the column
- Because we are usually dealing with samples that contain more than one sample component, a term describing the separation of peaks is needed.
- The separation factor describes the relative position of two peak maxima
- It is equal to the ratio of the time each component spends on the packing material

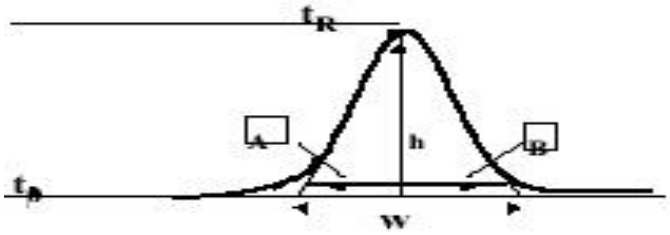
$$\alpha = \frac{tR(B)}{tR(A)}$$

This equation is more often seen as the ratio of the capacity factors

$$\alpha = \frac{k(B)}{k(A)}$$

Capacity factor (k')

It is the measure of how well the sample molecule is retained by the column during an isocratic separation. It is affected by solvent composition, separation and aging and temperature of separation.



Retention Factor

$$K = \frac{tR - t0}{t0}$$

Where, tR = retention volume at the apex of the peak (solute) and

t0 = void volume of the system.

Peak Width

Due to the nature of the chromatographic equipment a dilution of the injected sample solution occurs when the sample molecules migrates through the column. As a consequence the zone containing the sample molecules broadens continually during its passage through the column. The detector will register a peak with a certain width. The measure for the peak width is the height of a theoretical plate.

System Suitability Parameters and Recommendations

Parameter	**Recommendation**
	Capacity Factor (k') The peak should be well-resolved from other peaks and the void volume, generally k'>2.0
Repeatability	RSD </= 1% for N >/= 5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard, etc.
Tailing Factor (T)	T of </= 2
Theoretical Plates (N)	In general should be > 2000

STATISTICAL PARAMETERS

Linear regression

Once a linear relationship has been shown to have a high probability by the value of the correlation coefficient 'r', then the best straight line through the data points has to be estimated. This can often be done be done by visual inspection of the calibration graph, but in many cases it is far more sensible to evaluate the best straight line by linear regression (the method of least squares).

The equation of straight line is

$$y = mx + c$$

Where, y the dependent variable is plotted as result of changing x, the independent variable.

To obtain the regression line 'y on x' the slope 'm' of the line and the intercept 'c' on the y axis are given by the following equation.

$$m = \frac{N \Sigma xy - (\Sigma x)(\Sigma y)}{N \Sigma x2 - (\Sigma x)2} \text{ and } \qquad c = \frac{(\Sigma y)(\Sigma x2) - (\Sigma x)(\Sigma xy)}{N \Sigma x2 - (\Sigma x)2}$$

Correlation coefficient

To establish whether there is a linear relationship between two variables x1 and y1**,** use Pearson's correlation coefficient r.

$$r = \frac{n \Sigma x1y1 - \Sigma x1y1}{\{[n \Sigma x12 - (\Sigma x1)2]\,[n \Sigma y12 - (\Sigma y1)2]\}1/2}$$

Where n is the number of data points.

The value of r must lie between +1 and -1, the nearer it is to +1, the greater the probability that a definite linear relationship exists between the variables x and y, values close to +1 indicate positive correlation and values close to -1 indicate negative correlation values of 'r' that tend towards zero indicate that x and y are not linearly related (they made be related in a non-linear fashion).

Standard deviation

It is commonly used in statistics as a measure of precision statistics as a measure of precision and is more meaningful than is the average deviation. It may be thought of as a root-mean-square deviation of values from their average and is expressed mathematically as

$$S = \sqrt{\frac{\sum_{i=1}^{i=n} (x_i - \bar{x})}{N - 1}}$$

Where,

S is standard deviation.

If N is large (50 or more) then of course it is immaterial whether the term in the denomination is N -1 or N

Σ = sum

$\bar{x}$ = Mean or arithmetic average

$x - \bar{x}$ = deviation of a value from the mean

N = Number of observations

Percentage relative standard deviation (%RSD)

It is also known as coefficient of variation CV. It is defined as the standard deviation (S.D) expressed as the percentage of mean.

$$\%\ RSD = \frac{S.D}{\bar{x}} \times 100$$

Where,

S.D = standard deviation,

$\bar{x}$ = Mean or arithmetic average.

The variance is defined as S^2 and is more important in statistics than S itself. However, the latter is much more commonly used with chemical data.

Standard error of mean (S.E.)

Standard error of mean can be defined as the value obtained by division of standard deviation by square root of number of observations. It is mathematically expressed as

$$S.E. = \frac{S.D.}{\sqrt{n}}$$

Where,

S.D = Standard deviation

n = number of observations.

2. LITERATURE REVIEW

❖ ***Zarna Dedania et al . ,*** [17] (2009) developed an RP-HPLC method for simultaneous estimation of omeprazole and ondansetron in combined tablet dosage forms. The separation was carried out on RP C_{18} (250×4.6,5µ) using mobile phase consisting of Methanol:Acetonitrile (90:10). The retention times for omeprazole and ondansetron was found to be 5.39 and 11.08 min respectively. The detection was carried out at 218 nm using PDA detector. The linearity concentration was in the range of 4-20µg/ml of Omeprazole and Ondansetron respectively. The method has been validated as per ICH guidelines.

❖ ***Kiriti S Topagi et al . ,*** [18] (2010) have developed and validated HPLC method for simultaneous determination of drotaverine hydrochloride and omeprazole in tablet dosageforms. Seperation was achevied on HiQsil using UV detector. The mobile phase consists of n-heptane : dichloromethane : methanolic ammonia(5%) : methanol (50:25:1:4) at flow rate of 1.0ml/min. The retention times of drotaverine hydrochloride and omeprazole were found to be 6.0 and 8.1 respectively. The linearity of drotaverine and omeprazole were found in the concentration range of 10-50µg/ml and 2.5-12.5µg/ml.

❖ ***Nayan M Jagani et al . ,*** [19] (2012) developed simple,sensitive dual wavelength spectrophotometric method for simultaneous estimation of Omeprazole and Cinitapride tartarate in combined capsule dosage forms. The determination was carried out at 288 nm and 312.9 nm. The linearity results were obtained in the concentration range of 10-30 µg/ml and 1.5-4.5 µg/ml for omeprazole and cinitapride. The sutability of the method for quantitative determination was proved by validation.

❖ ***Patel G.H et al . ,*** [20] (2012) developed and validated an HPLC method for simultaneous estimation of Cinitapride and Pantaprazole in pharmaceutical dosage forms. Chromatographic separation was achieved isocratically on inertsil C_{18} (250×4.6, 5µ). The mobile phase consisting of acetonitrile : phosphate buffer (80:20 v/v, pH 6.8).The Flow rate was 1.0 ml/min. The uv detection was carried out at 278 nm. The retention times times of Cinitapride and Pantaprazole was 3.18 min and 4.72

min respectively. The method is linear within the range of 1.5-12 µg/ml and 20-160µg/ml for Cinitapride and Pantaprazole .The LOD of Cinitapride and Pantaprazole was 0.064 µg/ml and 0.78 µg/ml while LOQ was 0.205µg/ml and 2.38µg/ml respectively.

❖ ***Y.V. Rami Reddy et al . ,***[21] (2012) have done work on development and validation of HPLC method for simultaneous determination of Omeprazole and Domperidone in tablets .The mobile phase consists of acetonitrile : phosphate buffer (60:40v/v pH 7) at flow rate of 1.0 ml/min and separation was achieved on C_{18} Nucleosil column using internal standard diphenylamine.The retention times of omeprazole and domperidone are 3.64 and 4.31 respectively.

❖ ***Hemant Kumar Jain et al . ,*** [22](2012) developed RP-HPLC method for simultaneous estimation of Pantaprazole and Cinitapride in capsule formulation. The separation was carried out on chromatopak peerless LC-C_{18} column with mobile phase consisting of methanol :phosphate buffer(98:02 % v/v, pH 7). The flow rate was 0.6 ml/min and detection was carried at 280 nm. The retention times of pantaprazole and cinitapride are 4.8 and 6.2 min respectively. Linearity for pantaprazole and cinitapride was found in the range of 250-560 and 18-42 µg/ml.

❖ ***Devika P Chauhan et al . ,*** [23] (2012) developed and validated an HPTLC method for simultaneous estimation of cinitapride and omeprazole. The determination was carried on silicagel 60 gf254 hptlc plates using mobile phase consisting of toluene : ethylacetate : methanol : acetic acid (6:3:1:01v/v/v/v). The absorbance of spots was measured by densitometry at 289 nm. The Rf of cinitapride and omeprazole are 0.48 and 0.75 respectively. The linearity of cinitapride and omeprazole were found in the concentration range of 100-600ng and 15-90 ng/band respectively.

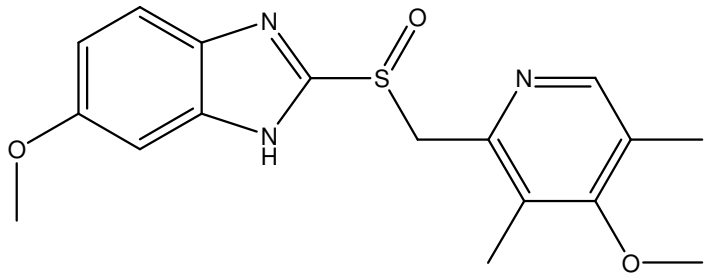
❖ ***Y.G. Makani et al . ,*** [24] (2012) have done and development and validation of first order derivative spectrophotometric method for simultaneous estimation omeprazole and cinitapride in combined dosage forms. The detection was carried out at 263.2 for omeprazole and253.8 for cinitapride. The method was linear in the range of 7-42µg/ml for omeprazole and 1-6µg/ml for cinitapride. The method is valdated as per ICH guidelines.

3.DRUG PROFILE

3.1 DRUG PROFILE

(a) Omeprazole

Molecular struture :



Molecular Formula : $C_{17}H_{19}N_3O_3S$

Chemical Name : 6-methoxy-2-{[(4-methoxy-3,5-dimethylpyridin-2yl)methane] Sulfinyl}-1H-1,3 benzodiazole

Molecular Weight : 345.4g/mole

Category : Anti-ulcer agent,proton pump inhibitor

Physical properties :

Colour : White

Melting point : 156^0c

Solubilty : Water

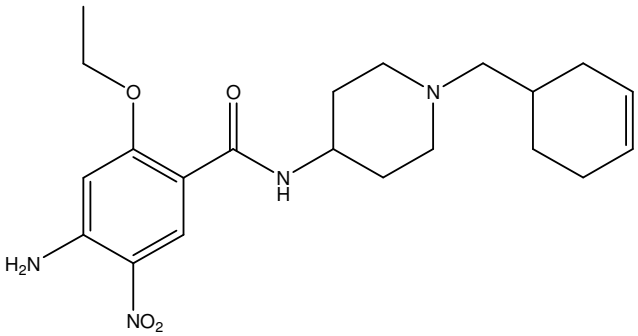
Clinical pharmacology :

Mode of action :

Omeprazole is a proton pump inhibitor that suppresses gastric acid secretion by specific inhibition of the H^+/K^+-APTase in the gastric parietal cell .By acting specifically on the proton pump ,Omeprazole blocks the final step in acid production thus reducing gastric acidity

(b) Cinitapride

Molecular structure:



Molecular formula : $C_{21}H_{30}N_4O_4$

Molecular weight : 402.49g/ml

Category : Antiemetics

Chemical name : (RS)-4-amino-N-[1-(1-cyclohex -3-enylmethyl)-4 **-** piperidyl]-2-ethoxy-5-nitro-benzamide

Physical properties

State : Solid

Colour : White

Melting point : 152-154^{0}c

Solubility : Acetonitrile ,Methanol

Clinical pharmacology:

Mode of action:

Acts via inhibition of serotonergic 5-HT_2 and Dopaminergic D_2 receptor and Stimulation of serotonergic 5-HT_4 receptors in the neuronal synapses of the myenteric plexi

AIM AND OBJECTIVE

Literature review survey revealed that sevaral methods have been reported for simultaneous estimation of Omeprazole and Cinitapride individually or in combination with other methods so far HPTLC, HPLC methods,have been developed. However no sensitive HPLC methods have been developed for simultaneous estimation of Omeprazole and Cinitapride.

Aimed to develop analytical method development and validation for Omeprazole and Cinitapride in pharmaceutical dosage form.

Objective:

To develop simple, rapid, and sensitive chrmatographic mehod for determination of

Omeprazole and Cinitapride in pharmaceutical dosage forms.

- Quantitative determination ,a trace level when necessary, i.e accurate ,precise and reproducible in any laboratory setting.
- Ease of use ,ability to be automated ,high sample through put, and rapid turn around time.
- To develop a method it is necessary to consider the properties of the analyte of interest that may be used to advantage and to established optimal ranges of analyte parameter values.
- It is important that method development be performed using only analytical standard that have been well identified and characterized and whose purity is already known.
- The developed method is to be validated according to ICH and USP guidelines.

5. EXPERIMENTAL

5.1. MATERIALS AND INSTRUMENTS

5.1.1. Instruments:

Table No 2: Table showing list of the Instruments used

S. No	Name of the Instrument	Manufacturer	Model
1.	HPLC	Water	Alliance-2695 PDA Waters-2996
2.	Electronic balance	Shimadzu	AY 220
3.	Digital pH meter	Digisun Electronics	7007
4.	Centrifuge	Thermolab	R_8C
5.	Ultrasonicate water bath	_____	Ultrasonicate SECOUS

5.1.2. Reagents and chemicals:

Table No 3: Table showing list of the chemicals used

S. No	Name	Grade	Manufacturer/ Supplier
1.	Omeprazole & Cinitapride working standard	**-**	-
2.	Potassium dihydrogen phosphate	HPLC	Merck
3.	Acetonitrile	HPLC	Merck
4.	Milli Q Water	HPLC	**-**

5.2. METHOD DEVELOPMENT AND OPTIMIZATION OF OMEPRAZOLE AND CINITAPRIDE

1. SELECTION OF DETECTOR WAVE LENGTH:

The wave length selection is made at 215 nm since all the two compounds shows maximum absorbance in UV spectrum as reported in the literature.

2. OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

a. Selection of mode of separation:

As the drug was polar in nature, RP-HPLC method was preferred.

Procedure:

Preparation of Buffer solution:

Dissolve 13.6 g of Potssium dihydrogen phosphate into a 1000 ml beaker with HPLC water. The pH was 6.5 .

Preparation of mobile phase:

Mix a mixture of above buffer 600mL (60%) and 400 mL of Acetonitrile HPLC (40%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vaccum filtration.

Preparation of diluent:

Dissolve 13.6 g of Potssium dihydrogen phosphate into a 1000 ml beaker with HPLC water. Transfer 500 ml of above solution and 500 ml of acetonitrile in 1000 ml beaker, mix and sonicate for 15 minitues.

Preparation of Standard Solution:

Accurately 20mg of Omeprazole & 15mg of Cinitapride were weighed and transferred into 20ml volumetric flask, about 10ml of NaoH was added and sonicated for 5 minutes to dissolve it. The volume was made up diluent. The solution was filtered through 0.45µm membrane filter.

From this pipette out 5.0ml of solution and transferred into 100ml of volumetric flask and the volume was made up with diluent(stock solution).

From this 5.0ml of solution was pipette out and transferred into 25ml of volumetric flask and the volume was made up with diluent. The solution was filtered through 0.45µm membrane filter(standard solution).

Inject 50 µl of the standard solution into the chromatographic system and measure the area for the Omeprazole & Cinitapride peaks and calculate the %Assay by using the assay formula.

TRIAL&ERROR METHODS:

TRIAL -1:

CHROMATOGRAPHIC CONDITIONS:

Column : Kromosil C_{18} (4.6 x 150mm, 5 µm, Make: ACE)

Detector : 215nm

Flow rate : 1.0 ml/min

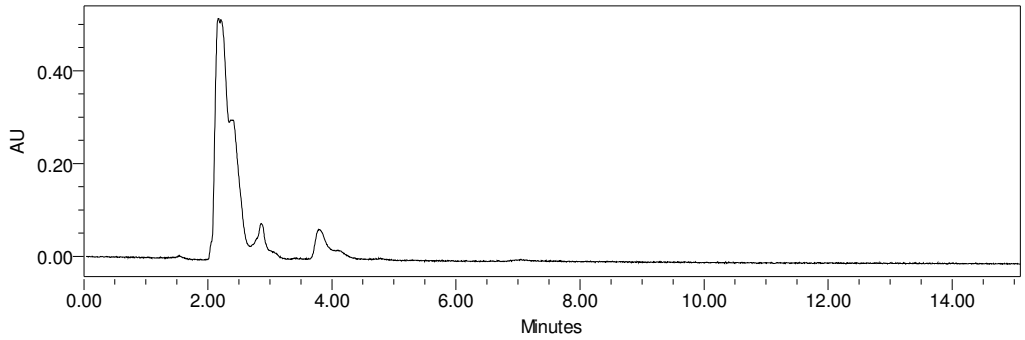
Injection volume : 50µl

Run time : 14min

Mobile Phase : Buffer (pH: 6.5): Acetonitrile (50: 50)

Trail-1

Fig No: 5.2.1



Results of Trial-1: Peak shapes are not good, so another trail is made by changing the column

TRIAL- 2:

CHROMATOGRAPHIC CONDITIONS:

Column : Hypersil BDS C_{18} (4.6 x 150mm, 5 µm, Make: ACE)

Detector : 215nm

Flow rate : 1.0ml/min

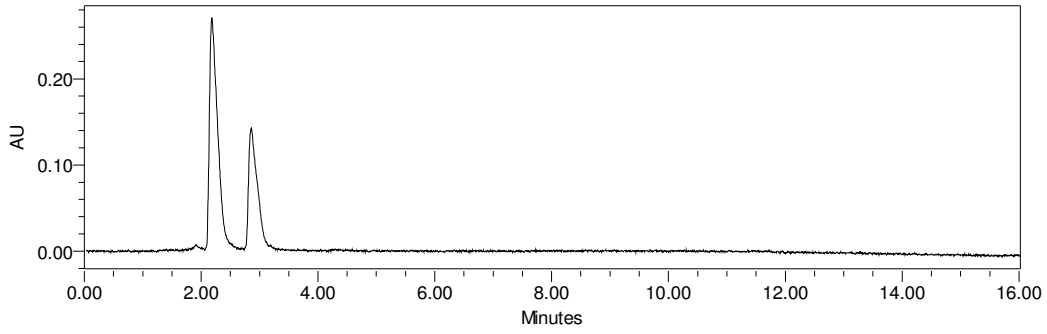
Injection volume : 50µl

Run time : 16min

Mobile phase : Buffer (pH-.6.5): Acetonitrile(50:50)

Trail – 2

Fig No: 5.2.2



Results of Trial-2: Peaks are merged, so another trial is made by changing the mobile phase ratio.

Trail-3:

CHROMATOGRAPHIC CONDITIONS:

Column : Hypersil BDS C_{18} (4.6 x 150mm, 5 µm, Make: ACE)

Detector : 215nm

Flow rate : 1.0ml/min

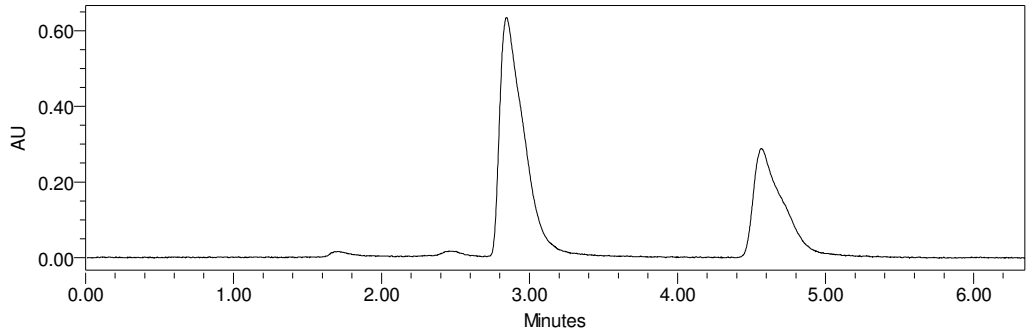
Injection volume : 50µl

Run time : 6min

Column temperature : 40°c

Mobile phase : Buffer (pH-6.5): Acetonitrile (60:40)

Fig No:5.2.3



Results of Trial-3: Peak shape was good but theoretical plates are not passed, so another trail is made.

Final method:

Column : Hypersil BDS C_{18} (4.6 x 250mm, 5 µm, Make: ACE)

Detector : 215nm

Flow rate : 1.0ml/min

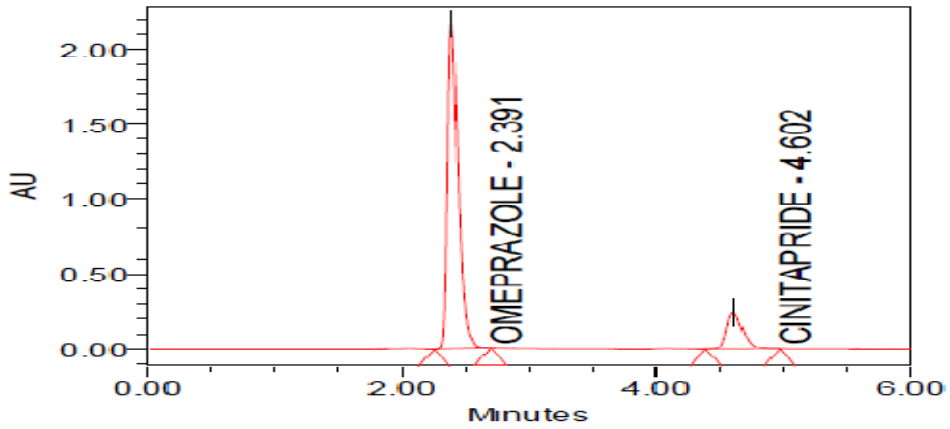
Injection volume : 50µl

Run time : 6min

Column temperature : 40°c

Mobile phase : Buffer (pH-.6.5): Acetonitrile (60:40)

Fig No:5.2.4



Results of Trial-4(Final Optimized Method):

RT's were observed at 2.391 (Omeprazole), and 4.602 (Cinitapride). The peaks are sharply resolved and hence the trial-4 method is optimized for analysis.

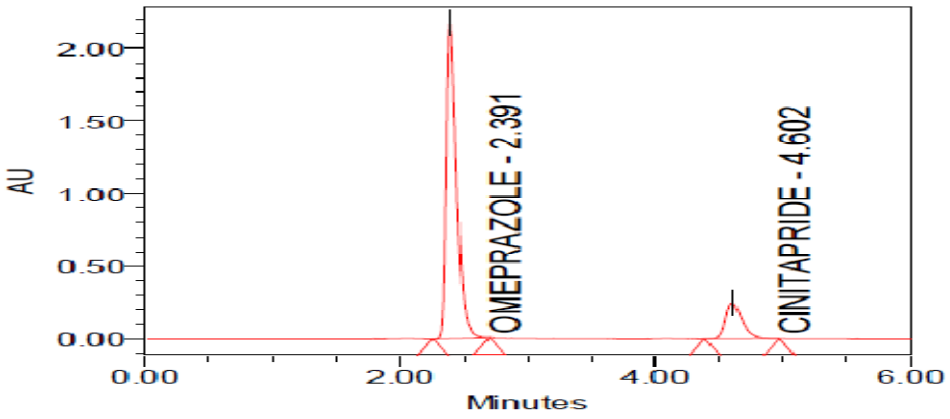
5.2.1 ANALYTICAL METHOD-OPTIMISATION

Aim: The present study is to develop a new reverse phase liquid chromatographic method for simultaneous determination of Omeprazole & Cinitapride in capsule dosage form.

Table No : 4 Table showing Optimized Chromatographic Parameters

OPTIMIZED CHROMATOGRAPHIC CONDITIONS	
Mode of separation	Isocratic elution
Mobile phase	Solvent-A: Potassium dihydrogen phosphate buffer pH-6.5 Solvent-B: Acetonitrile
Column	Hypersi BDS (4.6 x 250mm, 5 µm, Make: ACE)(60:40)
Flow rate	1.0 mL/ min
Detection Wavelength	215 nm
Injection volume	50 µl
Column oven temperature	40°C
Run time	6 min

Fig No: 5.2.5 Chromatogram showing peaks of standard solution of Omeprazole and Cinitapride



Peak name	**RT**	**Area**	**Plate count**	**Resolution**	**Tailing**
Omeprazole	2.391	12514807	4025	11.313	1.544
Cinitapride	4.602	2150791	6382		1.356

5.2.2 QUANTITATIVE DETERMINATION OF THE DRUG BY USING THE DEVELOPED METHOD

Sample : Omeprazole and Cinitapride

Label claim : 20,3 mg

Standard Solution :

Accurately 20mg of Omeprazole and 15mg of Cinitapride were weighed and transferred into 20ml volumetric flask, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up diluent. The solution was filtered through 0.45µm membrane filter.

From this pipette out 5.0ml of solution and transferred into 100ml of volumetric flask and the volume was made up with diluent

From this 5.0ml of solution was pipette out and transferred into 25ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter

Inject 50 µl of the standard solution into the chromatographic system and measure the area for the Omeprazole and Cinitapride peaks and calculate the %Assay by using the assay formula.

Sample Solution Omeprazole & Cinitapride

20 Omeprazole and Cinitapride capsules(Burpex) were weighed and the average weight was calculated. Accurately the sample equivalent to 20mg of Omeprazole & 15 mg of Cinitapride was weighed and transferred into 20ml volumetric flask about 10ml of Sodium hydroxide was added and sonicated for 5 minutes to dissolve it content. The volume was made up with diluent. The solution was filtered through 0.45µm membrane filter.

5.0ml of above solution was pipetted out and transferred into 100ml of volumetric flask and the volume was made up with diluent.

5.0ml of stock solution was pipetted out and transferred into 25 ml of volumetric flask and the volume was made up with diluent. The solution was filtered through 0.45µm membrane filter.

Inject 50 µl of the standard solution into the chromatographic system and measure the area for the Omeprazole and Cinitapride peaks and calculate the %Assay by using the assay formula.

Assay formula:

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \text{average weight of tablet}$$

Where,

AT = Peak Area of sample solution.

AS = Peak Area of standard solution.

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

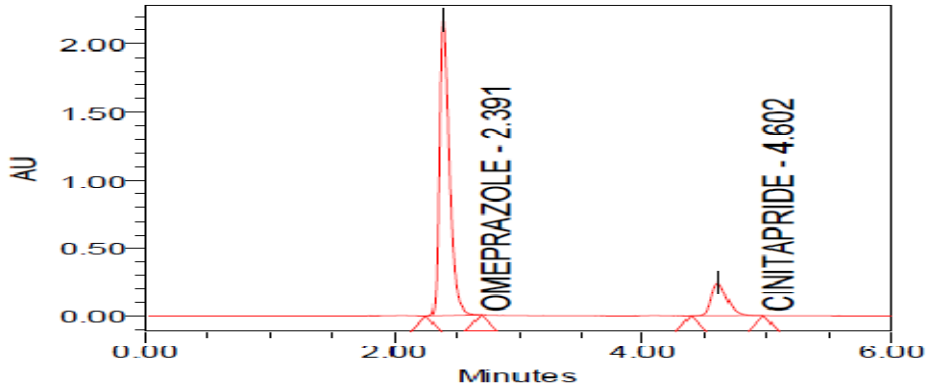
DT = Dilution of sample solution

Acceptance criteria: The limit of assay is in between the 98% - 102%

The chromatograms are as shown in Fig No 5.2.6 and 5.2.7 and assay results are tabulated and are as shown in Table No: 5

Chromatogram showing peaks of standard solution of Omeprazole and Cinitapride.

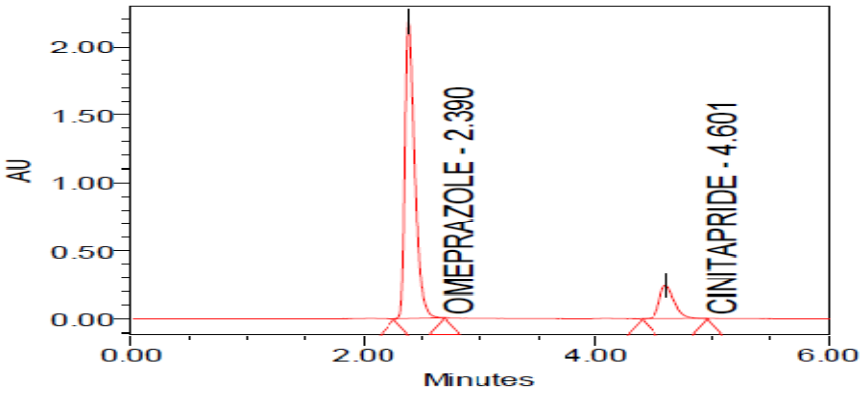
Fig No 5.2.6



Peak name	RT	Area	Plate count	Resolution	Tailing
Omeprazole	2.391	12514807	4025	11.313	1.544
Cinitapride	4.602	2150791	6382		1.356

Chromatogram showing peaks of test solution of Omeprazole and Cinitapride.

Fig No : 5.2.7



Peak name	RT	Area	Plate count	Resolution	Tailing
Omeprazole	2.390	12530126	3976	11.331	1.575
Cinitapride	4.601	2149391	6365		1.356

Table showing Assay Results of Omeprazole & Cinitapride :

Table No: 5

S. No	Compound name	Assay value
1.	Omeprazole	98.56%
2.	Cinitapride	99.1%

5.3 VALIDATION

Definition: Validation is a process of establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce meeting, its predetermined specifications and quality attributes.

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below.

- Accuracy
- Precision
- Specificity
- Linearity & Range
- Robustness
- System suitability testing

After method development, the validation of the current method has been performed in accordance with USP requirements for assay determination (Category-I: analytical methods for quantitation of active ingredients in finished pharmaceutical products) which include accuracy, precision, selectivity, linearity and range, robustness and ruggedness.

5.3.1 SYSTEM SUITABILITY TESTING

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated and the parameters like tailing factor, retention time, theoretical plates per unit, resolution factor are determined and the results are tabulated and are as shown in Table No: 6

Method

Preparation of Standard solution:

Standard Solution Omeprazole and Cinitapride:

Accurately 20mg of Omeprazole and 15mg of Cinitapride were weighed and transferred into 20ml volumetric flask, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up diluent. The solution was filtered through 0.45µm membrane filter.

From this pipette out 5.0ml of solution and transferred into 100ml of volumetric flask and the volume was made up with diluent .

From this 5.0ml of solution was pipette out and transferred into 25ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 50 µl of the standard solution into the chromatographic system and measure the area for the Omeprazole and Cinitapride peaks and calculate the %Assay by using the assay formula.

Acceptance criteria:

a. The column efficiency is not less than 2000 theoretical plates.
b. The tailing factor for the analyte peak is not more than 2.0.
c. The relative standard deviation for the replicate injections should not more than 2.0%.

Chromatograms showing system suitability testing of Standard Solutions of Omeprazole and Cinitapride.

Fig No : 5.2.8

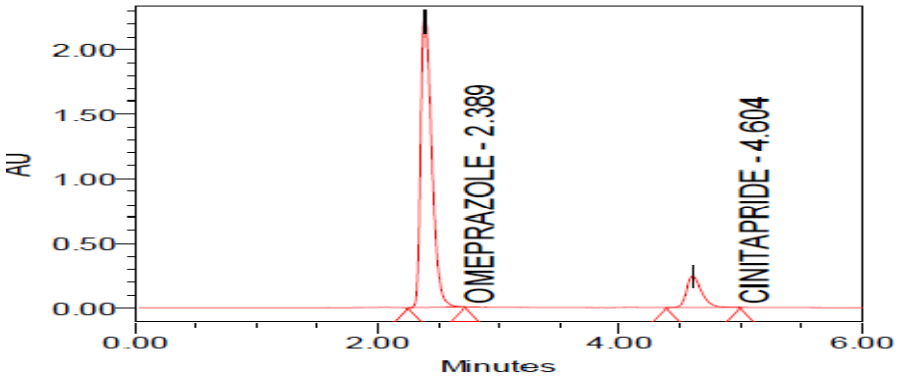


Fig No : 5.2.9

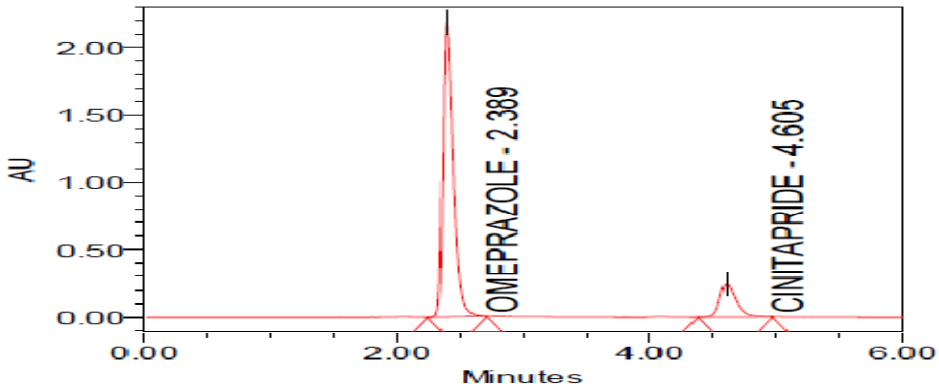


Fig No : 5.2.10

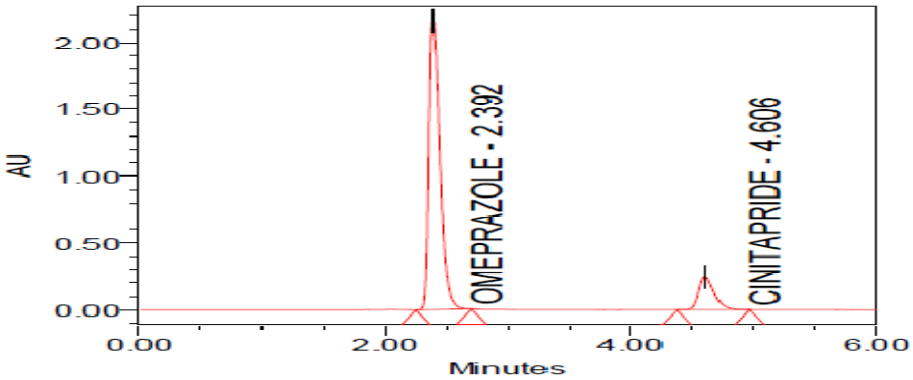


Fig No: 5.2.11

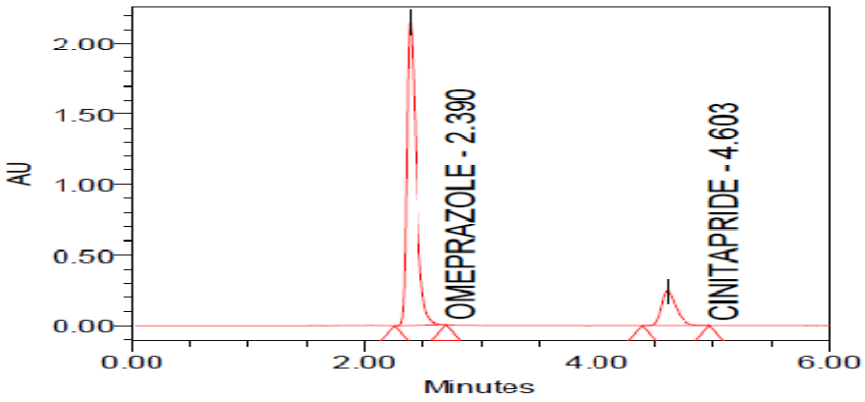


Fig No : 5.2.12

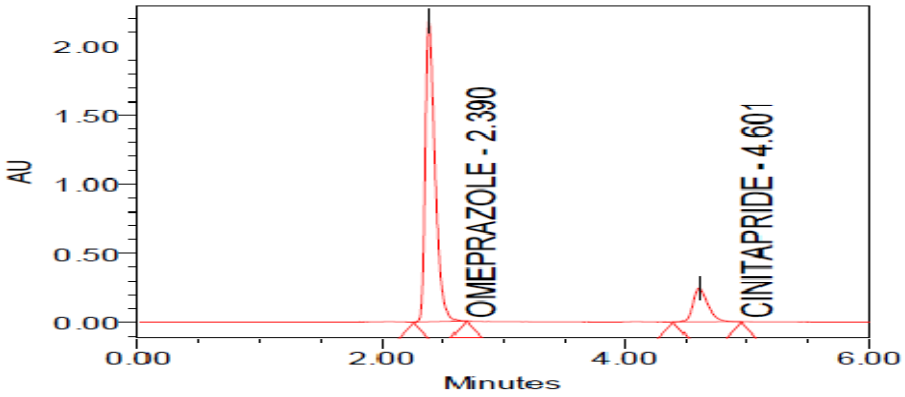


Table showing list of system suitability parameters of Omeprazole and Cinitapride.

Table No : 6

Parameters	**Omeprazole**	**Cinitapride**
Tailing factor	1.5	1.3
Retention time	2.39	4.603
Theoretical plates per unit	3985.6	6413.2
% RSD	0.32	0.21

5.3.2. ACCURACY

The accuracy of an analytical method is the closeness of that results obtained by that method to the true value. Accuracy may often be expressed as percent recovery by the assay of known added amount of analyte.

Determination:

The accuracy of the analytical method was determined by applying the method to the analyzed samples, to which known amounts of analyte had been added. The accuracy was calculated from the test results as the percentage of analyte recovered by the assay.

Procedure:

Preparation of Standard solution :

Accurately 20mg of Omeprazole and 15mg of Cinitapride were weighed and transferred into 20ml volumetric flask, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up diluent. The solution was filtered through 0.45µm membrane filter.

From this pipette out 5.0ml of solution and transferred into 100ml of volumetric flask and the volume was made up with diluent .

From this 5.0ml of solution was pipette out and transferred into 25ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter .

Preparation of 50% sample solution:

Accurately 10mg of Omeprazole and 7.5mg of Cinitapride were weighed and transferred into 20ml volumetric flask, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up diluent. The solution was filtered through 0.45µm membrane filter.

From this pipette out 5.0ml of solution and transferred into 100ml of volumetric flask and the volume was made up with diluent .

From this 5.0ml of solution was pipette out and transferred into 25ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter .

Preparation of 100% solution:

Accurately 20mg of Omeprazole and 15mg of Cinitapride were weighed and transferred into 20ml volumetric flask, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up diluent. The solution was filtered through 0.45µm membrane filter.

From this pipette out 5.0ml of solution and transferred into 100ml of volumetric flask and the volume was made up with diluent .

From this 5.0ml of solution was pipette out and transferred into 25ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter

Preparation of 150% solution:

Accurately 30 mg of Omeprazole and 22.5mg of Cinitapride were weighed and transferred into 20ml volumetric flask, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up diluent. The solution was filtered through 0.45µm membrane filter.

From this pipette out 5.0ml of solution and transferred into 100ml of volumetric flask and the volume was made up with diluent

From this 5.0ml of solution was pipette out and transferred into 25ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter

Inject 50µl of placebo and standard solutions of Accuracy -50%, Accuracy -100% and Accuracy -150% solutions into HPLC. Now calculate the amount obtained and amount added (API) for Omeprazole and Cinitapride samples.

Calculate the concentration in µg/ml in the spiked placebo in all the above cases by comparing with the standard solution. Calculate the individual recovery and mean recovery values. The chromatograms are as shown in Fig. No: 5.2.13-5.2.15 and the results are tabulated and shown in Table No: 7

Formula:

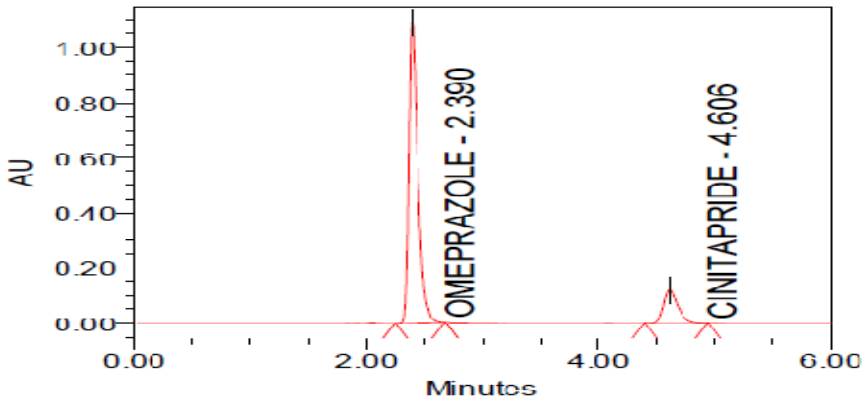
$$\%\text{recovery} = \frac{\text{(Amount recovered)}}{\text{(Actual amount added)}} \times 100$$

Acceptance criteria:

Percentage recovery in all the cases should be between 98.0 and 102.0 %.

Chromatogram showing Accuracy 50% of Omeprazole & Cinitapride.

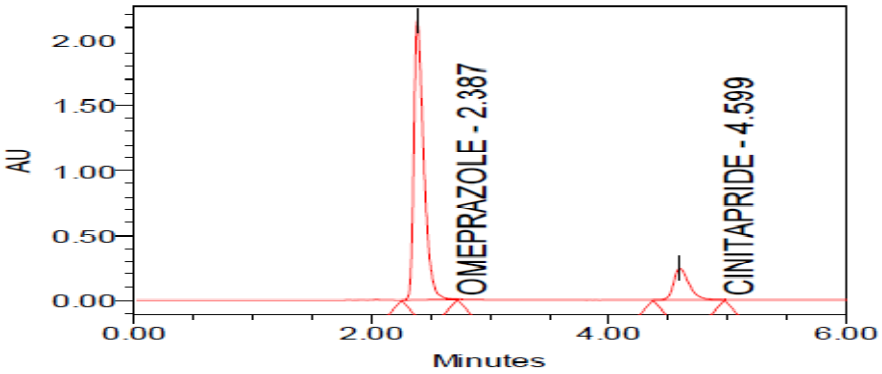
Fig No : 5.2.13



Peak name	RT	Area
Omeprazole	2.390	6205503
Cinitapride	4.606	1057276

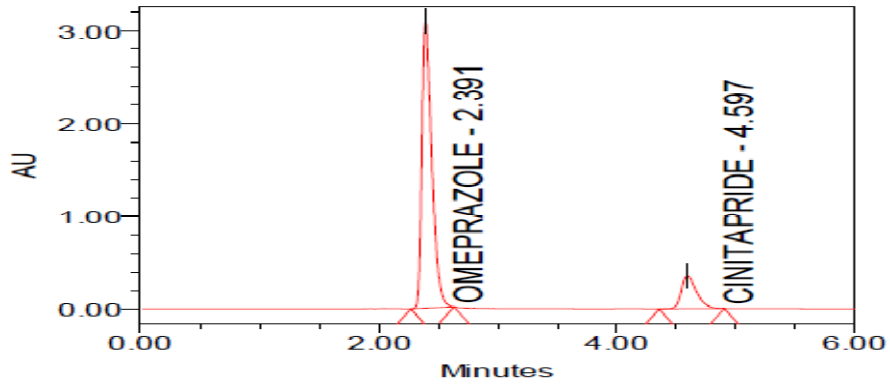
Chromatogram showing Accuracy -100% of Omeprazole and Cinitapride

Fig No :5.2.14



Peak name	RT	Area
Omeprazole	2.387	12281570
Cinitapride	4.599	2138513

Chromatogram showing Accuracy – 150% of Omeprazole and Cinitapride
Fig No : 5.2.15



Peak name	RT	Area
Omeprazole	2.391	17879545
Cinitapride	4.597	3138845

Table showing results of % Recovery studies for Omeprazole and Cinitapride

Table No : 7

Inj. Sample	Spike Level	Amount present in µg	Amount recovered	% recovered	Mean recovery	%RSD	Acceptance Criteria
Omeprazole	50 %	5	4.97mg	99.4%	99.16%	0.373	98-102%
	100 %	10	9.83mg	98.3%			
	150 %	15	14.97mg	99.8%			
Cinitapride	50 %	3.75	3.72mg	99.2%	99.3%	0.254	98-102%
	100 %	7.5	7.43mg	99.0%			
	150 %	10.25	10.23mg	99.8%			

5.3.3 PRECISION

Precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of a homogenous sample. Precision of analytical method is usually expressed as the standard deviation and relative standard deviation.

Determination:

The precision of the analytical method was determined by assaying sufficient number of samples and relative standard deviation was calculated.

The precision of the instrument was determined by assaying the samples consecutively, number of time and relative standard deviation was calculated.

A) System Precision:

Preparation of Standard Solution :

Accurately 20mg of Omeprazole and 15mg of Cinitapride were weighed and transferred into 20ml volumetric flask, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up diluent. The solution was filtered through 0.45µm membrane filter.

From this pipette out 5.0ml of solution and transferred into 100ml of volumetric flask and the volume was made up with diluent

From this 5.0ml of solution was pipette out and transferred into 25ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter

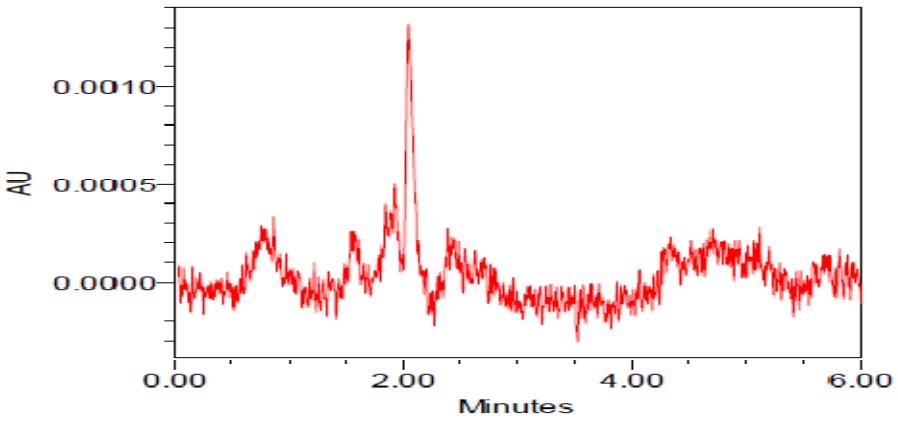
Inject 50µl of the blank solution and the standard solution of for six times and calculate the %RSD for the area of six replicate injections.The chromatograms are as shown in Fig No: 5.2.17-5.2.22 and the results are tabulated shown in Table No:8.

Blank solution: Mixture of phosphate buffer (pH6.5) and acetonitrile in ratio of 60:40 was filtered and degassed.

System Precision:

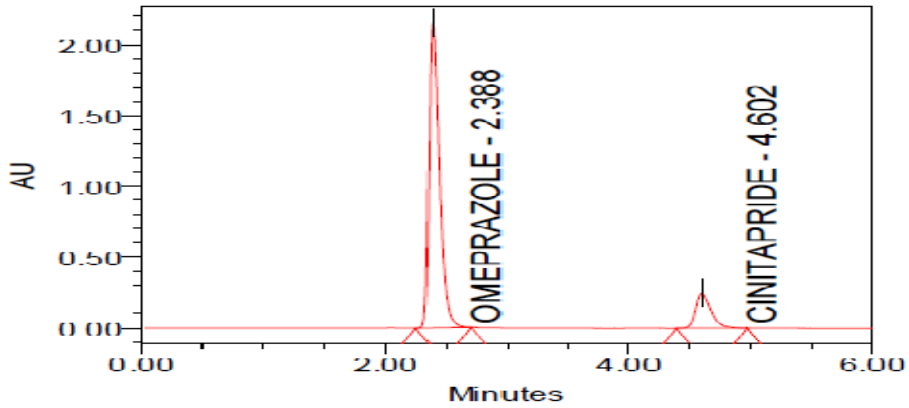
Chromatogram showing Precision-blank solution.

Fig No : 5.2.16



Chromatogram showing Precision of Standard Preparation-1 for Omeprazole and Cinitapride

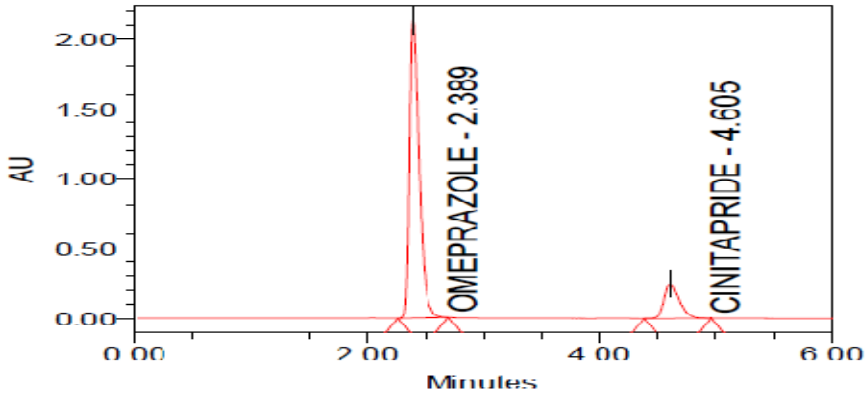
Fig No : 5.2.17



Peak name	RT	Area
Omeprazole	2.388	12490568
Cinitapride	4.602	2137140

Chromatogram showing Precision of Standard Preparation-2 for Omeprazole and Cinitapride

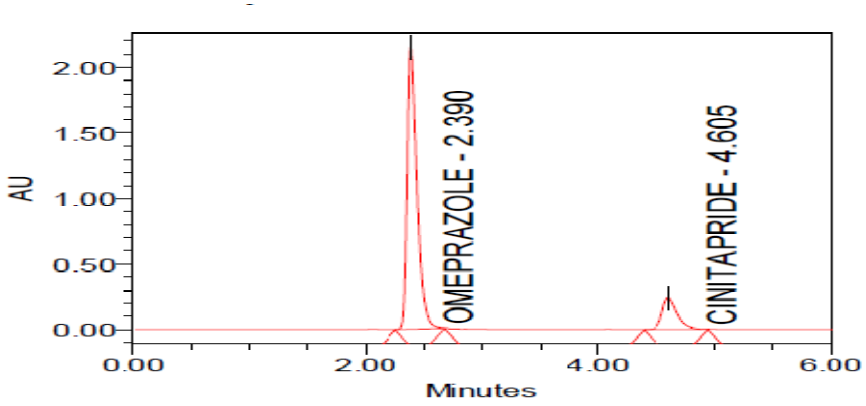
Fig No : 5.2.18



Peak name	RT	Area
Omeprazole	2.389	12514086
Cinitapride	4.605	2149337

Chromatogram showing Precision of Standard Preparation-3 for Omeprazole and Cinitapride

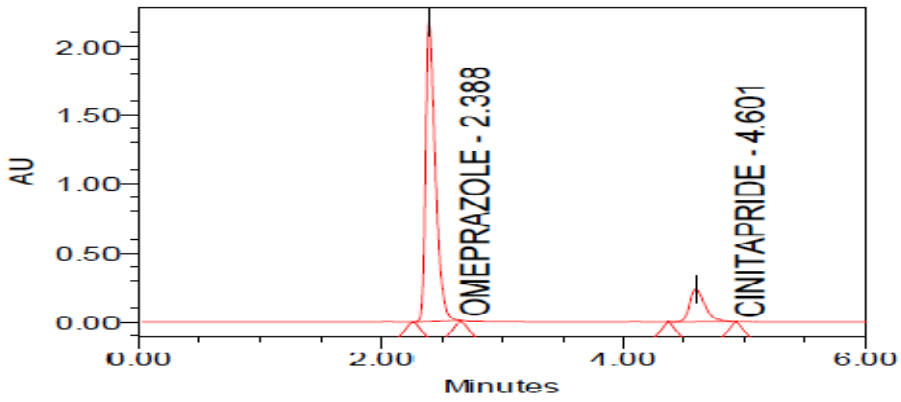
Fig No : 5.2.19



Peak name	RT	Area
Omeprazole	2.390	12358502
Cinitapride	4.605	2122528

Chromatogram showing Precision of Standard Preparation-4 for Omeprazole and Cinitapride

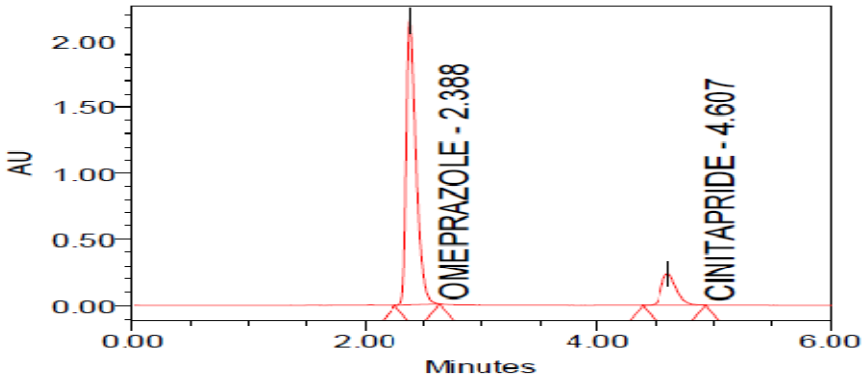
Fig No :5.2.20



Peak name	RT	Area
Omeprazole	2.388	12356161
Cinitapride	4.601	2121834

Chromatogram showing Precision of Standard Preparation-5 for Omeprazole and Cinitapride

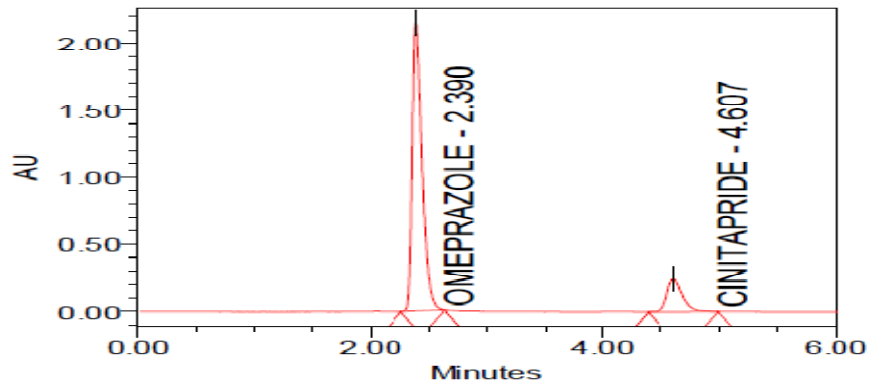
Fig No :5.2.21



Peak name	RT	Area
Omeprazole	2.388	12342535
Cinitapride	4.601	2119680

Chromatogram showing Precision of Standard Preparation-6 for Omeprazole and Cinitapride

Fig No :5.2.22



Peak name	RT	Area
Omeprazole	2.390	12391131
Cinitapride	4.607	2152385

B) Method Precision:

Preparation of Sample Solution:

Accurately pipette out 5ml of the sample (equivalent to 20mg of Omeprazole and 15mg of Cinitapride) into a 100ml volumetric flask and 70ml of diluent was added and mixed well and made up to the mark with diluent. Mix well and filter through 0.45µm filter.

Pipette out 5.0ml of Omeprazole and Cinitapride from stock solution and transferred into 25ml of volumetric flask and the volume was made up with diluent

Inject 50µl of the blank solution and six replicate injections of sample solution for six times and calculate the %RSD for the area of six replicate injections.

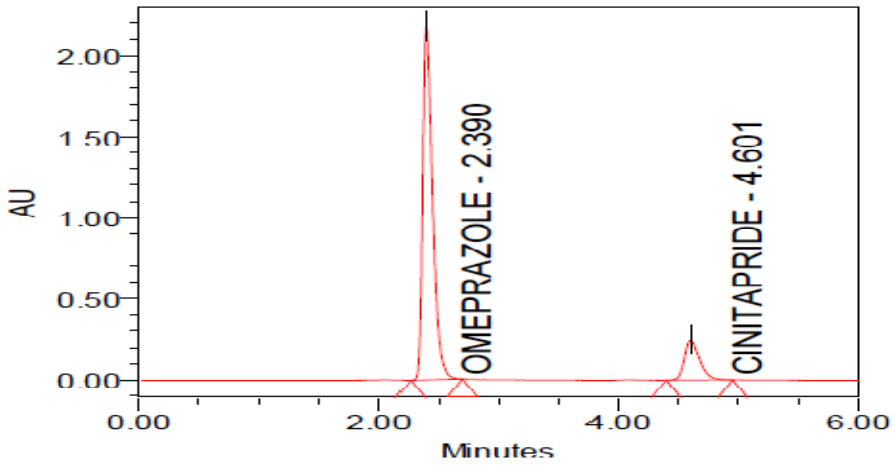
%RSD Formula: $(\sigma/\mu)*100$

Acceptance criteria: % Relative standard deviation (%RSD) for the areas of Omeprazole and Cinitapride from the standard chromatograms should not be more than 2.0

The method precision was determined by preparing the sample from the tablet formulation for five times and six successive injections of 50µl of working sample solution were injected and the chromatograms were recorded and shown in Fig No:5.2.23 and method precision data are shown in table. 8

Chromatogram showing Method Precision of Omeprazole and Cinitapride

Fig No : 5.2.23



Peak name	RT	Area
Omeprazole	2.390	12372131
Cinitapride	4.601	2142685

Table showing Results of Precision for Omeprazole and Cinitapride

Table No : 8

Parameter	System Precision		Method Precision	
Average Area	**Omeprazole**	**Cinitapride**	**Omeprazole**	**Cinitapride**
	12408830	2152385	12352130	119524
SD	74532	14612	74621	15752
%RSD	0.68	0.72	0.64	0.82

Intra Day Precision:

Table No : 9

Day	Peak area of Omeprazole	Mean	% RSD	Peak area of Cinitapride	Mean	% RSD
1	12523307	12527767	0.03	2149391	2150732	0.16
	12530126			2149433		
	12529869			2153372		
2	12452352	12452576	0.01	2129671	2129482	0.29
	12465893			2129984		
	12439482			2128792		
3	11469432	11445887	0.3	2091937	2092015	0.13
	11466336			2094382		
	11401894			2089726		

Inter day Precission:

Table No 10 :

Time	Peak area of Omeprazole	Mean	% RSD	Peak area of Cinitapride	Mean	% RSD
0th hour	12523307	12527767	0.30	2149391	2150899	0.10
	12530126			2149433		
	12529869			2153372		
3rd hour	12512352	12516922	0.28	2146871	2147216	0.16
	12518931			2147984		
	12519482			2146792		
6th hour	12469432	12465887	0.38	2121937	2124615	0.13
	12466336			2124382		
	12461894			2127526		

5.3.4 SPECIFICITY:

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Procedure:

Preparation of placebo :

Placebo was prepared by mixing all the excipients without active ingredients.

Accurately weighed quantity of placebo was transferred into 20ml volumetric flask, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up with diluent. The solution was filtered through 0.45µm membrane filter.

From this 5.0ml of solution was pipetted out and transferred into 100ml of volumetric flask and the volume was made up with diluent.

From this 5.0ml of solution was pipetted out and transferred into 25ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 50 µl of Standard solution for six times into the HPLC system and chromatograph.

Compare the chromatograms visually and check for any interference.

Preparation of Standard solution :

Accurately 20mg of Omeprazole and 15mg of Cinitapride were weighed and transferred into 20ml volumetric flask, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up diluent. The solution was filtered through 0.45µm membrane filter.

From this pipette out 5.0ml of solution and transferred into 100ml of volumetric flask and the volume was made up with diluent

From this 5.0ml of solution was pipette out and transferred into 25ml of volumetric flask and the volume was made up with diluent. The solution was filtered through 0.45µm membrane filter

Inject 20 µl of Standard solution for six times into the HPLC system and chromatograph. Compare the chromatograms visually and check for any interference.

Preparation of Standard + placebo:

Accurately weighed quantity of Placebo transferred into 20ml volumetric flask. To this accurately weighed quntatity of 20mg of Omeprazole and Cinitapride, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up with diluent. The solution was filtered through 0.45µm membrane filter.

From this 5.0ml of solution was pipette out and transferred into 100ml of volumetric flask and the volume was made up with diluent.

From this 5.0ml of solution was pipette out and transferred into 25ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 50µl of Standard solution for six times into the HPLC system and chromatograph.

Compare the chromatograms visually and check for any interference. The solution was filtered through 0.45m membrane filter. The solution was injected and the reports are given in table: 10

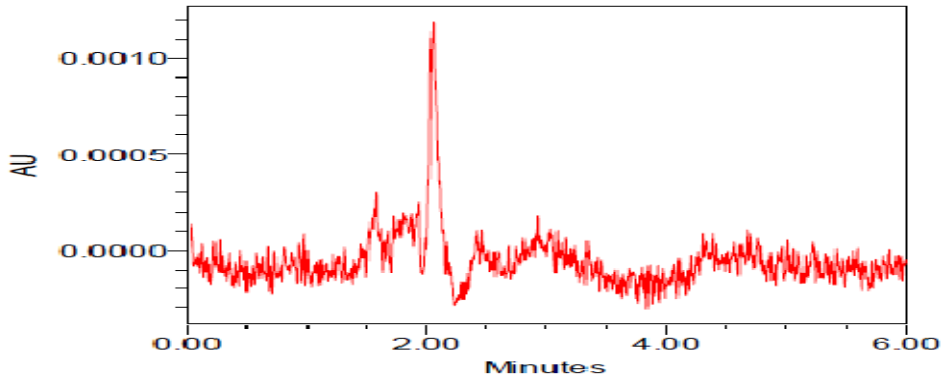
%RSD Formula: (σ /µ)*100

Acceptance criteria:

There should not be any peak in the blank and Placebo solution run at the retention time corresponding to Omeprazole and Cinitapride as in standard run.

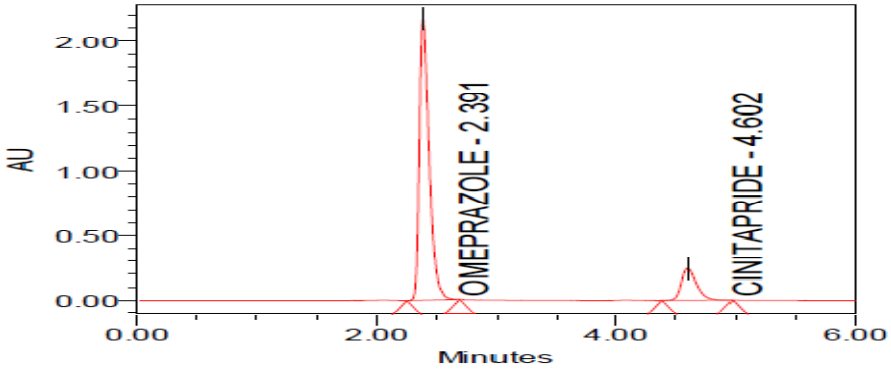
Chromatogram showing Specificity- Blank solution

Fig No : 5.2.24



Chromatogram showing Specificity-Standard-1 solution of Omeprazole and Cinitapride.

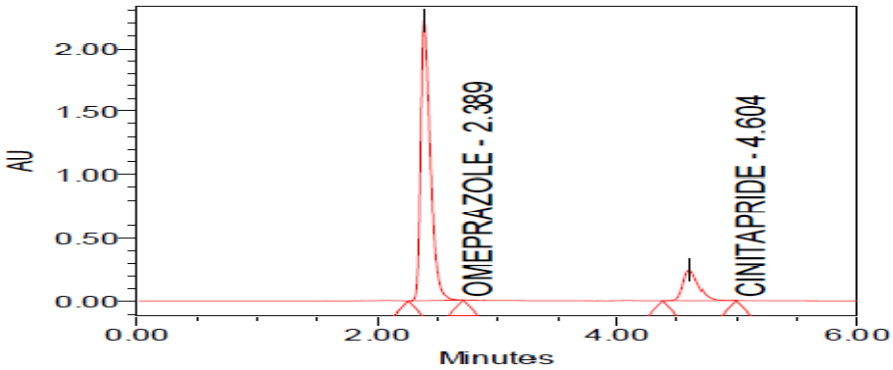
Fig No : 5.2.25



Peak name	RT	Area
Omeprazole	2.391	12523307
Cinitapride	4.602	2149391

Chromatogram showing Specificity- Standard-2 solution of Omeprazole and Cinitapride.

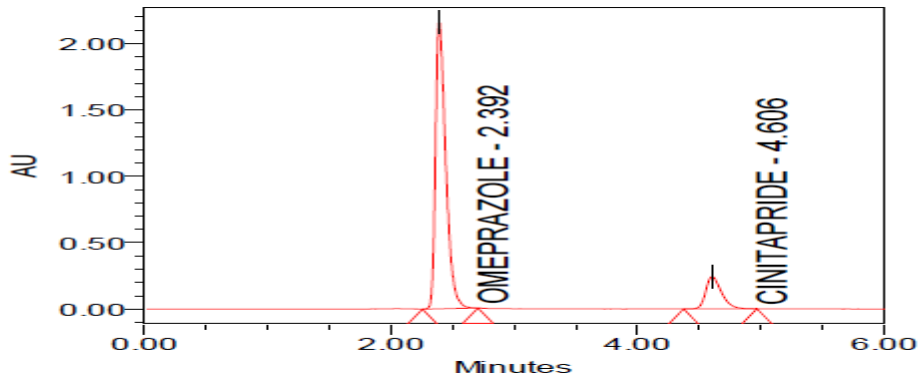
Fig No : 5.2.26



Peak name	RT	Area
Omeprazole	2.389	12604652
Cinitapride	4.604	2160105

Chromatogram showing Specificity-Standard-3 solution of Omeprazole and Cinitapride.

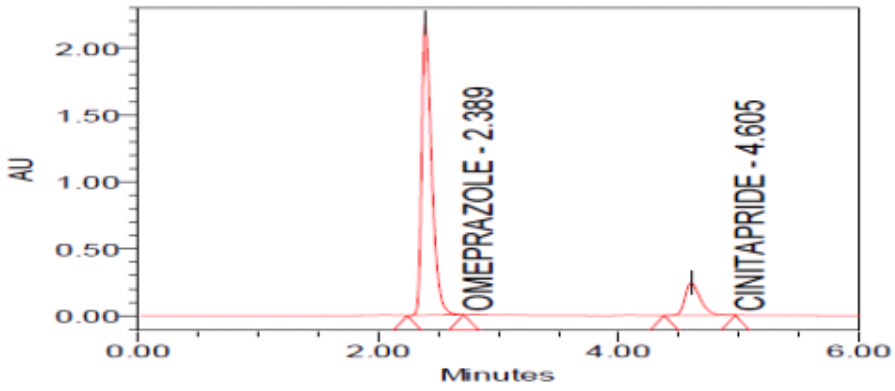
Fig No : 5.2.27



Peak name	RT	Area
Omeprazole	2.392	12529869
Cinitapride	4.606	2153372

Chromatogram showing Specificity- Standard-4 solution of Omeprazole and Cinitapride.

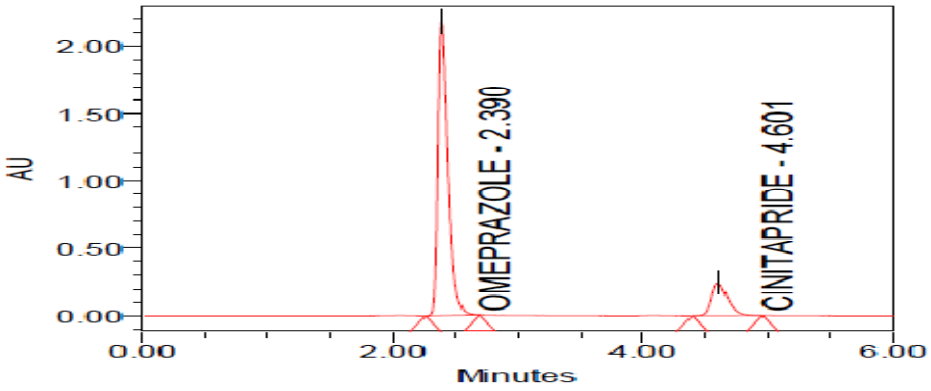
Fig No : 5.2.28



Peak name	RT	Area
Omeprazole	2.389	12553487
Cinitapride	4.605	2153144

Chromatogram showing Specificity- Standard-5 solution of Omeprazole and Cinitapride.

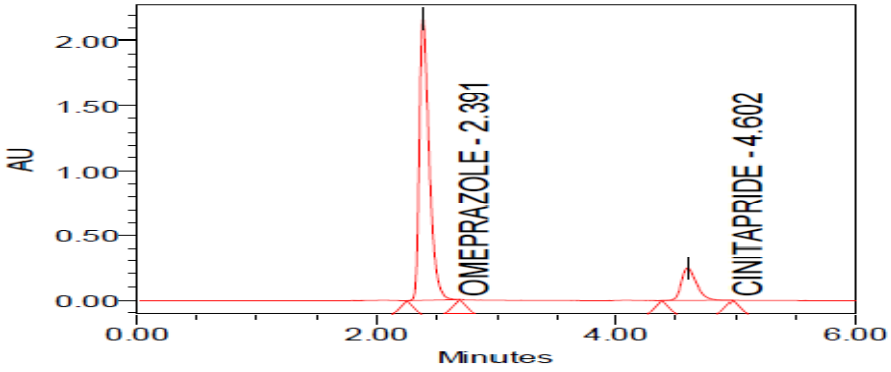
Fig No : 5.2.29



Peak name	RT	Area
Omeprazole	2.390	12530126
Cinitapride	4.601	2149433

Chromatogram showing Blank & Standard solution-1 overlay report of Omeprazole and Cinitapride.

Fig No :5.2.30



Peak name	RT	Area
Omeprazole	2.391	12523307
Cinitapride	4.602	2149391

Table showing results of Specificity of Omeprazole and Cinitapride.

Table No : 11

Parameter	Omeprazole	Cinitapride
Average area	12548288	2153089
SD	33530.0	4369.5
%RSD	0.35	0.26

5.3.5 LINEARITY&RANGE

LINEARITY: Linearity is the ability of the method to obtain test results that are directly proportional to the analyte concentration within a given range.

Range: Range of analytical procedure is the interval between the upper and lower concentration of analyte in the sample (including concentrations) for which it has been demonstrated that the analytical procedure has a sutable level of precision, accuracy, and linearity.

Procedure:

Preparation of Standard Stock Solution:

Accurately 20mg of Omeprazole and 15mg of Cinitapride were weighed and transferred into 20ml volumetric flask, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up diluent. The solution was filtered through 0.45µm membrane filter.

From this pipette out 5.0ml of solution and transferred into 100ml of volumetric flask and the volume was made up with diluent (stock solution).

Preparation of sample solutions:

From the above stock solution pipette out 2.5,3.75,5,6.25, and7.5ml respectively into individual 25 ml of volumetric flasks and diluted up to the mark with diluent to prepare of sample solutions respectively. Mix well and filter through 0.45µm filter.

Inject 50µl of blank solution and each linearity level standard solutions into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The results are tabulated shown in Table No: 11

$$Correl(X,Y) = \frac{\sum (x-\bar{x})(y-\bar{y})}{\sqrt{\sum (x-\bar{x})^2 \sum (y-\bar{y})^2}}$$

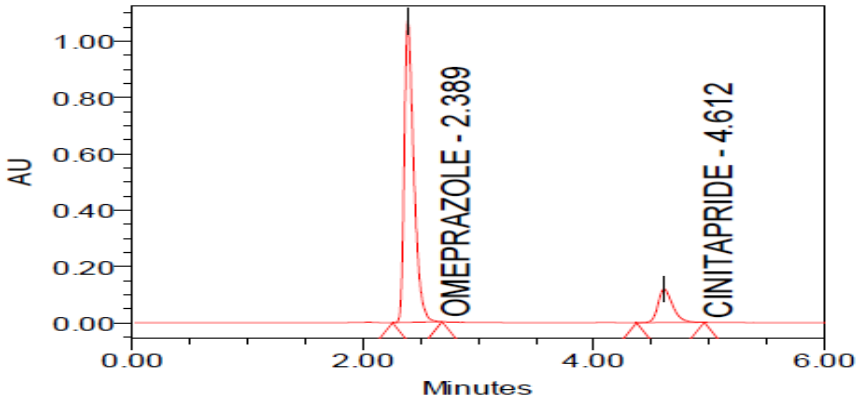
Acceptance criteria for Range:

- Correlation coefficient should not be less than 0.99%

The linearity data and analytical performance parameters of are shown in table and calibration curve of is shown Fig No: 5.2.31

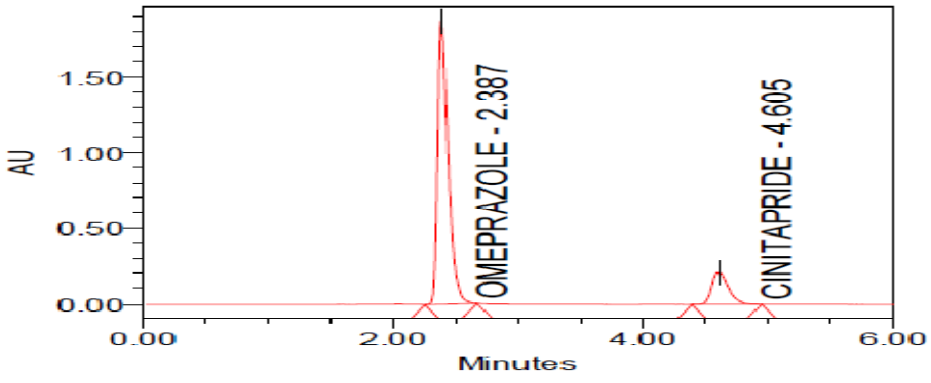
Chromatogram showing Linearity level-1 (50%) of Omeprazole and Cinitapride.

Fig No : 5.2.31



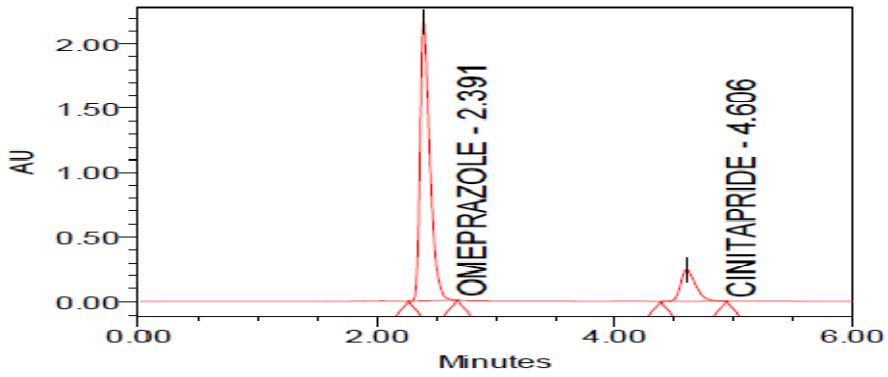
Chromatogram showing Linearity level-2 (75%) of Omeprazole and Cinitapride.

Fig No: 5.2.32



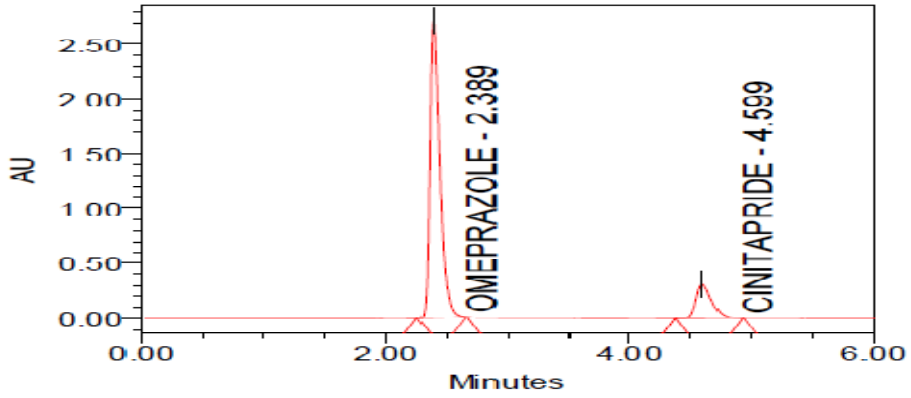
Chromatogram showing Linearity level-3 (100%) of Omeprazole and Cinitapride.

Fig No : 5.2.33



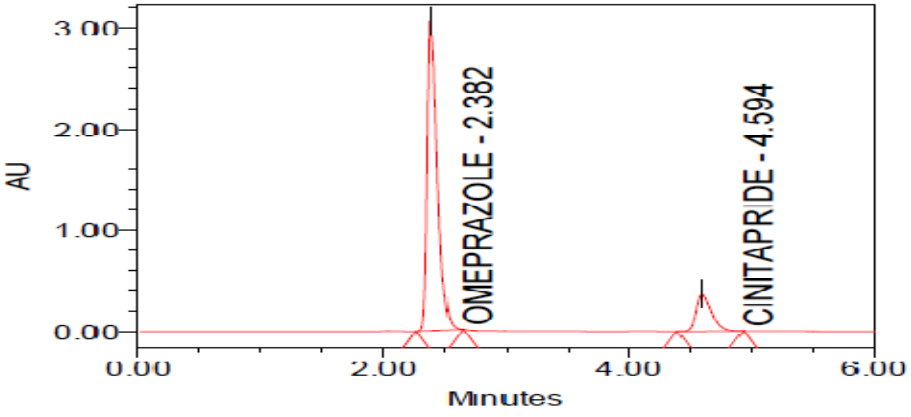
Chromatogram showing Linearity level-4 (125%) of Omeprazole and Cinitapride.

Fig No : 5.2.34



Chromatogram showing Linearity level-5 (150%) of Omeprazole and Cinitapride.

Fig No : 5.2.35



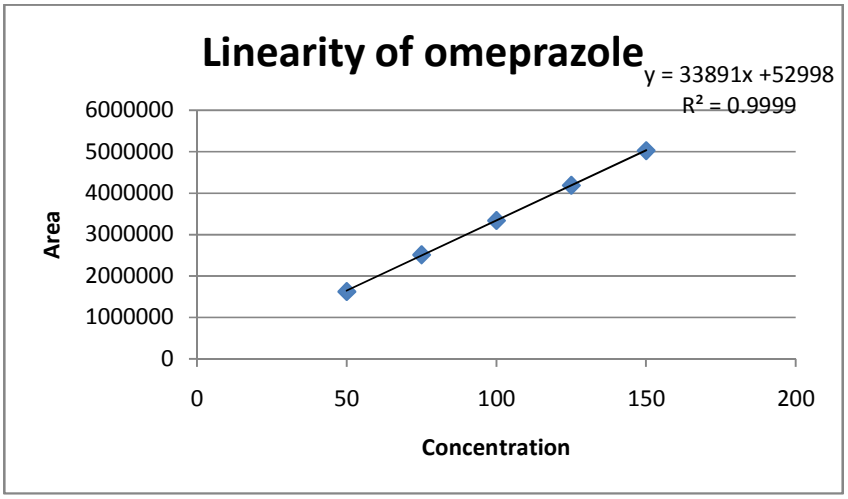
Linearity Results (for Omeprazole):

Table No : 12

S. No	Linearity Level	Concentration	Area
1	I	5µg/ml	6175171
2	II	7.5µg/ml	10622249
3	III	10µg/ml	12431211
4	IV	12.5µg/ml	15542577
5	V	15µg/ml	17822063
Correlation Coefficient			0.999

Figure showing Linearity graph of Omeprazole

Fig No : 5.2.36



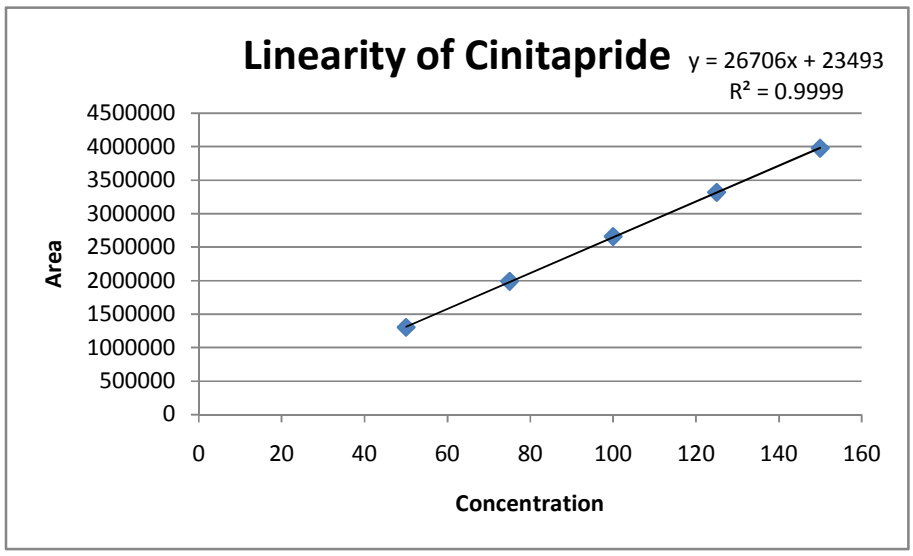
The correlation coefficient value was found to be 0.999

Linearity Results for Cinitapride:

Table No : 13

S. No	Linearity Level	Concentration	Area
1	I	3.75µg/ml	1049368
2	II	5.62µg/ml	1818704
3	III	7.5µg/ml	2138668
4	IV	9.35µg/ml	2710753
5	V	11.25µg/ml	3168558
Correlation Coefficient			0.999

Figure :5.2.37 showing linearity graph of Cinitapride



The correlation coefficient value was found to be 0.999

5.3.6 ROBUSTNESS

It is a measure of ability to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The robustness of an analytical method was determined by analysis of aliquots from homogenous lots by differing physical parameters that may differ but were still within the specified parameters of the assay for example change in physical parameters like flow rate, column temperature and mobile phase ratio.

A) the flow rate was varied at 0.9 to 1.1 ml/min.

Preparation of Standard Solution :

Accurately 20mg of Omeprazole and 15mg of Cinitapride were weighed and transferred into 20ml volumetric flask, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up diluent. The solution was filtered through 0.45µm membrane filter.

From this pipette out 5.0ml of solution and transferred into 100ml of volumetric flask and the volume was made up with diluent

From this 5.0ml of solution was pipette out and transferred into 25ml of volumetric flask and the volume was made up with diluent. The solution was filtered through 0.45µm membrane filter

Inject 50µl of the blank solution and the standard solution l for five times and analysed using varied flow rates (0.9ml, 1.1ml) along with method flow rate and temperature.calculate the %RSD for the area of five replicate injections.

%RSD of Omeprazole and Cinitapride assay under these conditions is calculated and the results are shown in below.

PROCEDURE

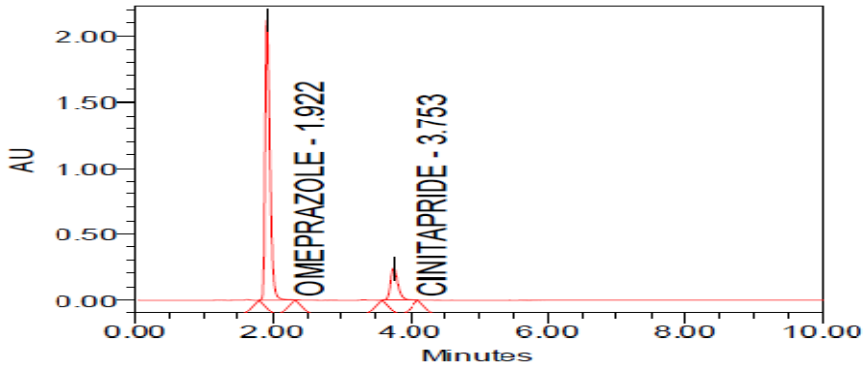
a)The flow rate was varied at ±0.1 ml/min.

Standard solution 70 µg/ml was prepared and analysed using the varied flow rates along with method flow rate and the chromatograms were recorded. The results are shown in table 14.

Flow variation-1 (1.1mL/min):

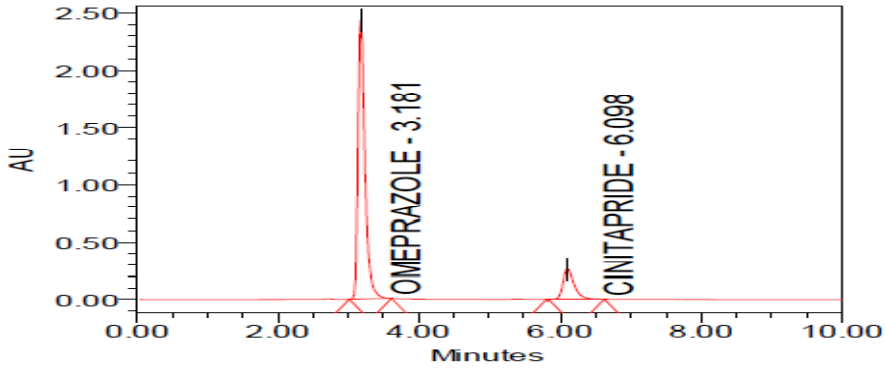
Chromatogram showing Robustness-Flow variation (1.1ml/min) of Omeprazole and Cinitapride

Fig No : 5.2.38



Robustness flow rate (0.9ml):

Fig No :5.2.39



B) Temperature

Preparation of Standard Solution:

Accurately 20mg of Omeprazole and 15mg of Cinitapride were weighed and transferred into 20ml volumetric flask, about 10ml of sodium hydroxide was added and sonicated for 5 minutes to dissolve it. The volume was made up diluent. The solution was filtered through 0.45µm membrane filter.

From this pipette out 5.0ml of solution and transferred into 100ml of volumetric flask and the volume was made up with diluent

From this 5.0ml of solution was pipette out and transferred into 25ml of volumetric flask and the volume was made up with mobile phase. The solution was filtered through 0.45µm membrane filter.

Inject 50µl of the blank solution and the standard solution of 500 µg/ml for five times and analysed using the varied Mobile phase composition along with the actual mobile phase composition in the method and calculate the %RSD for the area of five replicate injections. The chromatograms are as shown in Fig No:5.2. 40-5.2.43 and the results are tabulated shown in Table No: 14.

%RSD Formula: (σ /µ)*100

Acceptance criteria:

Relative standard deviation (RSD) of areas of Omeprazole and Cinitapride from five standard chromatograms in all the flow rate variation and mobile phase composition should not be more than 2.0 %.

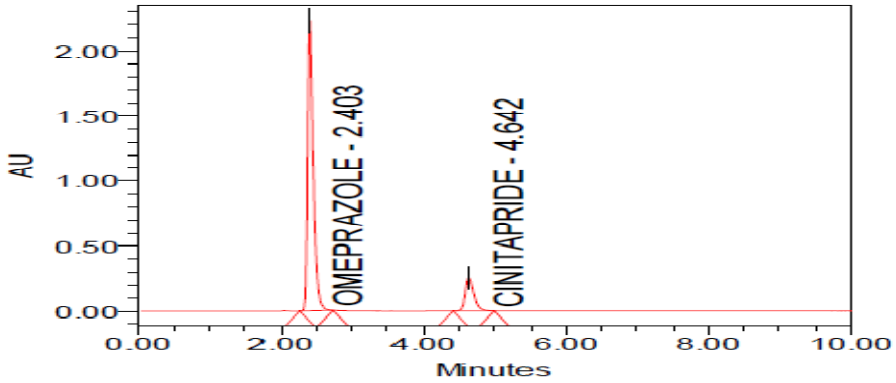
The Temperature was varied from ±5%:

Standard solution 10,7.5 µg/ml was prepared and analysed using the varied temperature the chromatograms were recorded.

The graphs are shown in fig 5.2.39and 5.2.40 and the results are shown in table no : 14

ROBUSTNESS TEMPERATURE 45^0C

Fig No :5.2.40



ROBUSTNESS TEMPERATURE 35^{0}C

Fig No: 5.2.41

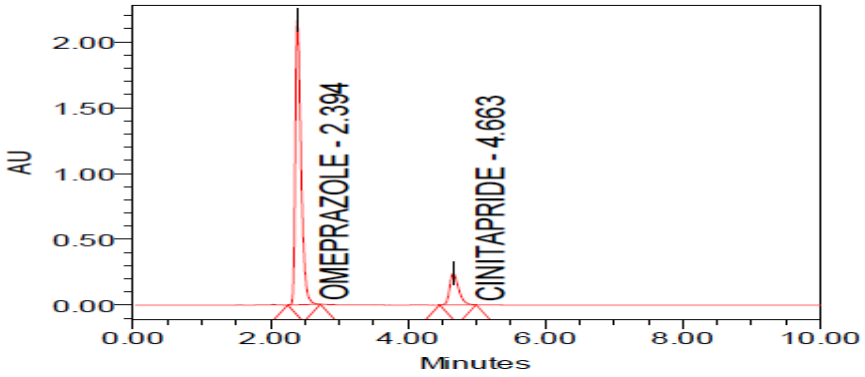


Table showing Robustness results for change in flow rate and Temperature of Omeprazole and Cinitapride .

Table No : 14

Flow rate	**Inj. Sample**	**Area**	**Plate count**	**Tailing**	**RT**	**% RSD**
1.1ml/min	Omeprazole	10160397	3811	1.50	1.922	0.925
	Cinitapride	1744825	6235	1.31	3.753	
0.9ml/min	Omeprazole	16773944	5275	1.21	3.181	0.873
	Cinitapride	2895363	7434	1.31	6.098	
Temperature						
45^{0}C	Omeprazole	12581162	4195	1.60	2.403	
	Cinitapride	2165745	7353	1.32	4.642	0.452
35^{0}C	Omeprazole	12656208	3867	1.60	2.394	
	Cinitapride	2153255	6331	1.37	4.663	0.780

5.3.7 LOD & LOQ:

LIMIT OF DETECTION :

Limit of detection is the lowest concentration of the analyte that can be detected by injecting decreasing amount, not necessarily quantity by the method, under the stated experimental conditions. The minimum concentration at which the analyte can be detected is determined from the linearity curve by applying the formula.

LOD = 3/s/n ratio x drug concentration

The lowest concentration of Omeprazole that can be detected was determined from standard curve was 0.005µg/ml.

The lowest concentration of Cinitapride that can be detected was determined from standard curve was 0.03µg/ml.

LIMIT OF QUANTIFICATION :

Limit of quantitation is the lowest concentration of the analyte in a sample that can be estimated quantitatively by injecting decreasing amount of drug with acceptable precision and accuracy under the stated experimental conditions of the method .Limit of quantification can be obtained from linearity curve by applying the following formula.

LOQ = 10/s/n ratio x drug conc.

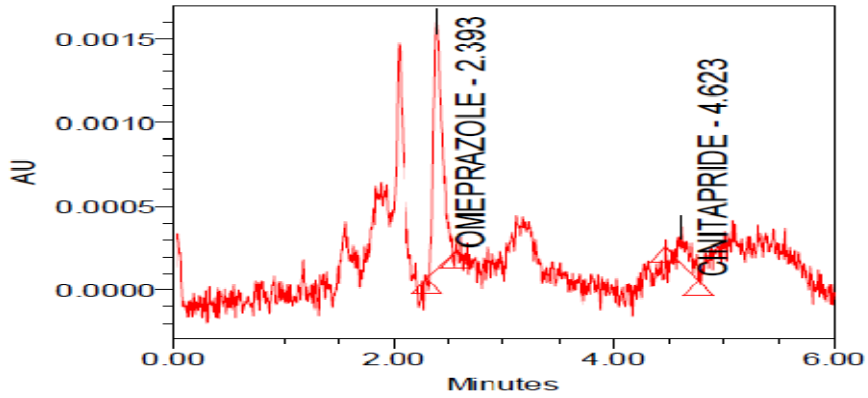
The lowest concentration at which peak can be quantified is called LOQ. It was found to be 0.02µg/ml for Omeprazole and for Cinitapride was found to be 1.4µg/ml.

Table no. 15 LOD & LOQ results showing for Omeprazole and Cinitapride:

Sample	LOD	LOQ
Omeprazole	0.005µg/ml	0.02µg/ml
Cinitapride	0.03µg/ml	1.4µg/ml

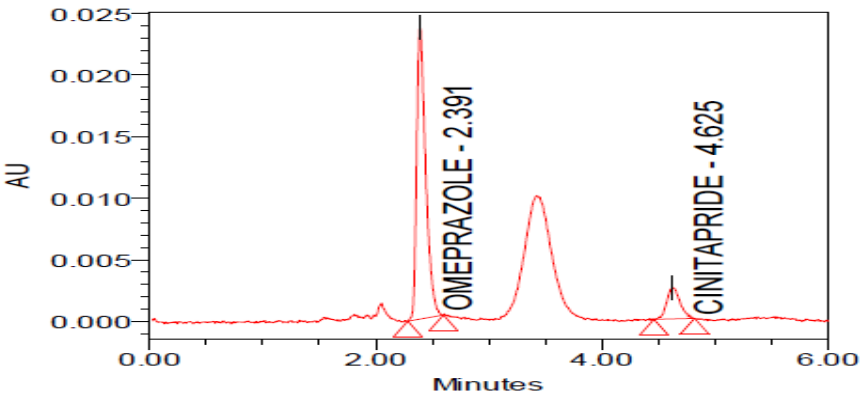
Chromatogram for limit of detection :

Figure no: 5.2.42



Chromatogram for limit of quantification :

Figure no: 5.2.43



5.3.8 DEGRADATION STUDIES :

ACID (0.1N HCL):

Test preparation:

20 Omeprazole and Cinitapride capsules (Burpex)were weighed and the average weight was calculated. Accurately the sample equivalent to 20mg of Omeprazole & 15 mg of Cinitapride was weighed & transferred into 20ml volumetric flask then add

10mlof acid(0.1N HCL) ,shake the flask for 30min and sonicated for 1 hr to dissolve it contentand then add 10 base (0.1N NaoH). The solution was filtered through 0.45µm membrane filter.

5.0ml of above solution was pipetted out and transferred into 100ml of volumetric flask and the volume was made up with diluent.

5.0ml of stock solution was pipetted out and transferred into 25 ml of volumetric flask and the volume was made up with diluent. The solution was filtered through 0.45µm membrane filter. Inject 50 µl of test solution into HPLC system.

BASE(0.1N NaOH):

Test preparation:

20 Omeprazole and Cinitapride capsules(Burpex) were weighed and the average weight was calculated. Accurately the sample equivalent to 20mg of Omeprazole and 15 mg of Cinitapride was weighed & transferred into 20ml volumetric flask then add10 base (0.1N NaoH).,shake the flask for 30min and sonicated for 1 hr to dissolve it contentand then add10mlof acid(0.1N HCL) . The solution was filtered through 0.45µm membrane filter.

5.0ml of above solution was pipetted out and transferred into 100ml of volumetric flask and the volume was made up with diluent.

5.0ml of stock solution was pipetted out and transferred into 25 ml of volumetric flask and the volume was made up with diluent. The solution was filtered through 0.45µm membrane filter. Inject 50 µl of test solution into HPLC system.

PEROXIDE:

Test preparation

20 Omeprazole and Cinitapride capsules(Burpex) were weighed and the average weight was calculated. Accurately the sample equivalent to 20mg of Omeprazole & 15 mg of Cinitapride was weighed & transferred into 20ml volumetric flask and add 5ml peroxide shake flask for 30 min sonicate for 1hr. The solution was filtered through 0.45µm membrane filter.

5.0ml of above solution was pipetted out and transferred into 100ml of volumetric flask and the volume was made up with diluent.

5.0ml of stock solution was pipetted out and transferred into 25 ml of volumetric flask and the volume was made up with diluent. The solution was filtered through 0.45µm membrane filter. Inject 50 µl of test solution into HPLC system.

LIGHT:

Test preparation

20 Omeprazole and Cinitapride capsules(Burpex) were weighed and the average weight was calculated. Accurately the sample equivalent to 20mg of Omeprazole and 15 mg of Cinitapride was weighed and kept under light for 3hr transferred into 20ml volumetric flask about 10ml of Sodium hydroxide was added and sonicated for 5 minutes to dissolve it content. The volume was made up with diluent. The solution was filtered through 0.45µm membrane filter.

5.0ml of above solution was pipetted out and transferred into 100ml of volumetric flask and the volume was made up with diluent.

5.0ml of stock solution was pipetted out and transferred into 25 ml of volumetric flask and the volume was made up with diluent. The solution was filtered through 0.45µm membrane filter. Inject 50 µl of test solution into HPLC system.

WATER:

Test preparation

20 Omeprazole and Cinitapride capsules(Burpex) were weighed and the average weight was calculated. Accurately the sample equivalent to 20mg of Omeprazole & 15 mg of Cinitapride was weighed & transferred into 20ml volumetric flask about 10ml of water was added and sonicated for 1hr to dissolve it content. The volume was made up with diluent. The solution was filtered through 0.45µm membrane filter.

5.0ml of above solution was pipetted out and transferred into 100ml of volumetric flask and the volume was made up with diluent.

5.0ml of stock solution was pipetted out and transferred into 25 ml of volumetric flask and the volume was made up with diluent. The solution was filtered through 0.45µm membrane filter. Inject 50 µl of test solution into HPLC system.

Acceptance criteria:

The net Degardation Should be between 1% to 50%.

Assay formula:

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{\text{average wt of capsule}}{\text{lable claim}} \times \text{working standard.}$$

Where,

AT = Peak Area of sample solution.

AS = Peak Area of standard solution.

WS = Weight of working standard taken in mg

WT = Weight of sample taken in mg

DS = Dilution of Standard solution

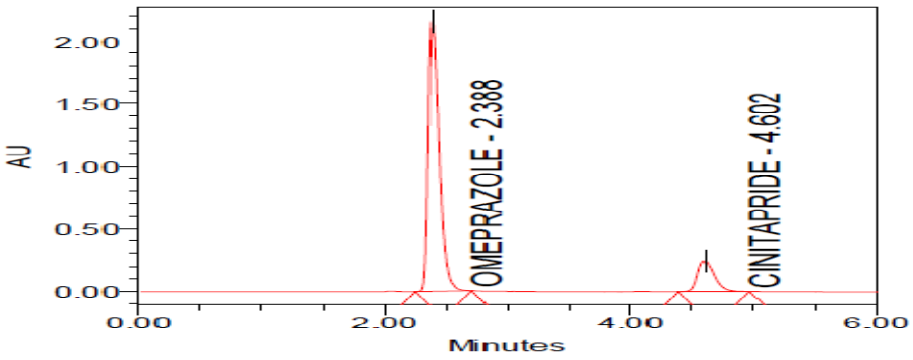
DT = Dilution of sample solution

Table no.16

Degradation studies showing for the Omeprazole and Cinitapride :

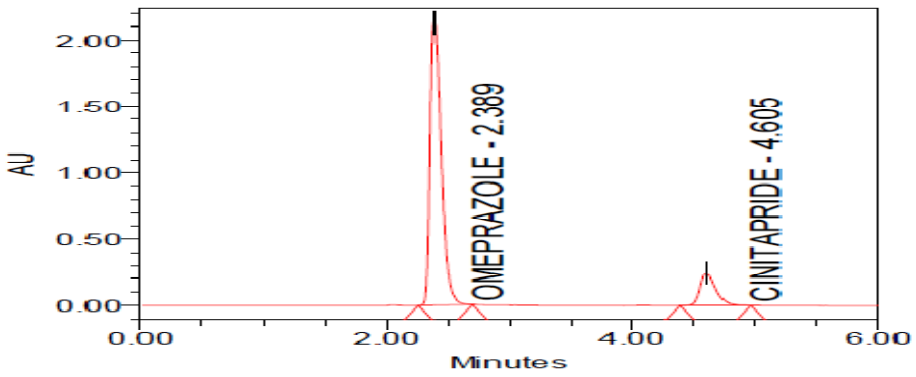
Test	Area of OME	Area of CIN	% Asaay of OME	% Assay of CIN	% deg of OME	% deg of CIN
Acid	11248673	1909516	89	89	10	11
Base	11401894	1368322	90	63	9	37
Peroxide	11469432	1260724	91	58	8	42
Light	11217957	1203319	89	56	10	44
Water	11466336	1981648	57	92	43	8
Avg assay			89	75	16	25

Chromatogram showing peaks of acid solution of Omeprazole and Cinitapride :
Figure no: 5.2.44



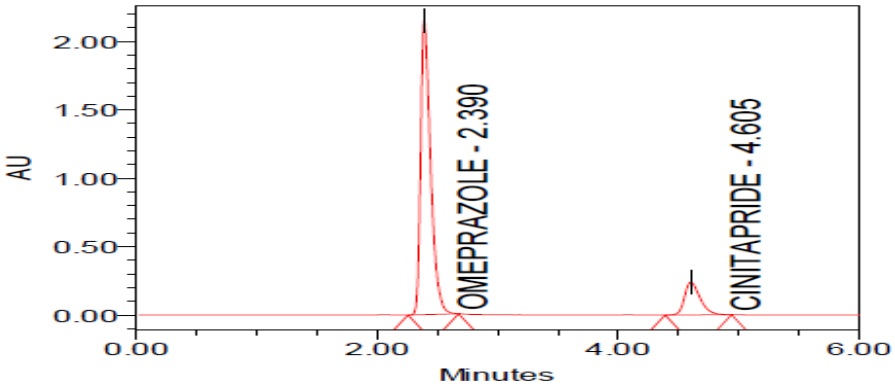
Chromatogram showing peaks of base solution of Omeprazole and Cinitapride :

Figure no: 5.2.45



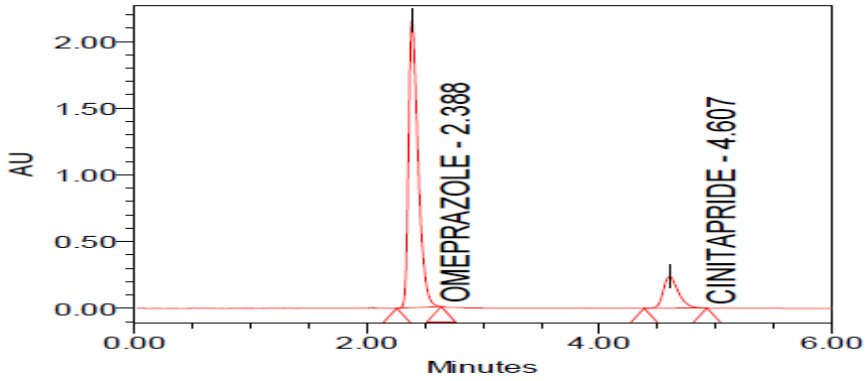
Chromatogram showing peaks of peroxide solution of Omeprazole and Cinitapride:

Figure no: 5.2.46



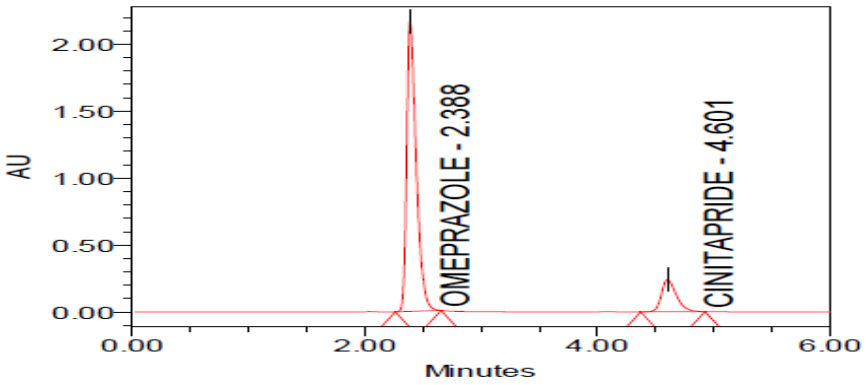
Chromatogram showing peaks of light solution of Omeprazole and Cinitapride:

Figure no: 5.2.47



Chromatogram showing peaks of water solution of Omeprazole and cinitapride :

Figure no: 5.2.48



6. RESULTS AND DISCUSSION

In the present work a new method development and validation was carried out for the estimation of Omeprazole and Cinitapride by RP-HPLC technique. The wavelength selection was made at 215nm since all the two compounds maximum absorbance at 215 nm.

Method development:

For the method development several trials were carried out and reported. These leads to the optimized chromatographic conditions for the estimation of Omeprazole and Cinitapride in pharmaceutical dosage forms by Hypesil BDS C_{18} (4.6×250mm,5µm) column eluted with mobile phase potassium dihydrogen phosphate buffer : acetonitrile (60:40) at a flow rate of 1 ml/ min and a detection wavelength of 215 nm with injection volume of 50 µl at 40°C temperature afforded the best results.

FIXED CHROMATOGRAPHIC CONDITION

Table No: 17

OPTIMIZED CHROMATOGRAPHIC CONDITIONS	
Mode of separation	isocratic elution
Mobile phase	Solvent-A: potassium dihydrogen phosphate pH-6.5 Solvent-B: acetonitrile(60:40)
Column	Hypesil BDS C_{18} (4.6 x 250mm, 5 µm)
Flow rate	1 mL/ min
Detection Wavelength	215 nm
Injection volume	50 µl
Column oven temperature	40°C
Run time	6 min

Results of Trial-1:

Peak shapes are not good, so another trail is made by changing the column

Results of Trial-2:

Peaks are merged, so another trial is made by changing the mobile phase ratio.

Results of Trial-3:

Peak shape was good but theoretical plates are not passed, so another trail is made.

6.1. METHOD VALIDATION:

After method development, the validation of the current method has been performed in accordance with USP requirements for assay determination (Category-I: analytical methods for quantitation of active ingredients in finished pharmaceutical products) which include accuracy, precision, selectivity, linearity and range, robustness and ruggedness.

Table showing Summary of results of method validation for Omeprazole and Cinitapride:

Table :18

S.NO	Parameter	Limits	Observation	
			OME	CIN
1	Specificity	No interference	No interference	No interference
2	System precision Method precision	RSD NMT 2.0%	0.60 0.59	0.68 0.65
3	Linearity range	Correlation coefficient NMT – 0.999	0.999	0.999
4	Accuracy	% Recovery range 98 –10 2 %	99.6	100.2
5	Limit of Detection	Signal noise ratio should be more than 3:1	0.005 µg/ml	0.03 µg/ml
6	Limit of Quantitation	Signal noise ratio should be more than 10:1	0.02 µg/ml	1.4 µg/ml
7	Similariy factor	NMT 2	0.9838	0.9646
8	Number of Theoretical Plates	NLT 2000	6382	4025
9	Robustness Change in flow and temperature		No effect on system suitability parameters	No effect on system suitability parameters
10	Forced degradation	Well within the limit	Pass	Pass

System suitability: From the system suitability studies it was observed that % RSD of retention times were found to be 0.2 (Omeprazole) & 0.3% (Cinitapride). USP tailing factor was found to be 1.5(Omeprazole) &1.3 (Cinitapride). Average theoretical plates are found to be 6365 (Omeprazole) & 3983 (Cinitapride). All the parameters were within the limit.

Linearity: From the Linearity data it was observed that the method was showing linearity in the concentration range of 5-15µg/ml for Omeprazole & 3.75-11.25 µg/ml for Cinitapride. Correlation coefficient was found to be 0.999 for Omeprazole & 0.999 for Cinitapride.

Precision: The RSD of peak area for standard chromatograms of system precision were found to be 0.60% for Omeprazole & 0.68% for Cinitapride, and in method precision it was found to be 0.59% for Omeprazole & 0.65% for Cinitapride. It passes method and system precision.

Accuracy: The percentage recovery of Omeprazole and Cinitapride were 99.6% and 100.2%.The percentage recovery of the two drugs were within the limit.

SPECIFICITY: The chromatograms of standard and sample are identical with nearly same retention time. No interference due to placebo and sample at the retention time of analyte which shows that the method was specific.

ROBUSTNESS : As the % RSD of retention time and asymmetry were within limits for variation (± 0.1ml)flow rate and (±5^{0}C) temperature.

As the % RSD of retention time and asymmetry were within limits for variation in flow rate (± 0.1 ml). Hence the allowable flow rate should be within 0.9 ml to 1.1 ml and temperature should be 35-45^{0}C.

FORCE DEGRADATION STUDIES: The percentage degradation of Omeprazole and Cinitapride parameters like acid, base, peroxide, water and light studies were found to be within the limits.

7. CONCLUSION

- A new method is developed for Simultaneous Estimation of Omeprazole andCinitapride by RP-HPLC method. The sample preparation is simple and the analysis time is short. The analytical procedure is validated as per ICH guidelines and shown to be accurate, precise and specific.

- This method represents a fast and sensitive analytical procedure for the simultaneous quantization of Omeprazole and Cinitapride. The method is amenable to the routine analysis of large numbers of samples with good precision and accuracy.

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