VALIDATED METHOD DEVELOPMENT FOR THE QUANTIFICATION OF CINITAPRIDE AND PANTOPRAZOLE BY SPECTROPHOTOMETRY AND RP-HPLC IN BULK AND ORAL DOSGE FORM

A dissertation submitted to

The Tamilnadu Dr. M.G.R. Medical University

Chennai – 600 032.

In partial fulfillment of the requirements
for the award of the degree of

MASTER OF PHARMACY
IN
PHARMACEUTICAL CHEMISTRY

Submitted by

Reg. No. 26108332

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY
COLLEGE OF PHARMACY
MADRAS MEDICAL COLLEGE
CHENNAI – 600 003

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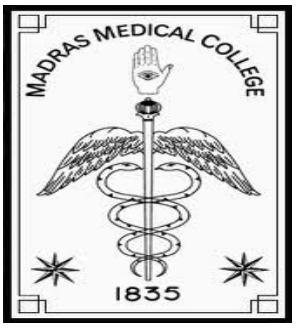

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CERTIFICATE

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CONTENTS

LIST OF ABBREVIATIONS

S.No	ABBREVIATION	EXPANSION
1.	CNP	Cinitapride
2.	PNP	Pantoprazole
3.	Avg.	Average
4.	LOD	Limit of Detection
5.	LOQ	Limit of Quantification
6.	µg	Microgram
7.	mg	Milligram
8.	mL	Millilitre
9.	R.S.D	Relative standard deviation
10.	Rt	Retention time
11.	Std.	Standard
12.	SD	Standard deviation
13.	UV	Ultra-Violet Spectroscopy
14.	Wt.	Weight
15.	HPLC	High Performance Liquid Chromatography
16.	RP-HPLC	Reverse phase High Performance Liquid Chromatography
17.	AAC	Acetyl acetone
18.	EAA	Ethyl acetoacetate
19.	ATC	Ammonium thiocyanate
20.	Abs	Absorbance
21.	Conc.	Concentration
22.	1,10-PHT	1,10- Phenanthroline

INTRODUCTION

Analytical chemistry is the branch of chemical science which deals with the identification of components of a mixture by separating them and then analyzing how much percentage of each component is present in the mixture, in other words, it is a branch of chemistry dealing with any type of technique which may yield any sort of information about a chemical system occurring in nature or of artificial or synthetic origin.

Analytical chemistry involves two basic or major divisions.

I. Qualitative analysis:

It involves the establishment of chemical composition of a material of natural or synthetic origin i.e., it deals with the identification of atoms, ions and molecules that make the material being analyzed. Two essential requirement of a qualitative analytical method are

Specificity

Ability to detect the presence of an unknown element in the presence of other elements. Example: iron in presence of nickel, manganese, chromium etc.

Sensitivity

Smallest quantity of the element that can be detected by the given method. It is expressed in magnitudes of the order of 1mgm (one millionth part of a gram)

II. Quantitative analysis

It involves the estimation or determination of the amount of element on molecule qualitatively detected present in the compound being analyzed. In addition to specificity and sensitivity quantitative analysis is characterized by accuracy.

Significance of Analytical Chemistry

- ❖ The identification of gases which was once considered as a single substance was of great significance in chemical analysis.
- ❖ The chemical composition of a matter is determined.
- ❖ Structure elucidation of a compound is possible.
- ❖ Helps to analyze which component of a mixture or which part of the chemical structure was responsible for the reaction

Significance of Pharmaceutical analysis

- ✓ Structure elucidation of newly synthesized drug or isolated from natural origin.
- ✓ Provides information on the identity, purity, content and stability of starting materials, excipients and active pharmaceutical ingredients. (Pharmaceutical analysis – Metrohm 2009)
- ✓ Pharmacokinetic studies of drugs become easier
- ✓ Studies involving metabolites of the drugs was possible
- ✓ Impurity profiling of the drug
- ✓ Stability indicating degradation studies

- ✓ Chiral separation of enantiomers and analyzing which enantiomer is responsible for the bio activity
- ✓ Bio analytical studies became simpler and easy to perform by the emergence of hyphenated analytical techniques
- ✓ To check the purity of the raw material thus making cost effective synthesis of drugs.
- ✓ Structure elucidation of proteins and creation of protein data bank. In medicine, analytical chemistry is the basis for clinical laboratory tests which help physicians diagnose disease and chart progress in recovery.
- ✓ In Industry, analytical chemistry provides the means of testing raw materials and for assuring the quality of finished products whose chemical composition is critical.
- ✓ Environmental quality is often evaluated by testing for suspected contaminants using the techniques of analytical chemistry (Anonymous www.acs-analytical.duq.edu/whatisanalyticalchem.html)
- ✓ The recent developments of computer automation and information technologies have innervated analytical chemistry to initiate a number of new biological fields. For example, automated DNA sequencing machines were the basis to complete human genome projects leading to the birth of ***genomics.*** Protein identification and peptide sequencing by mass spectrometry opened a new field of ***proteomics.***

Analytical chemistry plays important role in quality control and quality assurance. It helps to analyze whether a given raw material or finished product is of

good acceptable quality (quality assurance) and to control the quality of the analyte up to the standard so that the raw material could be further taken in to the manufaturing process and the finished product could be let in to the market.

Quality assurance is a wide-ranging concept covering all matters that individually or collectively influence the quality of a product. It is the totality of the arrangements made with the object of ensuring that pharmaceutical products are of the quality required for their intended use. Quality assurance therefore incorporates GMP

Quality control, commonly shortened to QC refers to all those processes and procedures designed to ensure that the results of laboratory analysis are consistent, comparable, and accurate and within specified limits of precision.

Methods of analytical chemistry

The compound of interest is separated by precipitation or extraction or distillation. Further they are subjected to qualitative or quantitative analysis.

- **Qualitative analysis**

  Separated components treated with reagents that yield product that could be recognized by color, boiling point, melting point, solubility in a series of solvents, odour, optical activity and refractive index.

- **Quantitative analysis**

  The separated components is quantitatively analysed by gravimetric or titrimetry.

Quantitative Analytical Techniques involve the following techniques (Willard, et al. 1986; Douglas, A. Skoog, et al.,2004; P.D.Sethi, 2001)

The different classical methods are

- ✓ **Titrimetric methods:** The volume of a solution containing sufficient reagent to react completely with the analyte is measured. It include acid – base, precipitation, redox, complexometric**,** diazotisation, aqueous and non aqueous titrations which involve either direct and indirect (back titration) methods
- ✓ **Gravimetric methods** determine the mass of the analyte or some compound chemically related to it by weighing mass of the analyte after extraction, derivative separation, residue after ignition.

Instrumental Methods

- ✓ **Spectrophotometric methods** are based on measurement of the interaction between electromagnetic radiation and analyte. It includes UV-Visible, Infra red, Raman, Mass, Nuclear magnetic resonance(NMR), fluorimetry, flame photometry, nephelo- turbidimetry.
- ✓ **Electro analytical methods** involve the measurement of electrical properties such as voltage, current, resistance etc. It include potentiometry, conductometry, amperometry, electrogravimetry etc
- ✓ **Separation methods** all types of chromatography like thin layer, paper, column, gas, High Performance Liquid and High Performance Thin Layer Chromatography.

- ✓ **Miscellaneous methods include**

  thermal analysis(based on heat of reaction), kinetic techniques (based on the kinetics of reactions of the analyte), enzyme assay

- ✓ **Hyphenated techniques** includes the combination of the above discussed methods like GC-MS (Gas chromatography – Mass spectrometry), LC-MS (Liquid chromatography – Mass spectrometry), GC-IR (Gas chromatography – Infrared spectroscopy), ICP-MS (Inductively coupled plasma–Mass spectrometry), LC-DAD-MS (Liquid chromatography – Diode Array Detector – Mass spectrometry), LC-MS-MS (Liquid chromatography – Mass spectrometry – Mass spectrometry).

Analytical Method Validation (Code Q2A- ICH Guidelines IP, 1996)

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. For pharmaceutical analytical methods guidelines from the United States Pharmacopeia (USP) International Conference on Harmonization (ICH), and Food and Drug Administration (FDA) provide a frame work for performing such validations.

Types of Validation (Sethi, 2008)

- **Prospective validation**

  This is employed when historical data of the productis not available. Such validation is conducted prior torelease of eithr new product or product made under revised / new manufacturing process where revision may affect the product characters.

- **Retrospective validation**

  This provides a review and evaluation of existing information for comparison when historical data is sufficient and readily available. Retrospective validation is acceptable only when specific test results generated by reliable analytical method on number of samples are available to allow statistical analysis.

- **Concurrent validation**

  This verifies the quality characteristics s of a particular batch and provides assurance that the same quality would be attained again when subsequent batches are manufactured and analyzed under similar conditions

Reasons for Validation

- Enables scientiststo evaluate procedures for checking complaints and taking remedial measures.
- To provide high degree of confidence that the same level of quality is consistently built in each unit of finished product from batch to batch.
- To take appropriate action, in case of non-compliance.
- Closer interaction with pharmacopoeial harmonization particularly with respect to determination of imprities and their limits.

Methods need to be validated or revalidated:

↳ Before their introduction into routine use

- Whenever the conditions change for which the method has been validated. e.g., instruments with different characteristics.

- Whenever the method is changed, and the change is outside the original scope of the method, the International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for human use has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics which includes.

    - Linearity
    - Specificity
    - Accuracy
    - Precision
        - Repeatability
        - Intermediate precision
        - Reproducibility
    - Limit of detection
    - Limit of quantification
    - Range
    - Ruggedness
    - Robustness

Linearity

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of analytes in samples. Acceptability of linearity data is judged by the correlation coefficient and intercept of the linear regression line for the response versus concentration plot. The range of correlation coefficient should be NLT -1 and NMT +1.

Specificity

It is the ability of the method to accurately measure the analyte response in the presence of all sample components like placebo formulation, synthesis intermediates, excipients, degradation products, process impurities etc., in comparison with the response of the standard analyte (or only the analyte).

Accuracy

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. Accuracy of the method is the closeness of the measured value to the true value.

Accuracy is calculated as the percentage of recovery by the assay of the known added amount of standard analyte in the previously analyzed sample (spiked sample), or as the difference between the mean and the accepted true value, together with confidence interval. Accuracy is usually performed in triplicate at three levels over a range of 50 to 150% of the target concentration.(Analytical chemistry 1996)

Precision

It may be measure of either the degree of reproducibility, intermediate precision or repeatability of the analytical method under normal operating conditions. It is usually expressed as SD (Co-efficient of variation) of a series of measurements.

Repeatability (intraday precision) refers to the use of analytical procedure within the laboratory over a short period of time using the same analyst with same instrument.

Reproducibility refers to the use of analytical procedure in different laboratories as in a collaborative study.

Detection Limit (LOD)

It is the lowest amount of analyte in the sample that produces a response detectable above the noise level of the system, but not necessarily quantified as an exact value. Based on the standard deviation of the response and the slope the detection limit (LOD) may be expressed as

$$LOD = \frac{3.3\sigma}{S}$$

Where,

σ - standard deviation of the response,

S – Slope of the calibration curve of the analyte.

Quantitation Limit (LOQ)

It is the lowest amount of analyte in the sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. If not determined experimentally it can be calculated as the analyte concentration that gives S/N= 10 (Signal to Noise ratio). The quantitation limit is expressed as the concentration of analyte (eg : percentage parts per million) of the sample.

$$LOQ = \frac{10\,\sigma}{S}$$

Where, σ - standard deviation of the response,

S – Slope of the calibration curve (of the analyte)

Range

Range of a method is the interval between the upper and the lower levels of analyte (including these concentrations) that have been demonstrated to be determined within a suitable level of precision, accuracy and linearity.

Sensitivity

The sensitivity of an analytical method is the capability of the method to discriminate small differences in concentration or mass of the test analyte. In practical terms, sensitivity is the slope of the calibration curve that is obtained by plotting the response against the analyte concentration or mass.

Ruggedness

Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. Ruggedness is determined by the analysis of aliquots from homogeneous lots in different laboratories.

Robustness

Robustness tests examine the effect that operational parameters have on the analysis results. For the determination of a method's robustness, a number of method parameters, for example, pH, flow rate, column temperature, injection volume, detection wavelength or mobile phase composition, are varied within a realistic range, and the quantitative influence of the variables is determined. If the influence of the parameter is within a previously specified tolerance, the parameter is said to be within the method's robustness range.

System suitability parameters for HPLC analysis:

The purpose of the system suitability test is to ensure that the complete testing system (including instrument, reagents, columns, analysts) is suitable for the intended application. System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. There are numerous guidelines which detail the expected limits for typical

chromatographic methods. In the current FDA guideline on "Validation of Chromatographic methods" the following acceptance limits are proposed as initial criteria.

Capacity Factor *(k')*

It is the measure of a sample peak in the chromatogram being specific for a given compound, a parameter which specifies the delay of a substance to be separated.

$$k' = \frac{t_r - t_0}{t}$$

Where,

t_r = retention time from time of injection to time of elution of peak maximum.

t_o = void volume

Resolution *(R_s)*

The resolution R_s is a function of column efficiency N and is specified to ensure that closely eluting compounds are resolved from each other to establish the general resolving power of the system and to ensure that internal standards are resolved from the drug.

$$R = \frac{2(t_1 - t_2)}{W_1 + W_2}$$

Where,

t_1 and t_2 = retention times of first and second adjacent bands.

W_1 and W_2 = width of peak

Limit = R_s of >2 between the peak of interest and the closest potential interfering peak is desirable.

Tailing Factor *(T)*

The tailing factor *T,* a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced.In some cases, values less than unity may be observed. As peak asymmetry increases, integration, and hence precision becomes less reliable.

$$T = \frac{W_{0.05}}{2f}$$

Where,

$W_{0.05}$ = width of peak at 5% height

f = Distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline.

Limit: ≤ 2 is preferable.

Theoretical plates *(N)*

The number of theoretical plates, *N* is a measure of column efficiency. For Gaussian peaks, it is calculated by the equations.

$$N = 16(t / w)^2 \text{ (or)} \quad N = 5.54(t / w_{1/2})^2$$

Where,

t = retention time of substance.

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

$w_{1/2}$ = 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.

The limits of system suitability are tabulated below (system stablility -online):

S.No	**System suitability parameters**	**Limits**
1.	Capacity factor	k'>2
2.	Injection Precision	RSD<1% for n > 5
3.	Resolution	Rs>2
4.	Tailing factor	T<2
5.	Theoretical plates	N > 2000

STATISTICS IN ANALYTICAL CHEMISTRY

It is the science of making effective use of numerical data relating to groups of individuals or experiments. It is the quantitative science, meaning that desired result is almost always numeric.

Quantitative results are obtained using devices or instruments that allow us to determine the concentration of a chemical in a sample from observable signal. One of the use of statistics in analytical chemistry is to provide an estimate of the likely value of that error, in otherwords, to establish the uncertainty associated with the measurement.

STATISTICAL PARAMETERS

The precision or reproducibility of the analytical method was determined by repeating the analysis and the following statistical parameters were calculated.

Mean

The Mean or average is obtained by dividing the sum of observed values by the number of observations (n).

$$\bar{x} = \frac{\sum X}{n}$$

Standard Deviation

Standard deviation (SD) is measure of data dispersion or variability. The standard deviation gives an idea of how close the entire set of data is to the average value. SD is also called root mean square deviation.

$$SD = \sum (X - \bar{X})^2 / \sqrt{n-1}$$

Relative Standard Deviation

The relative standard deviation (RSD) is also called co-efficient of variation. It is defined as

$$RSD = SD / X$$

$$\%RSD = \frac{SD}{X} \times 100$$

Regression Equation

A regression is a statistical analysis assessing the association between two variables. It is used to find the relationship between two variables. Regression equation **y = mx + c**

Where,

m – the slope of regression line;

c– the intercept point of regression line and the y axis

Standard Error

Standard error is a statistical term that measures the accuracy with which a sample represents a population. In statistics, a samplemean deviates from the actual mean of a population; this deviation is the standard error. Standard error (SE) is given by

$$SE = \frac{SD}{\sqrt{n}}$$

DRUG PROFILE

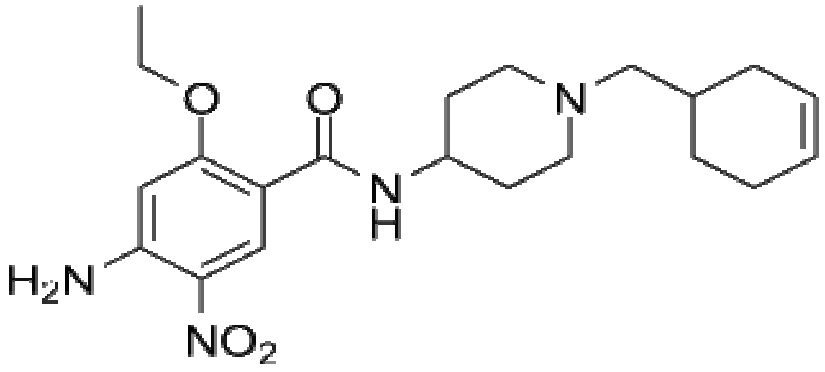
I. CINITAPRIDE (O' Neil MJ., 2007; S. C. Sweetman 2002)

Cinitapride is available in the form of cinitapride hydrogen tartarate which is a new gastro prokinetic agent. It is a substituted benzamide with 5-HT receptor antagonist and agonist activity. It is chemically described as 4-Amino-N-[1-(3-cyclohexen-1-ylmethyl)-4-piperidinyl]-2-ethoxy-5-nitrobenzamide hydrogen L-(+)-tartarate.

EMPIRICAL FORMULA : $C_{25}H_{36}N_{40}O_{10}$

MOLECULAR WEIGHT : **552.58.**

STRUCTURE



Cinitapride

DESCRIPTION

The tartarate salt of cinitapride is almost lemon yellow crystalline powder.It is freely soluble in methanol, soluble in water, ethanol, partially soluble in acetone, insoluble in carbon tetra chloride, chloroform.

SOLUBILITY

Cinitaprde hydrogen tartarate is freely soluble in methanol, Ethanol, acetonitrile; soluble in water, 0.1N hydrochloric acid and in chloroform, practically insoluble acetone, ether and precipitates in 0.1N sodium hydroxide.

MECHANISM OF ACTION

Cinitapride is a substituted benzamide gastroenteric prokinetic agent acting via complex, but synergistic effects on serotonergic 5-HT_2 (inhibition) and 5-HT_4 (stimulation) receptor and dopaminergic D_2 (inhibition) receptors in the neuronal synapses of the m yenteric plexus

INDICATIONS AND USAGE

Cinitapride is indicated for the treatment of gastrointestinal disorders associated with motility disturbances such as gastro esophageal reflux disease, non-ulcer dyspepsia and delayed gastric emptying.

DOSAGE AND ADMINISTRATION

The usual daily dosage for adults is 1mg of Cinitapride orally thrice a day 15 minutes before meals. The dose may be reduced, if required, depending on the patient's age and symptoms at the discretion of the physician.

PHARMACOKINETICS

Pharmacokinetic studies in man following oral and intramuscular administration have been made using doses substantially higher than the therapeutic

dose due to the absence of a sufficiently sensitive analytical method for the detection of plasma concentrations following very low doses of cinitapride. The absorption of cinitapride (12mg) following oral administration was rapid, with peak levels being achieved 2 h after dosing; absorption following intramuscular administration (4mg) was even more rapid, with peak levels (50% more that oral levels) being achieved 1 h after dosing.

CONTRAINDICATIONS

Cinitapride is contraindicated in patients with known hypersensitivity to cinitapride or any of the other constituents of the formulation.

DRUG INTERACTIONS

Anticholinergic agents like atropine, scopolamine etc, may reduce the action of cinitapride. Cinitapride can enhance the effect of medicines that are used for the treatment of illnesses of the nervous system and for insomnia. Cinitapride can also alter the absorption of some medicines e.g. digoxin as it simulates gastric emptying.

UNDESIRABLE EFFECTS

Cinitapride has usually been very well tolerated with the most common adverse events in clinical trials being drowsiness and diaorrhea. Extrapyramidal effects (involuntary muscular movements of the head, neck and tongue) have been reported occasionally. Very rarely, cutaneous reactions like eruptions, itching or angioedema and gynaecomastia have been reported.

OVERDOSAGE

The symptoms of overdose include drowsiness, confusion and extrapyramidal effects. Cinitapride does not cause QT prolongation. In case of excessive overdosage, the usual measures of gastric lavage and symptomatic therapy should be applied. The extrapyramidal effects should be treated with antiparkinsonians, anticholinergics or antihistaminics with anticholinergic properties.

II. PANTOPRAZOLE SODIUM SESQUIHYDRATE (www.drugbank)

Pantoprazole is an irreversible proton pump inhibitor drug used for short-term treatment of erosion and ulceration of the esophagus caused by gastro esophageal reflux disease. .Like other drugs of its class, reduces gastric acid secretion by inhibition of portion on the parietal cells. Pantoprazole inhibits H+/K+ ATPase pump function thereby reducing gastric acid secretion. It also has a role in the eradication of *H.pylori*. Chemically, pantoprazole is 6-(difluoromethoxy)-2-{[(3,4-dimethoxypyridin-2-yl)methane]sulfinyl}-1H-1,3-benzodiazole

EMPIRICAL FORMULA:

Pantoprazole free base **: $C_{16}H_{15}F_2N_3O_4S$**

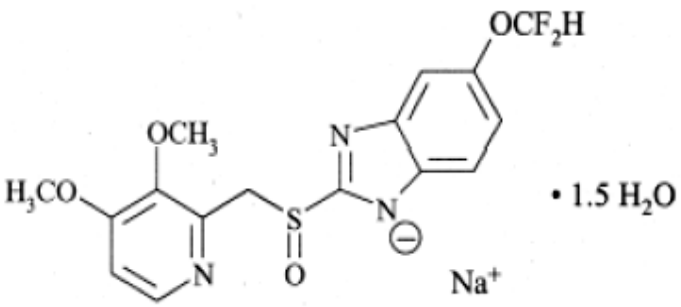
Pantoprazole sodium sesquihydrate **: $C_{16}H_{14}F_2N_3O_4SNa.\ 1.5\ H_2O$**

MOLECULAR WEIGHT:

Pantoprazole free base : **383.37 g/mol**

Pantoprazole sodium sesquihydrate : **432.38 g/mol**

STRUCTURE



Pantoprazole sodium sesquihydrate

DESCRIPTION:

The sodium salt of pantoprazole is almost white to off-white, crystalline powder.It is freely soluble in methanol, soluble in water, ethanol, partially soluble in acetone, insoluble in carbon tetra chloride, chloroform. Because of gradual degradation of pantoprazole sodium during heating, the melting point cannot be determined

MECHANISM OF ACTION

Pantoprazole is a proton pump inhibitor (PPI) that suppresses the final step in gastric acid production by forming a covalent bond to two sites of the (H+, K+)-ATPase enzyme system at the secretory surface of the gastric parietal cell. This effect is dose-related and leads to inhibition of both basal and stimulated gastric acid secretion irrespective of the stimulus. The binding to the (H+, K+)-ATPase results in a duration of antisecretory effect that persists longer than 24 hours.

PHARMACOKINETICS (Radhofer-Welte S.,1999)

Absorption : Well absorbed; peak plasma concentrations: Approx 2-3 hr

Oral bioavailability : Approx 77% with enteric-coated formulation (does not vary after single or multiple doses).

Distribution : Protein-binding: 98%; volume of distribution: 11-24L.

Metabolism : Hepatic; mainly by cytochrome *CYP2C19* and slightly by *CYP2D6* and *CYP2C0.*

INDICATIONS

Pantoprazole used for short-term treatment of erosion and ulceration of the esophagus caused by gastro- esophageal reflux disease. It is the drug of choice for the initial treatment of *H.pylori* along with other drugs.

DRUG INTERACTIONS

Pantoprazole shows interactions: Indinavir, Enoxacin, Clopidogrel, Ketoconazole, Topotecan, Cefditoren, Itraconazole, Dasatinib.

FOOD INTERACTIONS : Take without regard to meal

INCOMPATIBILITY : Incompatible with midazolam and zinc.

REVIEW OF LITERATURE

A review of literature was done for the past fifteen years to enumerate various methods available for the analysis of cinitapride and pantoprazole. In view of the literature cited for the quantification of cinitapride and pantoprazole in pharmaceutical dosage forms, there is no documentary evidence available.

Pantoprazole and Cinitapride in combined dosage form

Patel G.H., *et al.,* (2011) developed HPTLC method for the estimation of pantoprazole and cinitapride in combined dosage forms. Chromatographic separation of the drugs was performed on aluminium plates precoated with silica gel 60 F254 as the stationary phase and the solvent system consisted of ethyl acetate: methanol 9: 1*(v/v).* Densitometric evaluation of the separated zones was performed at 278 nm.

Paresh U. Patel., *et al.,* (2011) developed first derivative spectrophotometric analytical method for the simultaneous estimation of pantoprazole and cinitapride in combined dosage form. The drugs obeyed the Beer's law in the concentration range of 1-26 ug/mL. Zero crossing point of cinitapride was found to be 302 nm and zero crossing Point of Pantoprazole was 249 nm.

CINITAPRIDE

Alarcon de Lastra C., *et al*., (1998) studied the effects of cinitapride on gastric ulceration and secretion in rats. The study demonstrated a gastro protective nature of cinitapride not only through reduction of neutrophil toxicity but by an increased synthesis of free radical scavenging enzymes glutathione peroxidase. Further it

showed seratonergic dependent mechanism are also involved by 5-HT_2 blockade and 5-HT_1 receptor activation **.**

González Marti I., *et al*., (1998) determined cinitapride by differential pulse polarography and adsorptive stripping voltammetry (ASV) in Britton–Robinson buffer, with 3s detection limits of 1.3×10−8 and 8.4×10−10 M, respectively. ASV was applied for the determination of cinitapride in urine samples with an accumulation time of 65 s. A 3s detection limit was found to be 1.8×10−7 M with RSD of 1% was determined.

Matias E Manzotti., *et al*., (2007) determined the utility of prokinetic drugs

(cinitapride, cisapride) in improving symptoms and endoscopic lesions in patients with GERD esophagitis. They concluded that randomized controlled trials provided moderate-quality evidence that prokinetic drugs improve symptoms in patients with reflux esophagitis and low-quality evidence that they have an impact on endoscopic healing.

Helena Marquez., *et al*., (2011) developed a UHPLC method for the assessment of the metabolic profile of cinitapride. Metabolites were generated from the incubation of cinitapride with human liver microsomes. Cinitapride and its metabolites were separated by reversed-phase mode using a formate aqueous solution (pH 6.5) and acetonitrile as the components of the mobile phase. Limits of detection are about 0.03μmol/L, and repeatabilities were better than 0.06% for retention times and better than 3.5% for concentrations. The method was applied to characterize the in vitro cinitapride metabolism with human liver microsomes.

Marta Robert*., et al.***, (2007)** studied that the prokinetic cinitapride has no clinically relevant pharmacokinetic interaction and effect on QT during coadministration with ketoconazole. The safety and tolerability of the study treatments were also evaluated.

Shikha Roy M.N., *et al.*, (2008) determined free levels of cinitapride in human plasma by liquid chromatography- tandem mass spectrometry. Risperidone was used as the internal standard. The method was developed by extracting the sample and analyzed by high performance liquid chromatography coupled to electrospray tandem mass spectrometry API-4000 (LC-MS/MS). The method allowed an appropriate characterization of the pharmacokinetic profile of cinitapride at the therapeutic dose.

Thangabalan B., *et al.*, (2010) validated extractive spectrophotometric estimation of cinitapride in pure and its solid dosage form. These methods are based on the formation of chloroform soluble ion-association complexes of cinitapride with bromocresol green and with bromothymol blue in potassium hydrogen phthalate buffer PH- 4.0 with absorption maximum at about 414 and 416nm for BCG and BTB respectively.

Roy S.M.N., *et al.*, (2010) developed RP-HPLC method for the determination of cinitapride under stress induced degradation studies.. The drug was subjected to all stress conditions such as reduction, oxidation acidic and alkaline medium. Acetonitrile and phosphate buffer, pH 6.7 in the ratio (70:30 v/v) was used as the mobile phase and cinitapride detected at 260nm. Glimepride was used as an internal standard. The drug was found to degrade extensively in reduction conditions and mild

degradation in the presence of in alkaline, acidic and oxidative condition but the drug was found to be stable under thermal stress.

Syeda Humaire., *et al*., (2010) developed colorimetric method for the determination of cinitapride hydrogen tartarate in drug formulations. Six simple and sensitive spectrophotometric methods (A, B, C, D, E and F) have been developed for the quantitative estimation of cinitapride in bulk drug and pharmaceutical dosage forms. Method A and B is based on the oxidation followed by coupling reaction of cinitapride with 1, 10 phenanthroline and 2, 2' bipyridyl in presence of ferric chloride to form orange-red colored chromogens respectively. Methods are based on the diazotization of cinitapride with nitrous acid followed by its coupling *in situ* with N-(1-naphthyl) ethylenediamine dihydrochloride to form pinkish purple colored chromogen(C), with phloroglucinol to form orange colored chromogen (D), with diphenylamine to form pink colored chromogen (E) and with chromo tropic acid to form orange colored chromogen (F) respectively.

Syeda Humaira *et al*., (2011) developed a validated RP-HPLC method to determine cinitapride hydrogen tartarate in solid oral dosage forms. Gradient elution technique with mobile phase consisting of 0.1% formic acid in water and acetonitrile and UV detection wavelength of 268nm was utilized. The developed method was validated for precision which includes system precision and method precision, accuracy and linearity studies in the concentration range of 5-100 µg/mL with correlation coefficient of 0.9987. The accuracy (recovery) was between 97.32 and 100.82%.

Thangabalan B., *et al*., (2011) analysed spectrophotometrically the amount of cinitapride in tablet dosage form using 2.0 M sodium benzoate solution as the hydrotropic solubilizing agent.. Cinitapride showed λ max at 395 nm obeyed Beers law at 10-80µg/mL. The results were validated statistically and by recovery studies.

Suresh Ch.V., *et al*., (2011) developed a new visible spectrophotometric method for the determination of cinitapride in pharmaceutical dosage forms. They developed three methods involving charge transfer complex with chloranilic acid, oxidative coupling with MBTH and oxidation followed by complex formation with 1, 10 - phenanthroline in the ferric chloride. The complexes were found to show λ max at 550, 420, and 510nm respectively.

PANTOPRAZOLE

Patricia Poole., (2001) compared pantoprazole with histamine H2 -receptor antagonists (H_2RAs) and omeprazole in clinical trials of patients with grade II or III (Savary-Miller scale) reflux esophagitis. Studies comparing pantoprazole and H2RAs found that pantoprazole had consistently better healing rates and symptom control.

Abdel-Aziz M. Wahbi., *et al.,* (2002) applied compensation and chemometric methods (derivative, orthogonal and difference spectrophotometry) for the estimation of omepazole, lansoprazole and pantoprazole in their pharmaceutical preparations. The difference spectrophotometry was found to be unaffected by acid induced degradation products, thus could be applied for stability indicating assay.

Karljikovic–Rajic.K., *et al.,* (2003) developed a first-order UV-derivative spectrophotometry in the analysis of omeprazole and pantoprazole sodium salt and corresponding impurities. Applied zero-crossing method for the determination of omeprazole, omeprazole sulphone, pantoprazole sodium salt, and N-methylpantoprazole in methanol-ammonia 4.0% v/v. The method showed the impurity-drug intermolecular interactions, due to the possible intermolecular hydrogen bonds, confirmed by divergences of experimentally obtained amplitudes for impurities omeprazole sulphone and N-methylpantoprazole in comparison to expected values according to regression equations of calibration graphs.

Salama F., *et al*., (2003) validated spectrophotometric determination of omeprazole and pantoprazole sodium via their metal chelates. The procedures are based on the formation of 2:1 chelates of both drugs with different metal ions. The coloured chelates of omeprazole in ethanol are determined spectrophotometrically at 411, 399 and 523nm using iron (III), chromium (III) and cobalt (II) respectively.

Incilay Suslu., *et al.,* (2003) developed first derivative spectroscopy for the estimation of pantoprazole using 1: 9 ratio of water: methanol as the diluent; λmax of 295 nm and 303 nm for standard comparison and Zero crossing point respectively.

Lix Q., *et al.,* (2004) compared the inhibitory effects of the proton pump-inhibiting drugs omeprazole, esomeprazole, lansoprazole, pantoprazole, and rabeprazole on human cytochrome P450 activities. It was suggested that, although the inhibitory profiles of the five studied PPIs were similar, lansoprazole and pantoprazole are the most potent *in-vitro* inhibitors of CYP2C19 and CYP2C9, respectively. **Nafisur**

Rahman., et al., (2006) devised a kinetic spectrophotometric method based on the oxidation of pantoprazole with Fe(III) in sulfuric acid medium. The reaction was followed spectrophotometrically by measuring the increase in absorbance with time (1 - 8 min) at 725 nm. The initial rate method was adopted for constructing the calibration graph, which is linear in the concentration range of 5 - 90 µg mL-1.

Bardhan K., *et al.,* (D, (2007) performed a clinical trial comparing pantoprazole and esomeprazole to explore the concept of achieving 'complete remission' in gastro-oesophageal reflux disease. Thus, complete remission is a more reliable and clinically relevant endpoint of treatment.

Akheel Ahmed Syed., *et al.*, (2007) demonstrated a spectrophotometric determination of certain proton pump inhibitors using new reagents such as neocuproine and bathocuproine. Spectrophotometric methods were developed for the determination of antiulcer drugs namely omeprazole, lansoprazole, pantoprazole, rabeprazole and esomoprazole.

Basavaiah K., *et al.,* (2007) determined pantoprazole by colorimetric methods using bromate –bromide, methyl orange and indigo carmine as the colouring agents. The methyl orange complex showed λmax at 520 nm and indigo carmine complex at 610nm.

Syed AA., *et al.*, (2008) developed a spectrophotometric method for the determination of certain proton pump inhibitors belonging to the benzimidazole class of compounds. The method is based on the reaction of omeprazole, lansoprazole, pantoprazole, rabeprazole and esomeprazole with iron (III) and subsequent reaction

with ferricyanide under neutral condition which yields Prussian blue product with maximum absorption at 720-730 nm.

Pimpodkar N.V., *et al.,* (2008) developed visible spectrophotometric method by the oxidative coupling of pantoprazole by MBTH reagent in the presence of ferric ammonium sulphate and observed λ max at 504nm.

Urdigere Rangachar., *et al.,* (2008) developed visible spectrophotometric method by the redox and complexation method using N-bromosuccinimide and iron II thiocyanate and tiron reagents. Coupling of pantoprazole by MBTH reagent in the presence of ferric ammonium sulphate and measuring the absorbance at a λ max of 504nm.

Kanakapura Basavaiah., *et al.,* (2009) developed titrimetric and visible spectrophotometric methods for pantoprazole. Both methods involve reaction of pantoprazole with excess potassium permanganate in sulphuric acid and indirectly determining the amount of pantoprazole by titrating with iron II solution for titrimetry and measuring the absorbance at 545 nm for the spectrophotometry.

Kakde R.B., *et al.,* (2009) developed three-wavelength spectrophotometric method for simultaneous estimation of pantoprazole and domperidone in pharmaceutical preparations. The method utilizes a wavelength at which domperidone has zero absorbance for the determination of pantoprazole.

Basavaiah. K., *et al.,* (2009) determined pantoprazole by colorimetric methods using N-bromo succinimide, methyl orange and indigo carmine as the colouring agents.

The methyl orange complex showed λmax at 520 nm and indigo carmine complex at 610nm.

Paluru Rudra Mohan Reddy., ***et al.,*** **(2010)** developed a stability indicating fast LC method for the estimation of impurities of pantoprazole in tablet dosage forms. The method utilizes gradient elution technique, mobile phase consisting of ammonium acetate buffer – acetonitrile at a flow rate of 0.8mL/min and detected at 290 nm

Kalaichelvi R., ***et al.,*** **(2010)** developed a extractive colorimetric determination of pantoprazole sodium by acid-dye complexation method in solid dosage form using bromothymol blue dye for the complexation. The complex showed λmax at 428nm.

Basavaiah K., ***et al.,*** **(2010)** determined pantoprazole by colormetric methods using potassium permanganate as the colouring agent in neutral medium and measured absorbance at 350nm.

Krishna R. Gupta., ***et al.,*** **(2010)** developed simultaneous UV spectrophotometric method for the estimation of pantoprazole and itopride in capsule dosage form. Simultaneous equation method and absorbance ratio method have applied for the estimation.

Prasanna Reddy Battu., ***et al.,*** **(2011)** developed and validated a simple, selective, accurate High Performance Liquid Chromatographic (HPLC) method for the estimation of pantoprazole sodium sesquihydrate in pharmaceutical dosage forms and human plasma.Chromatographic separation was achieved isocratically on a C_{18}

column utilizing a mobile phase of acetonitrile/phosphate buffer (70:30, v/v, pH 7.0) at a flow rate of 0.8 mL/min and UV detection at 260 nm

Rajnish Kumar., ***et al.,*** **(2011)** developed UV spectroscopy for the estimation of pantoprazole in pharmaceutical dosage forms using distilled water as the diluent and λmax at 292 nm

Arunadevi S. Birajda., ***et al.,*** **(2011)** developed simultaneous estimation method for pantoprazole and mosapride in fixed dose combination by RP- HPLC and UV . The methods adopts 274 and 288.2 nm for the simultaneous estimation by UV and 30 mM ammonium sulphate buffer : acetonitrile (50:50v/v) as the mobile phase for RP- HPLC.

Kanakapura Basavaiah., ***et al.,*** **(2011)** developed titrimetric and visible spectrophotometric methods for pantoprazole. Both methods involve reaction of pantoprazole with excess ceric ammonium sulphate and indirectly determining the amount of pantoprazole by titrating with iron II solution for titrimetry and measuring the absorbance at 545 nm for the spectrophotometric method.

Juana Isabel Balderas., ***et al.,*** **(2011)** studied the bioavailability of two coated-tablet formulations of a single dose of pantoprazole 40 mg comparison in healthy mexican adult volunteers.

AIM AND OBJECTIVE OF WORK

Analysis of drugs plays a major role in the development, manufacture and therapeutic use of drug. Quantitative analysis of raw materials and the final product is necessary to ensure that they meet certain specifications and to ascertain the amount of each component in the final product.

Standard analytical procedures for newly designed and synthesized drugs are not available in any of the pharmacopoeias. So it becomes necessary to develop newer, accurate, specific, simple, easy to perform, reliable and economic analytical techniques for the estimation of the new drugs.

This thesis deals with pantoprazole (PNP) and cinitapride (CNP). Pantoprazole has been recently included in Indian Pharmacopoeia – 2010. Cinitapride is a very new drug, launched in 2007 and is not official in any of the pharmacopoeias. It has been very recently added in drug bank in 2012. The combination of the two drugs has been launched in 2011.Extensive literature survey reveals that only first derivative and HPTLC method for the determination of pantoprazole (PNP) and cinitapride (CNP) has been reported. There is no evidence for the estimation of PNP and CNP by UV-Visible spectrophotometry and RP-HPLC methods in bulk and in combined tablet dosage form. **So, an attempt has been made is to develop a validated, simple, easy to perform, accurate, cost effective and rapid spectrophotometric methods for the estimation of CNP and PNP in bulk and combined oral dosage form**.

PLAN OF WORK

1. Procurement of Gift samples of PNP and CNP.
2. Development of simple and accurate UV – Visible spectrophotometric methods.
    - Standard absorbance method
    - Area under curve
    - Second derivative spectroscopy
    - Visible spectrophotometry
        - Colorimetry (Diazotisation followed by coupling for CNP)
        - Colorimetry (Redox method for PNP)
3. Development of rapid and accurate RP-HPLC method using UV detection
4. Validation of proposed analytical methods.
5. Statistical analysis of developed analytical methods
6. Comparison of obtained results for the developed methods

MATERIALS AND METHODS

MATERIALS

DRUG SAMPLES

Cinitapride pure drug sample was generously gifted by M/S Zydus Cadila, Ankleshwar, Gujarat (India) and Pantoprazole by M/S Knis Laboratories, Chennai, TN (India).The combined capsule dosage form was acquired from the local market. The capsule contained within it enteric coated tablet of pantoprazole sodium sesquihydrate equivalent to 40 mg of PNP and extended release tablet of cinitapride hydrogen tartarate equivalent to 3 mg of cinitapride.

REAGENTS AND CHEMICALS

All the chemical used in the study were of analytical grade and procured from Merck India ltd.The chemicals used during the study are

S.no	Chemicals	Grade
1.	Methanol	Analytical grade
2.	Water	Double distilled water, HPLC grade for HPLC
3.	Methanol	HPLC grade for HPLC
4.	Acetonitrile	HPLC grade for HPLC
5.	Potassium dihydrogen orthophosphate	Analytical grade

6.	Potassium bromate	Analytical grade
7.	Potassium bromide	Analytical grade
8.	FAS	Analytical grade
9.	Ammonium Thiocyanate	Analytical grade
10.	1,10 phenanthroline	Analytical grade
11.	EAA, AAC	Analytical grade

INSTRUMENTS

1. Digital Balance
2. Shimadzu UV-Visible spectrophotometer, Model 1650PC
3. A Shimadzu Prominence LC-20AT with SPD-20A UV detector -HPLC
4. pH meter

ULTRA VIOLET- VISIBLE SPECTROSCOPY

General discussion

Ultraviolet spectroscopy is most frequently employed technique in pharmaceutical analysis. The ultraviolet region of the electromagnetic spectrum is used in the analysis which extends from 200-400 nm. It involves transitition of electrons of π orbitals and lone pairs (n = non bonding) so UV spectroscopy is of most use for identifying conjugated systems.

Choice of solvent (Sharma, 1994; Chatwal and Anand, 2000)

The most important requirement of the solvent are:

- It should solubilize the analyte freely.
- It should not itself absorb in the region of the analyte.
- It should not undergo association or dissociation with the analyte

The Absorption laws (Sharma YR, 2010)

There are two laws which govern the absorption of light by the molecules. Theyare,

- Lambert-Bouger's law
- Beer's law

Lambert-Bouger's law

It states that, when a beam of monochromatic radiation passes through a homogenous absorbing medium the ratio of decrease of intensity of transmitted

radiation with thickness of absorbing medium is directly proportional to the intensity of the incident radiation.

The law is given by: $\mathbf{I=I_0 10^{-ax}}$

Where, I_0 is the intensity incident light; I is the intensity of transmitted light.

Beer's law

This law states that, when a beam of monochromatic radiation is passed through a solution of an absorbing substance, the rate of decrease of intensity of transmitted radiation with concentration of the absorbing solution is directly proportional to the intensity of incident radiation. The law is given by:

$\mathbf{I=I_0 e^{-k'cx}}$

On combining these two laws ,the **Beer- Lambert law** is formulated

$\mathbf{\text{Log } I_0/I = } \boldsymbol{a\ .c.l} \mathbf{= A}$

Where, I_0 = intensity incident light; I = intensity of transmitted light

a = Molar extinction coefficient; c = concentration of solute in moles litre^{-1}

l = pathlength of the sample (usually 1 cm); A = absorbance

Quantitative analysis

The use of UV in quantitative analysis employs the method of comparing the absorbances of the standard and samples at selected wavelength. (Metreyi, 2008)

Assay of Substances in single Component Samples (Beckett and Stenlake, 2002)

The most important characteristics of photometric and spectrophotometric methods are high selectivity and ease of convenience. Quantitative analysis (assay of an absorbing substance) can be done using following methods.

- **Use of A (1%, 1cm) values**

This method can be used for the estimation of formulation or raw material when reference standard not available. The use of standard A (1%, 1cm) value avoids the need to prepare a standard solution of the reference substance in order to determine its absorption.

- **Use of calibration graph**

In this method a calibration curve is plotted using concentration (X-axis) Vs absorbance (Y-axis) with the value of five or more standard solutions. A straight line is drawn through maximum number of points. This line is called line of best fit. By interpolating the absorbance of the sample solution on the calibration chart, the concentration of the drug amount and percentage purity can be calculated. This is used in the new method development for the estimation of an analyte by UV- Visible spectrophotometry.

The amount present can be calculated using the formula

$$\text{Amount present} = \frac{\text{Sample absorbance}}{\text{Standard absorbance}} \times \text{Dilution factor} \times \frac{\text{Wt of Std}}{\text{Wt of Sample}} \times \text{Avg } wt$$

- For Area under Curve, instead of absorbance , Area is used.
- For Derivative spectrophotometry, Amplitude of negative maxima is used instead of absorbance.

VISIBLE SPECTROPHOTOMETRY

The visible spectrophotometry follows the same Beer- Lamberts law. But the abasorbance of the analyte is observed in the Visible region (380 nm to 800nm).Analytes which do not have absorbing chromophore in their structure are chemically derivatized by chromogenic reactions to form a coloured complex which show bathochromic shift towards the visible region. There different complexing methods and corresponding complexing agent.

1. Diazotisation followed by coupling reaction to form diazo dyes
2. Complexes involving redox reactions and reagents.
3. Acid dye techniques involving ion pair reagents like Bromothymol blue,Bromocresol green, Eriochrome black II. Methyl red etc
4. Reagents specific for phenols- 2,6 dichloro indo phenol
5. Reagents specific for primary amines- PDAB (para dimethyl amino benzaldehyde, MBTH (3-methyl 1,2 benzothiazoline hydrazone hydrochloride) etc.

While developing methods for estimation of analytes using UV –Visible spectrophotometry following criterias are considered

- Adherence to Beer's law.

- Stability of the drug in the solvent /diluents.
- Selectivity of the complexing agent in colorimetry.
- Stability of the complex, colour of the complex.
- Stability of absorbance with respect to time ,pH, temperature, ionic strength etc.

METHODS

I. ULTRA VIOLET SPECTROPHOTOMETRIC METHOD

SELECTION OF SOLVENT (IP,2010)

The solubility of CNP and PNP were studied as per Indian Pharmacopoeial standards. The solubility of the drugs was tested in both polar and non polar solvents. Both the drugs were found to be freely soluble in methanol. So methanol was selected for further studies on CNP and PNP by UV spectroscopy.

PREPARATION OF STANDARD STOCK SOLUTION

Weighed accurately about 50 mg each of standard CNP and PNP in two separate 50mL volumetric flasks. Dissolved in 10 mL of methanol and made up to volume with methanol to obtain stock solutions of concentration 1000 µg/mL of both CNP and PNP.

SELECTION OF ABSORPTION MAXIMA

The stock solutions of both CNP and PNP were appropriately diluted to obtain a concentration of 10 µg/ mL. The dilutions were scanned in ultra violet region (200-400nm) against methanol blank. CNP showed maximum absorbance at 262nm and PNP at 290 nm. Thus λ max for CNP and PNP were selected as 262 and 290 nm respectively and used during further studies for the estimation of CNP and PNP by UV spectrophotometry.

ESTABLISHMENT OF LINEARITY

CINITAPRIDE

The stock solution (1000 µg/ mL) of CNP was serially diluted with methanol to obtain dilutions ranging 4-20 µg/ mL. The final dilutions were scanned in ultraviolet region (200-400nm) against methanol blank. The λ max was found to be stable at 262nm. The absorbance of the dilutions was measured at the selected λ max, 262nm.

PANTOPRAZOLE

The stock solution (1000 µg/ mL) of PNP was serially diluted with methanol to obtain dilutions ranging 5-30 µg/ mL. The final dilutions were scanned in ultraviolet region (200-400nm) against methanol blank. The λ max was found to be stable at 290 nm. The absorbance of the dilutions was measured at the selected λ max, 290 nm.

SAMPLE ANALYSIS

The capsule dosage form contained PNP as enteric coated tablet and CNP as extended release tablet. Thus both the components were analyzed as separate entities

METHOD A: STANDARD ABSORBANCE METHOD

CINITAPRIDE

Twenty tablets of CNP were accurately weighed and crushed to fine powder. Tablet powder equivalent to 10mg of the CNP was weighed in a 100 mL

volumetric flask, shaken vigorously with sufficient quantity of methanol for half an hour. Finally the solution was made up to volume with methanol. The solution was well shaken and filtered through Whatmann filter paper (No.41). First few mL of the filtrate was discarded and an aliquot quantity of the filtrate was diluted to obtain a final concentration of 10µg/mL of CNP. The absorbance of the resulting solution was measured at 262nm against methanol blank.

PANTOPRAZOLE

Twenty tablets of PNP was accurately weighed and crushed to fine powder. Tablet powder equivalent to 50mg of PNP was weighed in a 100mL volumetric flask, shaken vigorously with sufficient amount of methanol for half an hour and finally made up to volume with methanol. The resulting solution was filtered through Whatmann filter paper (No.41). First few mL of the filtrate was discarded and sufficient quantity of the filtrate was diluted with methanol to obtain a final

concentration of 15µg/mL of PNP. The absorbance of the resulting solution was measured at 290 nm against methanol blank.

METHOD B: AREA UNDER CURVE

The AUC (area under curve) method involves the calculation of integrated value of absorbance between two selected wavelengths λ1 and λ2. The wavelength range is selected on the basis of repeated observations so as to get the linearity between area under curve and concentration. The standard spectra obtained in the linearity characterization and the sample spectra for both CNP and PNP obtained in method A were used. The AUC for CNP were determined between 239.8 and 296.4 for both standard and sample.

Similarly for PNP, the AUC between 248.4 and 314.0 nm nm for both standard and sample were determined. The calibration graph was plotted between AUC and concentration. The sample AUC was interpolated on the respective linearity chart and the concentration was determined.

METHOD C: SECOND DERIVATIVE

The zero order spectra obtained in the linearity characterization and method A for both CNP and PNP were derivatized to get second order spectra. CNP showed a negative maxima at 262nm and PNP at 290nm. The amplitude of the negative maxima were measured and plotted against concentration to determine the linearity. The sample amplitudes were interpolated on the respective linearity chart of the derivative spectra and the concentration was determined.

RECOVERY STUDIES

The recovery studies were carried out on spiked samples by adding predetermined amount of standard drugs to the respective preanalysed sample. About 20, 40 and 100% of standard drugs were added to the sample solutions and the absorbance was measured against methanol blank. The percentage recovered was calculated. The recovery studies were performed at three levels for all the three methods to confirm the accuracy of the above said methods.

II. VISIBLE SPECTROPHOTOMETRIC METHOD

Two different colorimetric methods were developed for the estimation of both Cinitapride and Pantoprazole each.

CINITAPRIDE

The developed visible spectrophotometry of CNP is based on the diazotization followed by coupling reaction. Method A involves the coupling of diazotized CNP with ethyl aceto acetate (EAA). Method B involves the coupling of diazotized CNP with acetyl acetone (AAC).

PREPARATION OF REAGENTS

- **Sodium nitrite solution (2%):**
  2gms of sodium nitrite dissolved in water and made up to 100 mL with distilled water.
- **Hydrochloric acid 0.5M:**
  42.5 mL of concentrated hydrochloric acid was made up to 1000 mL with distilled water.

- **Ethyl aceto acetate (EAA) (2%) solution:**

  2 mL of acetyl acetone was dissolved in 5mL of alcohol and made up to volume with water in a 100 mL volumetric flask.

- **Acetyl acetone(AAC) (2%) solution:**

  2 mL of acetyl acetone was dissolved in 5mL of alcohol and made up to volume with water in a 100 mL volumetric flask.

- **Sodium hydroxide 1 mol L^{-1}:**

  4gms of sodium hydroxide was dissolved and made up to 100 ml with distilled water.

METHOD A — USING ETHYL ACETO ACETATE (EAA)

SPECTRAL CHARACTERIZATION AND ESTABLISHMENT OF LINEARITY

A stock solution of CNP was prepared by dissolving accurately weighed quantity of standard CNP in 10 m l of methanol and made up to volume with water to obtain a concentration of 500 µg/ml. From the stock solution 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mL were transferred into six 50 mL volumetric flasks. The solutions were diazotized by the addition of 2 mL of 2% sodium nitrite solution and 2 mL of 0.5 M hydrochloric acid at a temperature of about 0-5° C for 5 minutes. Then 2 mL of 2 % EAA was added to each reaction mixture followed by the addition of 1 mL of 1M sodium hydroxide, when a reddish orange coloured chromogen was formed. Finally the solutions were made up to volume with distilled water and shaken well. The absorbance of the reddish orange coloured chromogen was scanned between 350-

800nm against distilled water blank. The chromogen gave maximum absorbance at 392.5 nm.

ANALYSIS OF FORMULATION

Twenty tablets of CNP from the capsules were accurately weighed and ground to fine powder. Tablet powder equivalent to 25 mg of CNP was accurately weighed and dissolved in 10 mL of methanol, made up to volume with water to obtain a concentration of 250 µg/mL. The solution was filtered through Whatmann filter paper No.41. First few mL of the filtrate was discarded, and then 6 mL of the filtrate was transferred to a 50 mL volumetric flask. The sample solution was diazotized, coupled with EAA using the same procedure as that of standard CNP. The absorbance of the resulting chromogen was measured at 392.5 nm.

METHOD B — USING ACETYL ACETONE (AAC)

SPECTRAL CHARACTERIZATION AND ESTABLISHMENT OF LINEARITY

A stock solution of CNP was prepared by dissolving accurately weighed quantity of standard CNP in sufficient amount of methanol and made up to volume with distilled water to obtain a concentration of 1000 µg/mL. From the stock solution 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL were transferred into six 50 mL volumetric flasks. The solutions were diazotized by the addition of 2 mL of 2% sodium nitrite solution and 2 mL of 1M hydrochloric acid at a temperature of about 0-5° C for 5 minutes. Then 2 mL of 2 % AAC was added to each reaction mixture followed by

the addition of 1M sodium hydroxide, when a reddish orange coloured chromogen was formed. Finally the solutions were made up to volume with distilled water and shaken well. The absorbance of the reddish orange coloured chromogen was scanned between 350-800nm against distilled water blank. The chromogen gave maximum absorbance at 399nm.

ANALYSIS OF FORMULATION

Twenty tablets of CNP from the capsules were accurately weighed and ground to fine powder. Tablet powder equivalent to 25 mg of CNP was accuratey weighed and dissolved in methanol and shaken well for 20 minutes, made up to volume with water to obtain a concentration of 250µg/mL. The solution was filtered through Whatmann filter paper No.41. First few mL of the filtrate was discarded, and then 6 mL of the filtrate was transferred to a 50 mL volumetric flask. The sample solution was diazotized, complexed with AAC similar to that of standard CNP and the absorbance of the resulting solution was measured at 399nm.

PANTOPRAZOLE

The visible spectrophotometric studies of PNP is based on the oxidation of PNP by excess of bromine and indirectly determining the amount of PNP using ferrous ammonium sulphate and 1,10 phenanthroline in Method A and ammonium thiocyanate in Method B.

PREPARATION OF REAGENTS

- **Potassium bromate – bromide mixture :**

  Dissolved 100 mg of potassium bromate and 1g of potassium bromide in 10 mL of water and made up to volume in a 100 mL volumetric flask. Appropriate dilution of the stock solution was made to obtain a concentration of and 35 µg/mL and 20 µg/mL to be used in method A and B respectively.

- **Ferrous ammonium sulphate:**

  Dissolved 400 mg of FAS in 50 mL of distilled water, added 1 mL of dilute sulphuric acid and made up to 100 mL in a volumetric flask. Further dilutions were made to obtain a concentration of 350 µg/mL and 400 µg/mL of FAS to be used in method A and B respectively.

- **1,10 – Phenanthroline:**

  Dissolved 250 mg of 1, 10 – Phenanthroline in distilled water with the aid of heat and made up to volume in 100 mL volumetric flask.

- **Hydrochloric acid ($5M^{-}$):**

  About 43 mL of concentrated hydrochloric acid in water was added in to 10mL of distilled water, made up to volume in 100 mL volumetric flask with distilled water.

- **Hydrochloric acid (1M)**

  About 85 mL of concentrated hydrochloric acid was added in to 100 mL of distilled water, made up to volume in 1000 mL volumetric flask with distilled water.

- **Ammonia solution (50 %)**

  About 50 mL of strong ammonia solution was added to 20 mL distilled water and made up to 100 mL with distilled water

- **Ammonium thiocyanate (3 mol L^{-1})**

  Dissolved 23g of ammonium thiocyanate in water and made up to 100 mL with distilled water.

- **Hydrochloric acid (5 mol L^{-1-})**

  About 15.4 mL of concentrated hydrochloric acid was added into100 mL water and made up to volume in 1000mL volumetric flask with distilled water.

PREPARATION OF STANDARD STOCK SOLUTION

A stock solution of PNP was prepared by dissolving accurately weighed quantity of standard PNP in 1M hydrochloric acid and made up to volume with the same hydrochloric acid to obtain a final stock concentration of 1000 µg/mL.

METHOD A— USING 1, 10 – PHENANTHROLINE

SPECTRAL CHARACTERIZATION AND ESTABLISHMENT OF LINEARITY

The stock solution was further diluted with distilled water to obtain a working standard solution of concentration 30 µg/mL. Transferred 1 – 4.5 mL of the working standard solution of PNP (30µg/mL) into six 25 mL volumetric flasks. To each flask was added 2 mL of potassium bromate – bromide mixture (35µg/mL) using a burette and 2 mL of hydrochloric acid (5M), stoppered immediately, shaken

well and kept aside for 5 minutes. To the reaction mixtures, about 2 mL of FAS was added (350 µg/mL), shaken well and again kept aside for fifteen minutes until the reaction is completed. This was followed by the addition of 2 mL of 1, 10-phenanthroline, when a blood red coloured chromogen is obtained. Finally the solutions were made up to volume with distilled water. The absorbance of the reddish coloured chromogen was scanned between 350-800nm against reagent blank. The chromogen gave maximum absorbance at 510 nm.

ANALYSIS OF FORMULATION

Twenty tablets of PNP from the capsules were accurately weighed and ground to fine powder. Tablet powder equivalent to 50mg of CNP was accurately weighed and shaken well with 1M hydrochloric acid for 20 minutes and made up to volume with 1M hydrochloric acid to obtain a concentration of 1000µg/mL. The solution was filtered through Whatmann filter paper No.41. First few mL of the filtrate was discarded and the filtrate was appropriately diluted to obtain a concentration of 30 µg/mL with water. Transferred 3 mL of the first dilution (30µg/mL) to a 25mL volumetric flask and the same procedure for standard PNP was followed. The absorbance of the resulting solution was measured at 510nm.

METHOD B—USING AMMONIUM THIOCYANATE

SPECTRAL CHARACTERIZATION AND LINEARITY ESTABLISHMENT

A stock solution of PNP was prepared by dissolving accurately weighed quantity of standard PNP in 1M hydrochloric acid and made up to volume with the same hydrochloric acid to obtain a final stock concentration of 1000µg/mL. The

stock solution was further diluted with distilled water to obtain a working standard solution of concentration 20 µg/mL. Transferred 0.5, 1, 1.5, 2, 2.5 and 3 mL of the working standard solution of PNP (20µg/mL) into six 25 mL volumetric flask. To each was added 2 mL of potassium bromate – bromide mixture (40µg/mL) using a burette and 2mL of hydrochloric acid (5M), stoppered immediately, shaken well and kept aside for 5 minutes. To the reaction mixtures, about 2 mL of FAS (400µg/mL) was added, shaken well and again kept aside for fifteen minutes until the reaction is completed. This was followed by the addition of 1mL of 3 mol L^{-1} ammonium thiocyanate when a blood red coloured chromogen is obtained. Finally the solutions were made up to volume with distilled water. The absorbance of the reddish coloured chromogen was scanned between 350-800nm against reagent blank. The chromogen gave maximum absorbance at 477 nm.

ANALYSIS OF FORMULATION

Twenty tablets of PNP from the capsules were accurately weighed and ground to fine powder. Tablet powder equivalent to 50 mg of PNP was accurately weighed and shaken well with 1M hydrochloric acid for 20 minutes and made up to volume with 1M hydrochloric acid to obtain a concentration of 1000µg/mL. The solution was filtered through Whatmann filter paper No.41. First few mL of the filtrate was discarded and the filtrate was appropriately diluted to obtain a concentration of 20µg/mL with distilled water. Transferred 1mL of the first dilution (20µg/mL) to a 25mL volumetric flask and the same procedure for standard PNP was followed. The absorbance of the resulting solution was measured at 477 nm.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The ability to separate and analyse complex samples is integral to the biological and medical sciences. The innovations and development in the classic column chromatography chromatographic techniqueshas offered major improvements in speed, resolving power, detection, quantification, convenience and applicability to new samples types.

Modern HPLC techniques became available in 1969, but not widely used in pharamaceuticals. Once HPLC systems capable of quantitative analysis became commercially available, their application in pharma industry became exclusive.

GENERAL PRINCIPLE OF CHROMATOGRAPHY

Generally chromatographic separation technique makes use of any of the four mechanism:

- **Adsorption :** Separation arises due to difference in affinity of compounds towards solid stationary phase generally makes use of Normal phase
- **Partition:** Separation arises due to difference in affinity of compounds towards liquid stationary phase that is immiscible with the eluent and coated on an inert support. Could be normal or reverse phase
- **Ion exchange** involves solid stationary phase called Ion exchange resin is used to separate mixture of similar charged ions.

- **Ion pair:** An alternative to ion exchange chromatography Reverse phase column is used to separate ionic compounds. It involves the addition of an organic ionic substance to the mobile which forms an ion pair with sample component of opposite charge.

- **Size exclusion:** A mixture of components with different molecular sizes are separated by using gels as the stationary phase with controlled pore size

- **Chiral phase:** Separation of enatiomers by using either chiral stationary phases or the chiral mobile phase

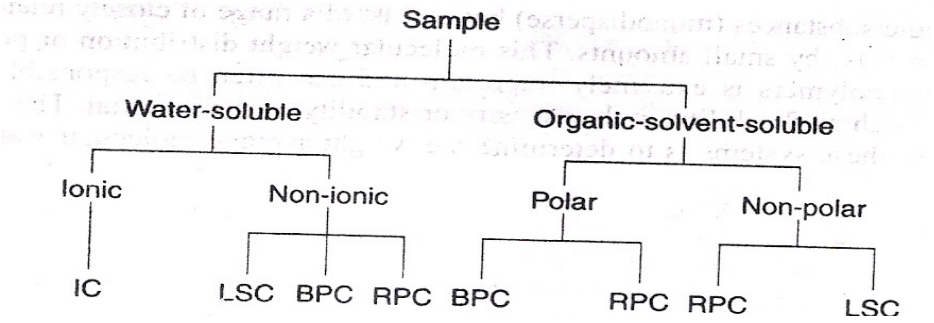
Modes of chromatography (Mendham, et al. 2002)

Normal phase chromatography, the stationary phase is more polar than the mobile phase which is a mixture of organic solvents without added water (eg : Isopropane with hexane) and the column packing is either an inorganic adsorbent (silica) or a polar bonded phase (cyano, diol, amino) on a silica support. Sample retention in normal phase chromatographic technique increases as the polarity of the mobile phase decreases.

Reverse phase chromatography is the chromatography in which the stationary phase is less polar than the mobile phase and the mobile phase is a mixture of organic and aqueous phase. Reverse phase chromatography is more convenient and rugged than the other form of liquid chromatography and is more likely to result in a satisfactory final separation. RP –HPLC columns are efficient, stable and reproducible.

Choice of column chromatography system

To select the most appropriate column type, understand the physical characteristics of the sample and the type of information required. The following chart shows a guard guide to selecting chromatographic methods for separating compound based on their relative molecular species.



IC- Ion exchange chromatography; LSC – Liquid solid chromatography

BPC – Bonded phase chromatography; RPC – Reverse phase chromatography

Choice and optimization of the mobile phase (James W. Munson 2001)

The power of HPLC in term of being able to resolve many compound is mainly due to the diversity of mobile phase (or) mobile solvent available. The mobile phase in HPLC however has a great influence on the retention of the solutes and the separation of component mixtures. The viscosities, compressibility, refractive index, UV cut off, Polarity, vapour pressure of the solvent are considered while selecting a mobile phase. The best mobile phase and its strength for a specific separation can be determined primarily by performing asilica TLC. The results are readily transferable to HPLC and k' (capacity factory) values can be

predicted if the stationary phase in both methods is the same product but with a different in particle size.

Separation/Elution technique: There are two types of elution technique

Isocratic elution technique: When the mobile phase compositions do not change throughout the course of run, it is said to be isocratic. This is the simplest technique and should be the method of first choice when developing a separation

Gradient elution technique: When the mobile phase composition is changed over time during the course of run, it is said to be gradient elution technique. The elution strength of the eluent is increased during the gradient run by changing the polarity, pH, or ionic strength. This technique is a powerful tool to separate mixtures of compounds with different retention.

Derivatisation:

Derivatisation involves chemical reaction that alters the molecular structure of the analyte of interest to improve detection. It is used to enhance the sensitivity and selectivity of detection when the available detectors are not satisfactory for the underivatised compounds. A good derivative should be stable, low background, convenience etc. Both UV and Fluorescent derivatives are used. Some of the derivatizing agents are: N-succinimidyl-*p*-nitrophenylacetate (SNPA), phenyl hydrazine, 3,5-dinitrobenzoyl chloride (DNBC), dansyl chloride (DNS-Cl), 4-bromomethyl-7-methoxy coumarin (BMC) and flourescamine (FC).

Quantitative Analysis

Quantification by HPLC basically involves measurement of peak height(or) peak area. Well resolved peak's peak area and peak height are proportional to the concentration. Similar to UV quantification, Beer's concentration is established for the standard analytes and calibration curve is plotted by taking peak height(or) peak area along X-axis and concentration along Y-axis. The plot should be a linear line essentially passing through the origin. There are three different quantification techniques.

1. External standard method:

This is the simplest and accurate method. Linearity is established for the standard drug (peak area vs. concentration) and beer's concentration is determined. A concentration of the test sample essentially lying in the Beer's range is also studied and interpolated on the calibration chart to attain the sample concentration. Concentration can be calculated using the following formula:

$$Conc\ (unknown) = \frac{Area\ (unknown)}{Area(known)} \times Conc\ (known)$$

2. Internal standard method

This method tends to yield the most accurate and precise results. In this method an equal amount of internal standard that is not present in sample is added to

both the sample and standard solutions. Quantification is achieved using the ratios of peak height (or) peak area of the component to the internal standard

3. Standard addition method

This is especially useful to determine the or identify problems due to interference from sample matrix, since it cancels out these effects. In this method the sample is divided in to two portions. To one portion a known amount of the analyte (spike) is added. The original and the original plus spike is analysed. The sample with spike shows a larger response than the original sample due to the additional amount of analyte.

Applications of HPLC in the pharma industry

Present applications of HPLC in pharmaceutical research and development are:

- To purify synthetic or natural products
- To characterize metabolites and in pharmacodynamic and kinetic studies.
- To assay APIs, impurities, degradation products and in dissolution assays

Hyphenated techniques/Hybrid Techniques

These have improved the ability to separate and identify multiple entities within a mixture. The techniques include HPLC – MS, HPLC – MS / MS, HPLC – IR, and HPLC – NMR. These techniques involve usual chromatography separation followed by peak identification using traditional detector such as UV, combined with further identification of the compound with MS, IR, NMR, and DAD.

III. REVERSE PHASE - HIGH PERFORMANCE LIQUID

INSTRUMENT

High Performance Liquid Chromatograph

1. Shimadzu prominence
2. UV-Visible Detector (SPD 20A)
3. Auto sampler
4. Isocratic (LC-20AT) pump
5. Rheodyne Valve injector with 20 µl fixed loop
6. Chromatographic Column- Phenomenex Gemini, C_{18}(5 µ) : 250x4.60 mm i.d

PREPARATION OF MOBILE PHASE

The mobile phase consisted of acetonitrile, methanol and phosphate buffer (pH-7) in the ratio of 40:20:40. Phosphate buffer was made from 0.2M potassium dihydrogen orthophosphate adjusted to pH-7 with triethylamine. The mobile phase was filtered through 0.2µ membrane filter and used throughout the study.

SELECTION OF WAVE LENGTH (IP, 2010)

A known concentration of standard CNP was prepared in methanol The solution was further appropriately diluted with the mobile phase containing acetonitrile, methanol and phosphate buffer (pH-7) in the ratio of 40:20:40%v/v to obtain a concentration of 10 µg/ml. The solution was then scanned in the UV region

in the UV spectrophotometer against the mobile phase as the blank. The λ_{max} was observed at 262nm. The λ_{max} was analysed using different ratios of mobile phase, different pH of the buffer. There was no significant change in the λ_{max}. So 262 nm was selected as the detection wavelength for the estimation CNP by RP-HPLC.

Similarly 10 µg/ml concentrated solution of PNP was prepared in mobile phase containing acetonitrile, methanol and phosphate buffer (pH-7) in the ratio of 40:20:40 to obtain a concentration of 10 µg/ml. The solution was then scanned in the UV region in the UV spectrophotometer against the mobile phase as the blank. The λ_{max} was observed to be 290nm. The λ_{max} was analysed using different ratios of mobile phase, different pH of the buffer. There was no significant change in the λ_{max}. So 290 nm was selected as the detection wavelength for the estimation PNP by RP-HPLC.

OPTIMIZATION OF THE MOBILE PHASE

Effect of different ratios of mobile phase

Both the drugs were studied by the RP-HPLC method using different ratios of mobile phase like 50:20:30%v/v, 20: 20: 60%v/v and 40:20:40%v/v of acetonitrile : methanol :phosphate buffer PH-7. The shape and symmetry of the peak, the retention times were good and acceptable in 40:20:40%v/v mobile phase. So mobile phase containing acetonitrile : methanol :phosphate buffer pH-7 in the ratio of 40:20:40%v/v was selected for the study of the drugs by HPLC.

Effect of pH of mobile phase

The different pH solutions were tried i.e. 6.5, 7.0, 7.5, and the chromatograms were recorded. On comparing the chromatogram obtained for the different pH solutions, at pH 7, the peak was very sharp for both CNP and PNP.

Effect of flow rate on separation

Using the above selected mobile phase (40:20:40%v/v of acetonitrile: methanol: phosphate buffer pH-7, separation of both CNP and PNP were tried in different flow rates i.e. 1.5 ml/min, 2.0ml/min, 2.5 ml/min. The chromatograms were recorded. At 2.0ml/min, the peak was very sharp and the retention time was stable at 6.5 minutes and 3.5 minutes for CNP and PNP respectively.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS

The following parameters were used for RP-HPLC analysis of CNP and PNP

Mode of operation : Isocratic

Stationary phase : C18 Column (250 mm × 4.6 mm i.d., 5 µ)

Mobile phase : Acetonitrile, Methanol and Phosphate buffer (pH-7)

Ratio : 40:20:40 %v/v

Detection wavelength : 262 nm (CNP) and 290 nm (PNP)

Flow rate : 2.0ml/min

Temperature : Ambient

Sample volume : 20 µl

Quantification method: External Standard Calibration Method

Determination of CNP and PNP in bulk and oral dosage form

Since the combined capsule dosage form contains CNP and PNP as separate tablets, they were studied as separate entities.

CINITAPRIDE

Preparation of standard solutions and Establishment of Linearity

Accurately weighed 30mg of standard CNP in 25 ml volumetric flask, dissolved in methanol and made up to volume in methanol to get a concentration of 0.6 mg/ml of cinitapride. The solution was filtered through 0.2µ membrane filter and used. Aliquot quantity of the standard solution (0.6 mg/ml) of CNP was serially diluted with the mobile phase to get a concentration range of 84µg/ml to132µg/ml. About 20µl of the dilutions were injected one by one in the 20µl loop. The eluate was detected at 262nm and the chromatogram was recorded. The peak area was plotted against concentration and the linearity was studied using regression analysis.

Analysis of formulation:

Twenty tablets of CNP from the formulation were weighed accurately. Tablet powder equivalent to 6mg of CNP was then accurately weighed, dissolved in methanol with the aid of ultrasonication for 15minutes and made up to volume (10ml) with the methanol. The solution was further diluted with mobile phase to obtain a concentration of 0.12 mg /ml.The solution was then filtered through 0.2µ membrane filter and injected into the column. The eluate was detected at 262nm and the chromatogram was recorded.

Recovery studies

To 5ml of preanalysed sample stock solution (0.6mg/ml) 2.5 ml of standard stock solution (0.6mg/ml) was added and made up to 25 ml in a volumetric flask.The solution was then filtered and injected in to the column. The eluate was detected at 262nm and the chromatogram was recorded.

PANTOPRAZOLE

Preparation of standard solutions and Establishment of Linearity

Accurately weighed 100mg of standard PNP in 50 ml volumetric flask, dissolved in mobile phase and made up to volume with the mobile phase to get a concentration of 2mg/ml of PNP. The solution was filtered through 0.2µ membrane filter and used further. Aliquot quantity of the standard solution (2mg/ml) of PNP was serially diluted with the mobile phase to get a concentration range of 140µg/ml to 220µg/ml. The dilutions were injected in the 20µl loop one by one; the eluate were then detected at 290nm and the chromatograms were recorded. The peak area was plotted against concentration and the linearity was assessed using regression analysis.

Analysis of formulation:

Twenty tablets of PNP from the formulation were weighed accurately. Tablet powder equivalent to 100mg of PNP was accurately weighed, transferred to 50 ml volumetric flask. Dissolved in mobile phase with the aid of ultrasonication for 15minutes and made up to volume with mobile phase to obtain a concentration of

2mg/ml. The solution was further diluted to obtain a concentration of 0.2mg/ml. The final dilution was then filtered through 0.2µ membrane filter and 20 µl was injected into the column. The eluate was detected at 290nm and the chromatogram was recorded.

Recovery studies

To 5ml of preanalysed sample stock solution (2mg/ml) 2.5 ml of standard stock solution (2mg/ml) was added and made up to 50 ml in a volumetric flask.The solution was then filtered and injected in to the column. The eluate was detected at 290nm and the chromatogram was recorded.

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION

Calibration of standard (CNP and PNP) was repeated for three times. The limit of detection and limit of quantification was calculated by using the average value of slope and standard deviation of intercept.

SYSTEM SUITABILITY STUDIES

The system suitability studies carried out as specified in ICH guidelines and USP. The parameters like tailing factor, asymmetry factor, number of theoretical plate, capacity factor were calculated.

RESULTS AND DISCUSSION

I. ULTRA VIOLET SPECTROSCOPIC METHOD

METHOD A- STANDARD COMPARISON METHOD

CNP was found to be freely soluble in methanol. It gave maximum absorption in methanol at 262 nm.(fig-1)

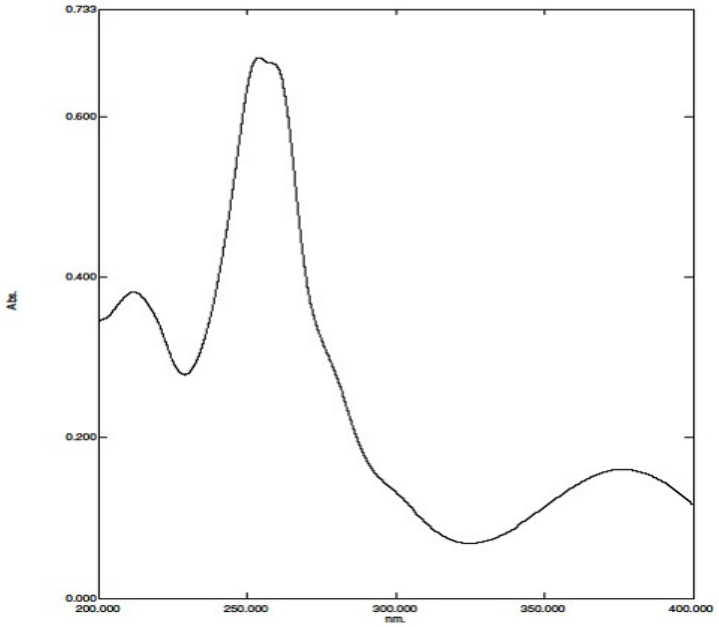


Fig 1: UV spectrum of CNP showing λmax at 262 nm

The standard drug was subjected to linearity studies from 2 to 100 µg/mL A five point calibration chart was plotted using absorbance along Y–axis and concentration along X–axis. It was observed that CNP obeyed Beer's law at 4–20 µg/mL. The overlain spectrum of the linearity studies are shown in fig-2. The absorbance with respect to concentration is presented table-1. Above 20µg/mL it showed negative deviation from Beer's law.

Table 1: Absorbance of CNP at 262 nm

S.No.	Concentration (µg/mL)	Absorbance*
1	4	0.224
2	8	0.449
3	10	0.561
4	12	0.672
5	16	0.896
6	20	1.121

*****_Each value is the mean of three determination_

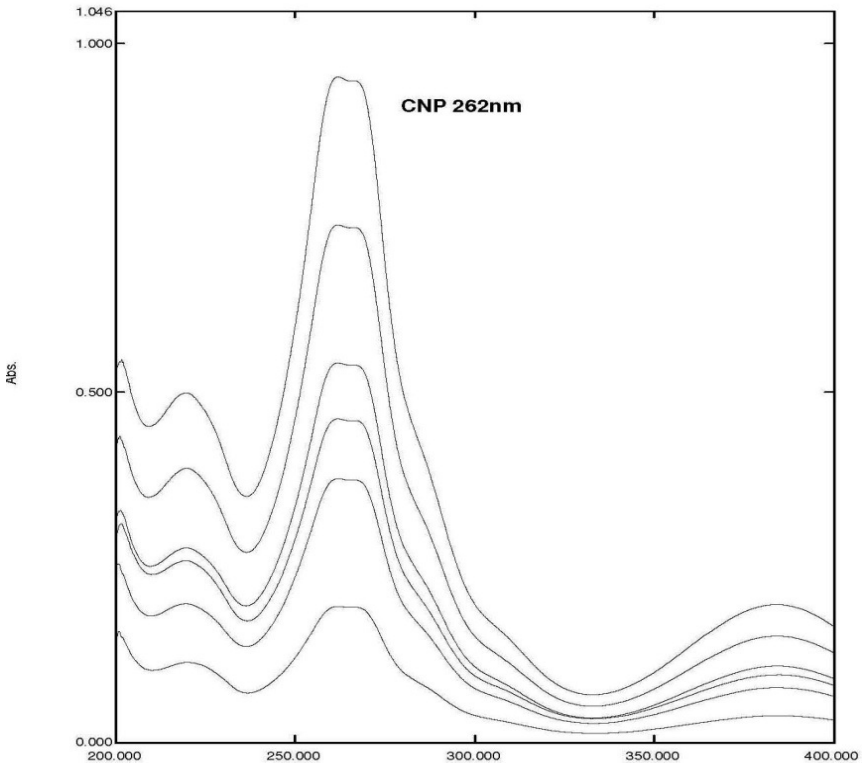


Fig 2: Overlain spectrum of CNP

The linearity was assessed by the regression analysis which is shown in fig-3. The correlation coefficient was found to be 1 which was within the limit. The formulation was quantified.

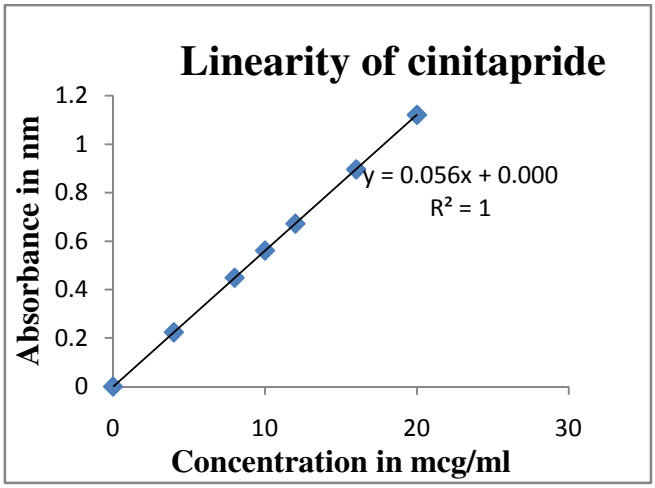


Fig 3: Calibration chart of CNP

Similarly, PNP was also found to be very freely soluble in methanol. PNP showed absorption maxima at 290 nm. (fig-4)

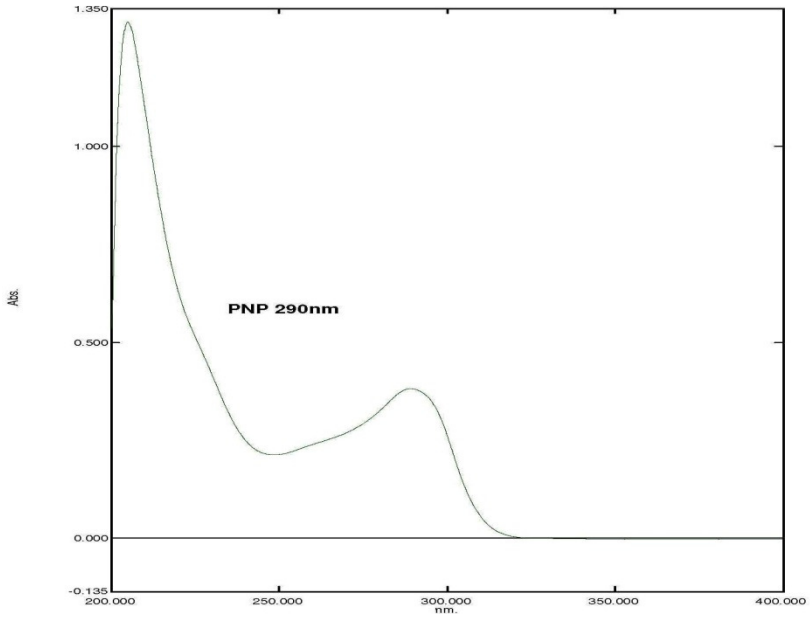


Fig 4: UV spectrum of CNP -λmax at 262 nm

The overlain spectrum of PNP is shown in fig-5.The absorbance corresponding to the concentration is tabulated in table-2. The five point calibration chart showed that the drug obeyed Beer's law in the concentration range of 5–30 µg/mL. The linearity was assessed by the regression analysis as shown in the calibration chart. (fig-6) The correlation coefficient was found to be 1 which was within the limit.

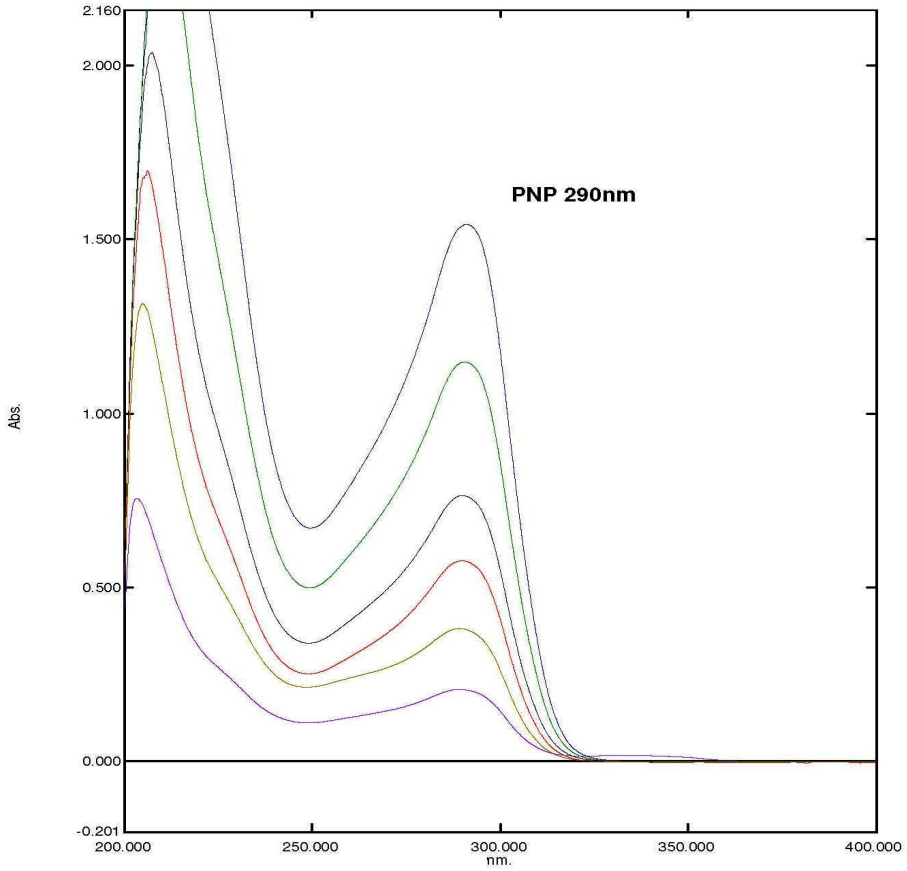


Fig 5: Overlain spectrum of PNP- λmax at 290 nm

Table 2: Absorbance of PNP at 290nm

S.No.	Concentration (µg/mL)	Absorbance at 290nm*
1	5	0.192
2	10	0.384
3	15	0.579
4	20	0.769
5	30	1.149

**Each value is the mean of three determination*

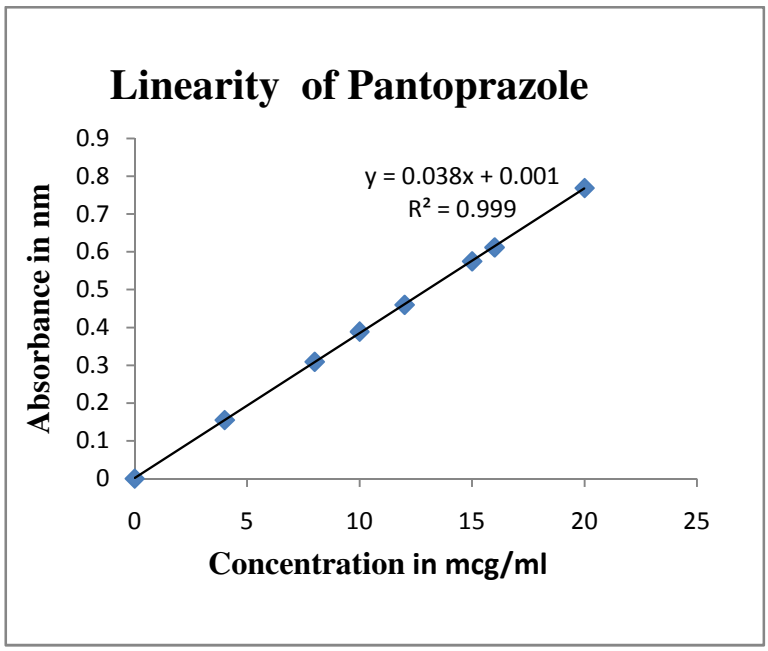


Fig 6: Calibration chart of PNP

SAMPLE ANALYSIS:

The capsule dosage form contains CNP and PNP as separate tablets. So the drugs are estimated as separate entities.

CINITAPRIDE

The formulation containing CNP was quantified. The sample concentration (8µg/mL) was so selected that it lies within the range of the linearity (4–20 µg/mL) of standard CNP. The analysis was performed in triplicate for three times and the mean of the three analyses was used for the calculation. The amount present and the percentage purity were calculated using the formula previously discussed. The percentage purity was found to lie between 99 and 101% and repeated analytical results confirms the precision of the developed method. The results of the sample analysis are shown in table-3

Table 3: Results of Assay of CNP

S.No.	Label claim	Amount present (in mg) ± SD*	RSD	% Purity ± SD	RSD
1.	3mg	3.05 ± 0.000004	0.143	101.85 ± 0.145	0.00143
2.		2.98 ± 0.000002	0.056	99.43 ± 0.056	0.00056
3.		2.98 ± 0.000003	0.097	99.59 ± 0.097	0.00097

**Each value is the mean of three determinations.*

PANTOPRAZOLE

The formulation containing PNP was quantified. The sample concentration (15 µg/mL) was so selected that it lies within the range of the linearity (5–30 µg/mL) of standard PNP. The analysis was performed in triplicate for three times and the mean of the three analyses was used for the calculation. The amount present and the percentage purity were calculated. The percentage purity was found to lie between 99% and 101%. The results of the sample analyses are shown in table - 4

Table 4: Results of Assay of PNP

S.No.	Label claim	Amount present (mg) ± SD*	RSD	% Purity ± SD	RSD
1.	40mg	39.9 ± 0.00004	0.0917	99.75 ± 0.0914	0.0009
2.		40.5 ± 0.00003	0.0675	101.29 ± 0.068	0.0007
3.		40.6 ± 0.00005	0.135	101.43 ± 0.137	0.0014

**Each value is the mean of three determinations.*

RECOVERY STUDIES

The recovery studies were performed on spiked samples at three levels (20%, 40%, 100%) using the standard addition technique. The results of the recovery analyses of CNP are tabulated in table-5 and that for PNP in table-6. The results of the recovery studies confirm the accuracy of the developed method. **Thus, it assures that the developed method shows no interference by the sample matrix.**

Table 5: Results of Recovery Studies of CNP

Expected % recovery	Amount of drug added (mg)		Total amount assayed (mg) *	Amount recovered (mg)	Assessed % Recovery	% Recovered ± S.D	RSD
	Sample	Std					
20%	3	0.6	3.62	0.61	20.31	101.55± 0.18	0.0089
40%		1.2	4.23	1.22	40.71	101.78± 0.45	0.0112
100%		3.0	6.05	3.04	101.24	101.24± 0.64	0.0063

*****Each value is the mean of three determinations.*

Table 6: Results of Recovery Studies of PNP

Expected % recovery	Amount of drug added (mg)		Total Amount assayed (mg) *	Amount recovered (mg)	Assessed % Recovery	% Recovered ± S.D	RSD
	Sample	Std					
20%	40	8	48.38	8.05	20.14	100.69± .398	0.02
40%		16	56.53	16.3	40.87	101.34± 0.87	0.02
100%		80	79.6	39.27	98.17	100.47 ± 0.73	0.01

*****Each value is the mean of three determinations.*

METHOD B – AREA UNDER CURVE

The AUC (area under curve) method involves the calculation of integrated value of absorbance, between the two selected wavelengths λ1 and λ2. The wavelength range is selected on the basis of repeated observations so as to get the linearity between area under curve and concentration. The AUC between two selected wavelengths is digitally calculated and given by the in-built software in the UV spectrophotometer. The spectra obtained in method A were used.

CINITAPRIDE

The AUC for CNP was determined by taking 239.8nm as λ_1 and 296.4nm as λ_2 in the spectra of the standard, sample and recovery studies. (fig-7) The AUC for different concentration are tabulated in table-7. Linearity was established by plotting AUC along X-axis and concentration along Y-axis

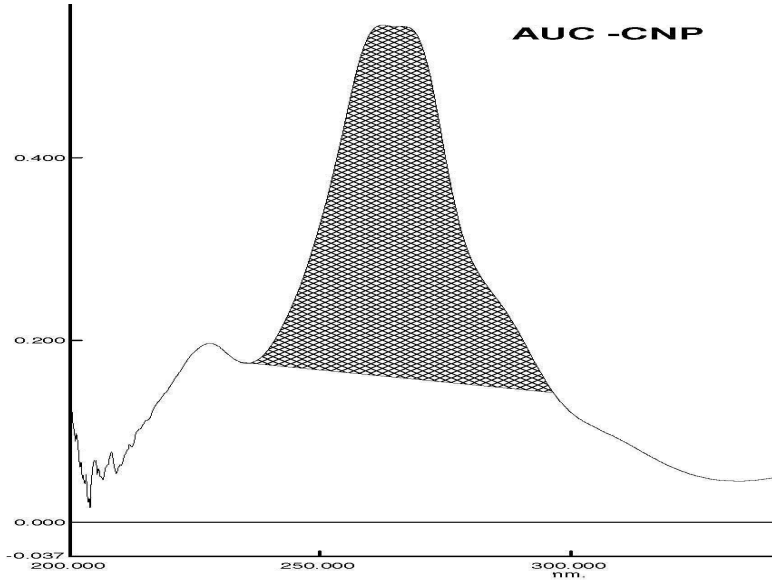


Fig 7: AUC of CNP

Table 7: Area Under Curves of CNP

S.No.	Concentration(µg/mL)	Area under curve*
1	4	3.696
2	8	7.314
3	10	8.699
4	12	10.559
5	16	13.967
6	20	17.894

**Each value is the mean of three determinations*

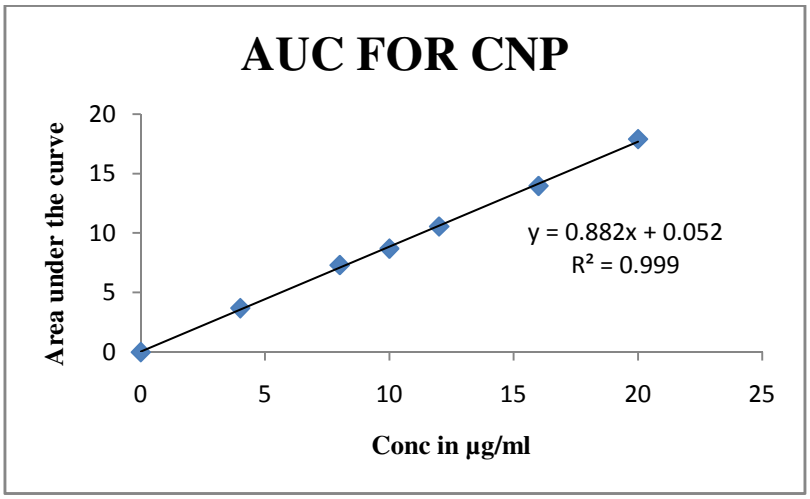


Fig 8: Calibration chart – AUC vs Conc of CNP

The charts were found to be linear and the linearity assessed using regression analysis as shown in fig-8. The correlation coefficient was determined to be 0.9990 for CNP. The sample AUCs were interpolated on the linearity charts to determine the concentrations. The amount present and the percentage purity were determined and

tabulated in table 8. The percentage purity was found to lie between 100% and 102%. The results of the repeated analysis confirm the precision of the method.

Table 8: Results of Assay of CNP using AUC

S.No.	Label claim	Amount present (in mg) ± SD*	RSD	% Purity ± SD	RSD
1.	3mg	3.04 ± .000028	0.0093	101.57 ± 0.945	0.0093
2.		3.02 0.000019	0.0062	100.68 ± 0.630	0.0062
3.		3.02 ± .000019	0.0062	100.68 ± 0.630	0.0062

**Each value is the mean of three determinations.*

PANTOPRAZOLE

Similar to that of CNP, the AUCs of the standard spectra of PNP were determined between 248.4 (λ_1) and 314.0nm as (λ_2) and shown in fig-9 and the AUC corresponding to concentration is given in table-9.

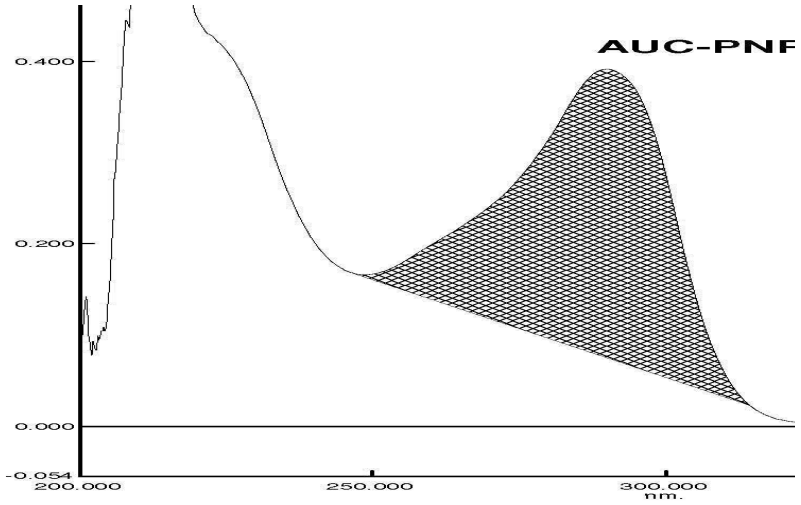


Fig 9: AUC of PNP

Table 9: Area under Curves of PNP

S.No.	Concentration (µg/mL)	Area under curve*
1	5	4.707
2	10	9.046
3	15	14.551
4	20	18.984
5	30	28.539

**Each value is the mean of three determinations*

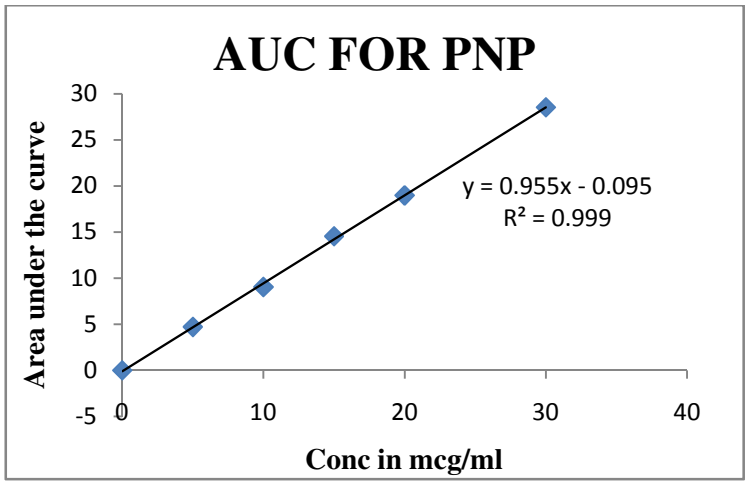


Fig 10: Calibration chart – AUC vs Conc of PNP

The linearity of response was assessed using regression analysis. The correlation coefficient was determined to be 0.9990 for PNP shown in fig-10. The sample AUCs were interpolated on the linearity charts to determine the concentrations. The amount present and the percentage purity were determined and shown in table-10. The

percentage purity was found to lie between 99% and 100%. From the SD and RSD values obtained, precision of the developed method is confirmed.

Table 10: Results of Assay PNP using AUC

S.No.	Label claim	Amount present (mg) ± SD*	RSD	% Purity ± SD	% RSD
1.	40mg	40.1 ±0.0004	0.0094	100.35 ± 0.939	0.009
2.		40.1 ± .0002	0.0054	100.35 ± 0.542	0.005
3.		39.6 ± .0002	0.0047	99.02 ± 0.469	0.0047

*****Each value is the mean of three determinations.*

RECOVERY STUDIES (CNP&PNP)

The AUC of the spectra obtained for the recovery studies (spiked samples at three levels 20%, 40%, 100%) of the samples (both CNP and PNP) were interpolated on the linearity chart of the respective drugs and the concentrations were determined. The recovery percentage was calculated using the formula

$$Recovery\ \% = \frac{Original\ amount - Standard\ Amount}{Standard\ Amount} \times 100$$

The results of recovery studies and recovery percentage for both CNP and PNP are tabulated in table-11&12 respectively.

Table 11: Results of Recovery Studies of CNP using AUC

Expected % recovery	Amount of drug added mg		Total amount assayed (mg)*	Amount recovered (mg)	Assessed % Recovery	% Recovered ± S.D	RSD
	Sample	Std					
20%	3	0.6	3.67	0.6	20.13	100.66 ± 0.59	0.029
40%		1.2	4.27	1.21	40.18	100.45 ± 0.59	0.014
100%		3.0	6.1	3.03	100.95	100.95 ± 0.29	0.002

*****Each value is the mean of three determinations.*

Table 12: Results of Recovery Studies of PNP using AUC

Expected % recovery	Amount of drug added mg		Total amount assayed (mg)*	Amount recovered (mg)	Assessed % Recovery	% Recovered ± S.D	% RSD
	Sample	Standard					
20%	40 mg	8	48.03	8.15	20.38	101.10 ± 0.313	0.0153
40%		16	56.00	16.13	40.31	100.80 ± 0.626	0.0155
100%		80	79.09	39.21	98.02	98.02 ± 0.717	0.0073

*****Each value is the mean of three determinations.*

The results of the recovery studies shows recovery % of 98 to 102% which is within the limit and the low RSD value confirms that the developed method is accurate for both CNP and PNP. Thus the developed method is accurate and precise, irrespective of the sample matrix.

METHOD C – SECOND DERIVATIVE (Beckett, 2007)

Derivative spectrophotometry involves the conversion of normal spectrum to its first, second or higher derivative spectrum. The second derivative (D^2) spectrum is the plot of the curvature of the D^0 spectrum or a plot of $d^2A/ d\lambda^2$ against λ. The characteristic of the second order spectra is that it represents a negative maximum just at the original λ max of the analyte. The amplitude of the negative maxima downwards is directly proportional to the concentration of the analyte.

CINITAPRIDE

The zero order spectra of CNP obtained in method A were derivatized to the second order derivative spectra. CNP shows negative maxima at 262 nm which is the λ max of CNP. The overlain second derivative spectra are shown in fig-11.

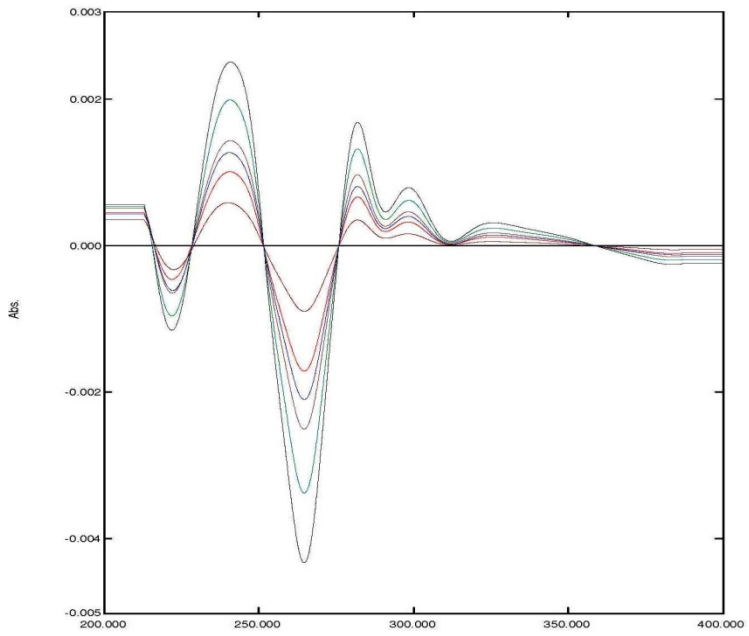


Fig 11: Overlain II Derivative spectrum of CNP

Graph was plotted using amplitude of the negative maxima along X-axis and concentration along Y –axis as shown in table-13 and fig-12. A linear graph obtained was analysed using regression analysis method to determine the linearity of amplitude maxima with concentration. It reveals the correlation coefficient to be 0.999.

Table 13: Amplitude and Concentrations-CNP

S.No.	Concentration(µg/mL)	Amplitude*
1	4	2
2	8	4
3	10	4.9
4	12	5.9
5	16	7.7
6	20	9.6

**Each value is the mean of three determinations*

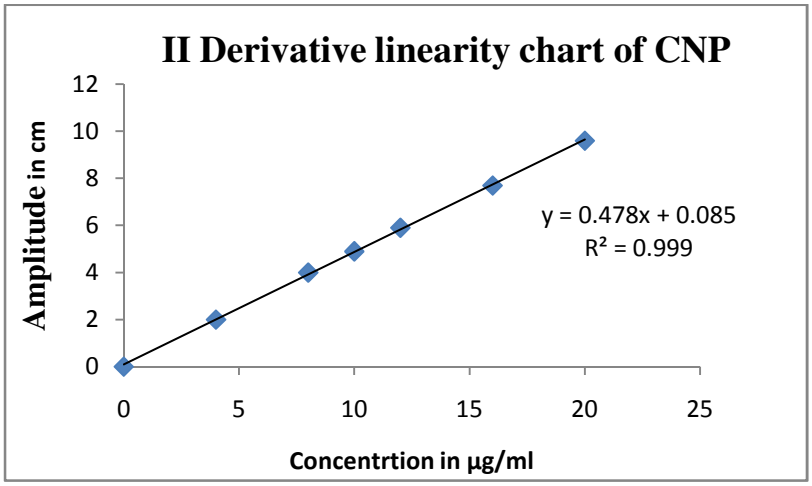


Fig 12: Calibration chart –Amplitude vs. Conc-CNP

The zero order spectrum of the sample was derivatized to second order. The sample concentrations were arrived by interpolation of their amplitudes in the linearity chart. The amount present was calculated and presented in table-14. The percentage purity of CNP was determined to lie between 99% and 102%. The assay was conducted thrice in triplicate. The results of the repeatability confirm that the method is precise.

Table 14: Results of Assay CNP using II derivative method

S.No.	Label claim	Amount present (in mg) ± SD*	RSD	% Purity ± SD	% RSD
1.	3mg	3.05 ± 0.000014	0.0046	101.91 ± 0.472	0.0046
2.		2.99 ± 0.000009	0.0031	99.80 ± 0.315	0.0031
3.		2.99 ± 0.000014	0.0047	99.91 ± 0.472	0.0047

**Each value is the mean of three determinations.*

PANTOPRAZOLE

Similarly, the zero order spectrum of PNP was derivatized to obtain second derivative spectrum. It showed a negative maximum at 290 nm. The overlain second derivative spectrum of PNP is shown in fig-13. Linearity was established using amplitude against concentration. The amplitudes of the negative maxima are given in table-15.

Table 15: Amplitude and Concentrations-PNP

S.No.	Concentration (µg/mL)	Amplitude*
1	4	0.9
2	8	1.8
3	10	2.2
4	12	2.6
5	16	3.5
6	20	4.3

**Each value is the mean of three determinations.*

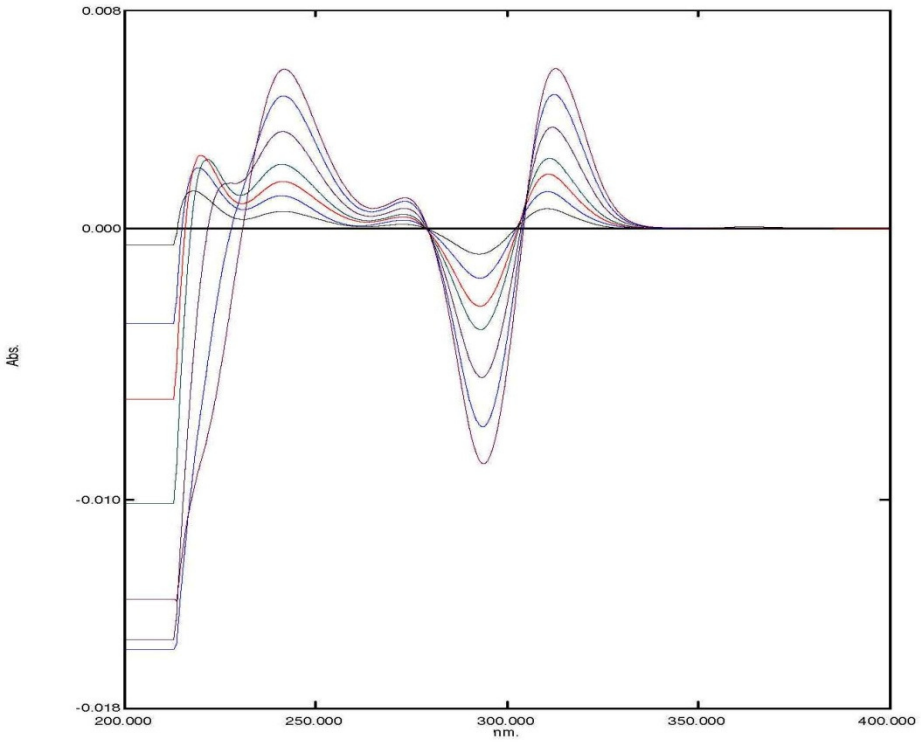


Fig 13: Overlain II Derivative spectrum of PNP

The linearity chart is shown in fig-14, which shows the correlation coefficient to be 0.9996.

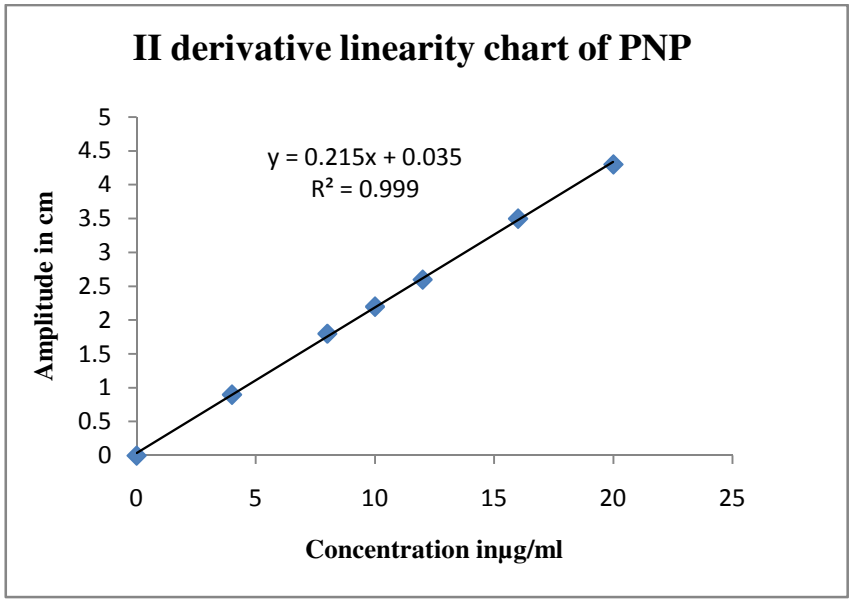


Fig 14: Calibration chart –Amplitude vs. Conc-PNP

Similarly, the sample spectrum was derivatized to the second order. The amplitude of the negative maxima was interpolated on the linearity chart to determine the sample concentration. The results of the analysis are shown in table-16. The percentage purity of the CNP was determined to lie between 99% and 102%. The precision of the method was analysed by conducting the assay thrice in triplicate. The results of the repeatability studies show that the method is precise.

Table 16: Results of Assay PNP using II Derivative method

S.No.	Label claim	Amount present (mg) ± SD*	RSD	% Purity ± SD	RSD
1.	40mg	39.5 ± 0.0001	0.0031	98.8 ± 0.313	0.0030
2.		40.0 ± .00009	0.0023	100.02 ± 0.234	0.0023
3.		40.0 ± .00019	0.0046	100.02 ± 0.469	0.0047

*****_Each value is the mean of three determinations._

RECOVERY STUDIES (CNP&PNP)

The recovery studies were performed for CNP and PNP. Recovery studies were performed based on stanadard addition technique. The zero order spectra obtained for recovery studies (20%, 40%, and 100%) in method A were derivatized. The amplitudes of the negative maxima were interpolated on the respective second derivative linearity charts and the concentrations were determined. Using the concentration, the recovery percentage was determined and tabulated in table-17 for CNP and table-18 for PNP. The low RSD value indicates that recovery was appreciable and confirms that the interference due to sample matrix is negligible. Thus the method is accurate for both CNP and PNP.

Table 17: Results of Recovery Studies of CNP - II Derivative method

Expected % recovery	Amount of drug added in mg		Total amount assayed (mg)*	Amount recovered (mg)	Assessed % Recovery	% Recovered ± S.D	RSD
	Sample	Standard					
20%	3mg	0.6	3.61	0.58	19.59	97.94 ± 0.295	0.015
40%		1.2	4.25	1.22	40.68	101.70 ± 0.511	0.0125
100%		3.0	6.06	3.03	101.04	101.04 ± 1.06	0.010

*****Each value is the mean of three determinations.*

Table 18: Results of Recovery Studies of PNP - II Derivative method

Expected % recovery	Amount of drug added in mg		Total amount assayed (mg)*	Amount recovered (mg)	Assessed % Recovery	% Recovered ± S.D	RSD
	Sample	Standard					
20%	40 mg	8	47.94	8.09	20.21	101.07±0.313	0.0155
40%		16	56.00	16.15	40.37	100.93±0.626	0.0155
100%		80	80.50	40.70	101.74	101.74 ± .939	0.0092

*****Each value is the mean of three determinations.*

Table 19: Optical parameters of CNP and PNP-UV Spectrophotometry

S.No	Optical Parameter	Cinitapride			Pantoprazole		
		Method			Method		
		A	B	C	A	B	C
1.	Wavelength λmax	262 nm	262nm	262nm	290 nm	290nm	290nm
2.	Molar absorptivity	22557.89	---	---	14746.97	----	----
3.	Beer's law limit µg/ml	4-20	4-20	4-20	5-30	5-30	5-30
4.	Regression equation	y = 0.056x + 0.000	y = 0.882x + 0.052	y = 0.478x + 0.085	y = 0.038x + 0.001	y = 0.955x - 0.095	y= 0.215x +0.035
5.	Slope	0.05602	0.882	0.478	0.03815	0.955	0.215
6.	Intercept	0.00029	0.052	0.085	0.001	-0.095	0.035
7.	Correlation coefficient	1.0	0.9990	0.999	1.000	0.9990	0.999
8.	Sandell's sensitivity	0.0178	----	----	0.0260	----	----
9.	LOD	0.03366	---	---	0.4144	----	----
10.	LOQ	0.10200	----	----	1.2560	----	---
11.	RSD	0. 4710	0. 9901	1.1310	0.8022	0.9548	0.4782

II. VISIBLE SPECTROPHOTOMETRIC METHOD

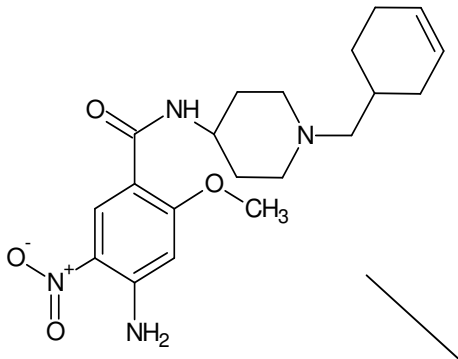
CINITAPRIDE (Chand Pasha, et al., 2010)

METHOD A USING ETHYL ACETO ACETATE (EAA)

The colorimetric method for estimation of CNP is based on the diazotization of the primary aromatic amino group in CNP. The diazotized CNP is then coupled with EAA in alkaline media. The diazotisation of drugs containing free amino group is a well known reaction.

In this method the primary aromatic amino group in CNP is diazotized using sodium nitrite solution and hydrochloric acid. Diazonium compounds are unstable and would decompose at elevated temperature, hence temperature was maintained below 5°C until the addition of sodium hydroxide. The amino group is converted into diazonium salt which on treatment with EAA undergoes coupling reaction to form a diazo couple. EAA is the coupling agent. The active methylene group present in it undergoes coupling reaction in alkaline condition. The diazo couple formed is waters soluble and coupling reaction takes place only in alkaline medium which is provided by the addition of sodium hydroxide solution. The diazo couple is reddish orange in colour for which the maximum absorbance was observed at 392.5 nm. The stability of the colour was determined by measuring the absorbance of the solutions at 15 minute intervals. The absorbance was found to stable for 3 hrs 15 minutes, after which the absorbance started decreasing. Thus the stability of the diazo couple was determined to be 3 hrs 15 minutes. So, all the studies were performed within 3 hrs of formation of the diazo couple. The reaction mechanism is given below:

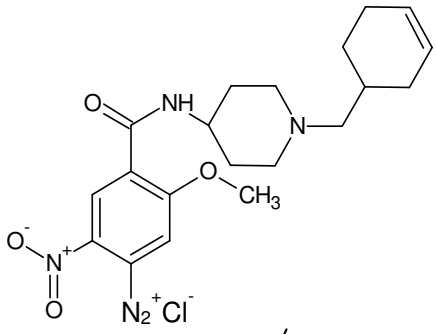
REACTION MECHANISM



Cinitapride

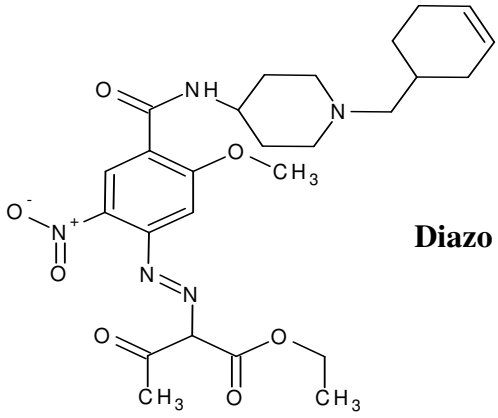
$NANO_2/HCl$

0-5°C



Diazonium salt of Cinitapride

$CH_3COCH_2COOCH_2CH_3$ NaOH



Diazo couple of CNP -EAA

OPTIMISATION OF THE REAGENTS

Diazotization and complexation reaction condition:

The conditions for the diazotization (Shahar Yar M) are well established to take place at 0-5°C. An elevated temperature leads to incomplete diazotization and decomposition of diazotized compound (Jim Clark) which could be assessed by the non linear response of the diazotized mixture in UV spectrophotometry at 410nm.The volume and strength of the sodium nitrite and hydrochloric acid used was optimized. It was found that an increase or decrease from 1-2mL and 1-4% of sodium nitrite brought about no change in response or very less absorbance. By trial and error it was established that 2 mL each of 2% sodium nitrite solution, 0.5M hydrochloric acid and 2% EAA were found to give good linear relationship between absorbance and concentration. Any change in the volume or strength of the above reagents showed deviation from the linearity. Similarly the strength and volume of sodium hydroxide were also optimized. It was observed that 1mL of 1M sodium hydroxide was optimal to ensure complete complexation and good colour intensity. Finally the concentration of the analyte was also studied. Lower the concentration (below 10µgm) lower was the colour intensity as a result, the absorbance was very less and did not show significant difference. Higher concentration (above 60µg/mL) showed deviation from the linearity. The absorbance of the couple with respect to concentration is shown in table-20. The absorbance gradually increased with respect to concentration

The overlain spectrum of CNP –EAA diazo couple is shown in fig-14.

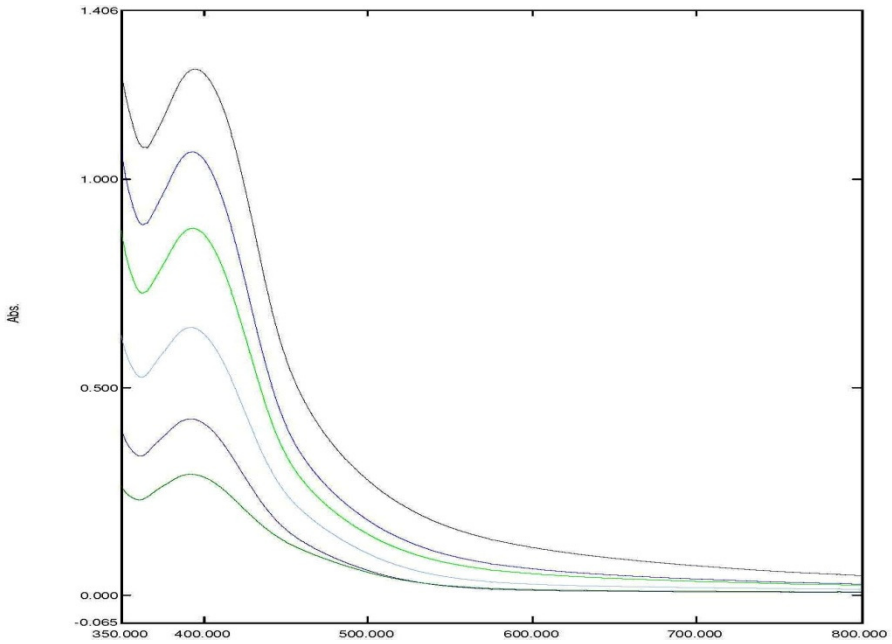


Fig 15: Overlain spectrum of CNP –EAA diazo couple

Table 20 : Absorbance of CNP –EAA complex

S.No.	Concentration (µg/mL)	Absorbance* at 392.5 nm
1	10	0.247
2	20	0.424
3	30	0.644
4	40	0.882
5	50	1.064
6	60	1.265

** Each value is the mean of three determination*

Thus 10-60µg/mL of CNP obeyed Beer's law and was selected for the study. The correlation coefficient was found to be 0.999 as shown in fig-16. Thus 10-60µg/mL of CNP obeyed Beer's law and was selected for the study. The correlation coefficient was found to be 0.998 as shown in fig-16. The linearity chart shows that the absorbance linearly increased with concentration. The linearity chart is used for the estimation of the drug in formulation.

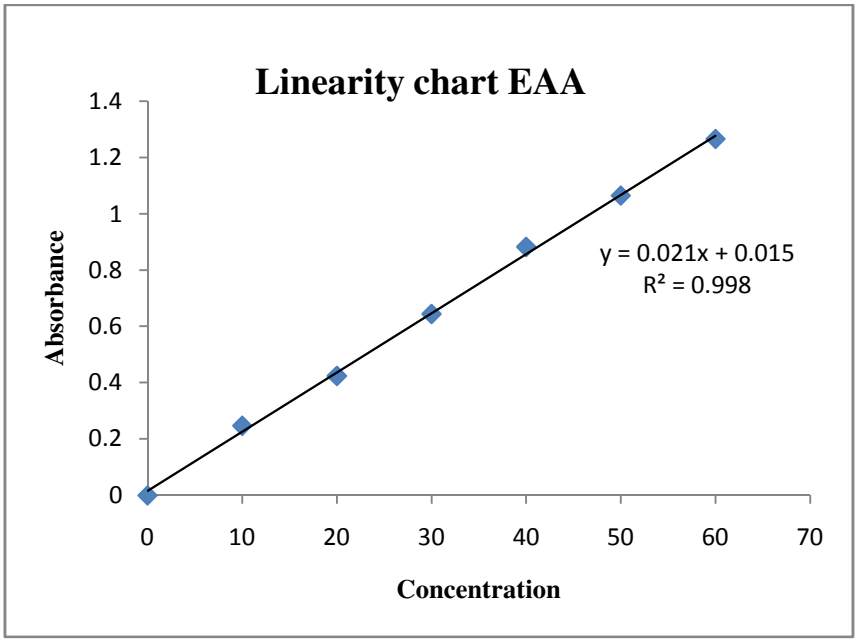


Fig 16: Calibration chart of CNP – EAA

ANALYSIS OF FORMULATION

About 30 µg/mL concentration of formulation was similarly analysed to that of the standard drug and the amount present and percentage purity was calculated presented in table- 21. Precision of the developed method has been ascertained by the

reproducibility of the assay results, when repeated thrice in triplicate. Since the results were found to be reproducible, the method is precise.

Table 21: Results of assay of CNP using EAA

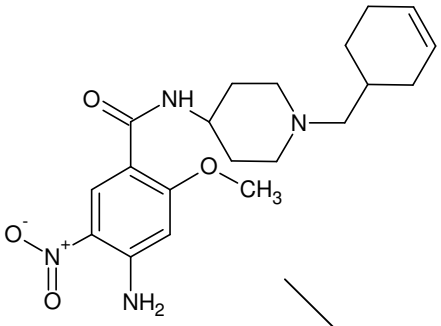
S.No.	Label claim	Amount present (mg) ± SD*	RSD	% Purity ± SD	RSD
1.	3mg	2.98 ± 0.000010	0.0034	99.17 ± 0.3373	0.0034
2.		2.99 ± 0.000008	0.0026	99.54 ± 0.2654	0.0026
3.		2.99 ± 0.000020	0.0068	99.17 ± 0.472	0.0068

** Each value is the mean of three determination*

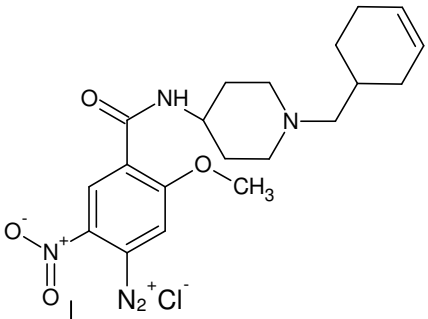
METHOD B — USING ACETYL ACETONE (AAC)

This method is also based on the diazotisation of CNP followed by coupling of the diazonium salt with a diketone. The diketone is acetyl acetone which acts as a coupling agent. The amino group is converted into diazonium salt which on treatment with AAC forms a diazo couple. The active methylene group in AAC undergoes coupling with the nitrogen of the diazo group in CNP. The coupling reaction takes place only in an alkaline medium which is provided by the addition of sodium hydroxide solution. The complex is reddish orange in colour for which the maximum absorbance was observed at 399nm. The mechanism of the reaction is hown below. The stability of the diazo couple was found to be 3 hrs 15 minutes. The mechanism of the complexation is shown in the next page.

REACTION MECHANISM

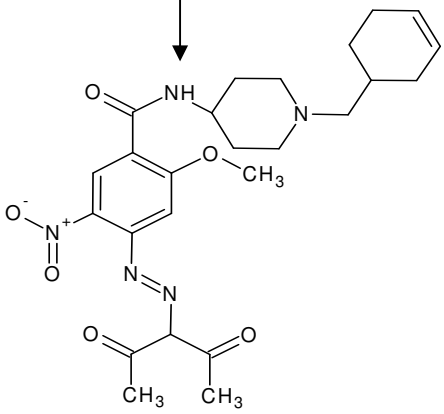


Cinitapride $NaNO_2/HCl$ 0-5°C



Diazonium salt of Cinitapride

$CH_3COCH_2COCH_3$ NaOH



Diazo couple of CNP –AAC (reddish orange chromogen)

OPTIMISATION OF THE REAGENTS

Diazotization and complexation reaction condition:

The optimizations of diazotization reagents are the same as that for the method A. In this method also 2mL of 2% sodium nitrite solution and 2ml of 0.5 M hydrochloric acid was sufficient for diazotising CNP completely at 0-5° C. By trial and error, it was established that 2mL of 2% AAC were found to give good linear relationship between absorbance and concentration. Any change in the volume or strength of AAC showed deviation from the linearity. Similarly the strength and volume of sodium hydroxide were also optimized. It was observed that 1mL of 1M sodium hydroxide was optimal to ensure complete coupling and good colour intensity. Below 0.5 ml of 1M sodium hydroxide, the intensity of colour of the diazo couple was not good and above 1 ml there was no change in the colour intensity. Finally the concentration of the analyte was also studied. Lower the concentration (below 10µgm) lower was the colour intensity as a result the absorbance was very less and did not show much difference in the absorbance. Higher concentration (above 50µg/mL) showed deviation from the linearity. Thus 10-50µg/mL of CNP obeyed Beer's law (table-22 & fig-18) and was selected for the study. The correlation coefficient was found to be 0.996.

The overlain spectra of CNP - AAC is shown in fig-17.

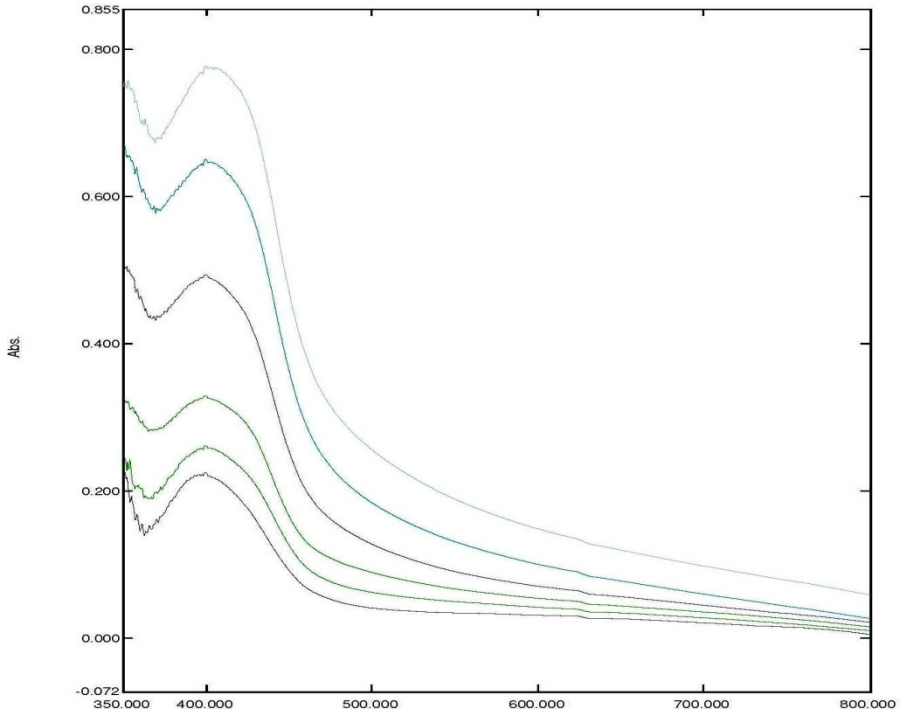


Fig 17: Overlain spectrum of CNP-AAC diazo couple

Table 22 : Absorbance of CNP –AAC

S.No.	Concentration (in µg/mL)	Absorbance*
1	10	0.169
2	20	0.330
3	30	0.494
4	40	0.651
5	50	0.776

*****_Each value is the mean of three determinations._

The linearity chart of CNP-EAA complex is shown in fig -18. It shows that the absorbanbce linearly increase with concentration.

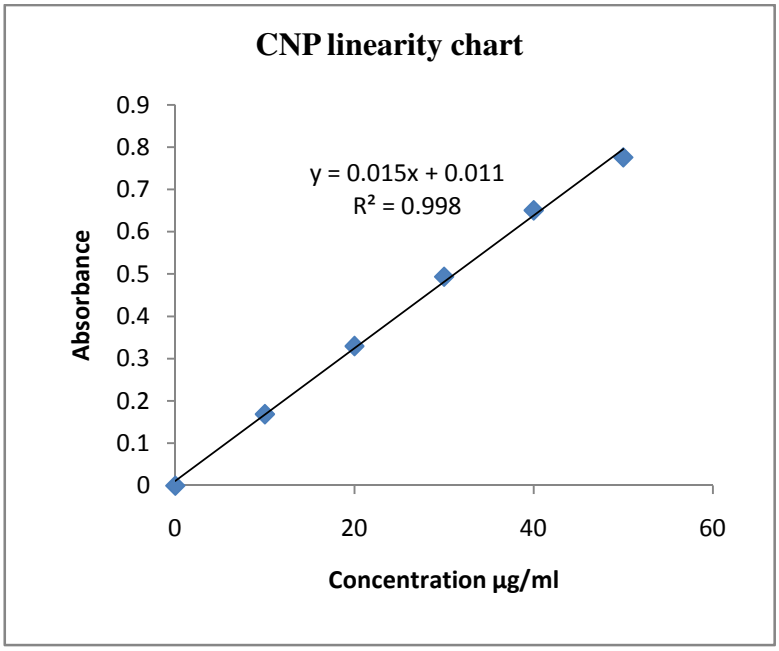


Fig 18: Calibration chart of CNP – AAC

ANALYSIS OF FORMULATION

Sample concentration was so selected for the study such that the final concentration (20 µg/mL) for the complexation lies within the Beer's range. Similar to the standard drug, the sample was allowed to undergo diazotization and coupling reaction. The percentage purity was calculated and presented in table–23. The percentage purity was found to lie between 99.8 to 101.9%.The precision studies was performed by analysing sample thrice in triplicate. The results of the repeatability studies ensure that the method is precise.

Table 23. Results of assay CNP using AAC

S.No.	Label claim	Amount present (in mg) ± SD*	RSD	% Purity ± SD	RSD
1.	3mg	3.02 ± 0.000027	0.0090	101.91 ± 0.472	0.0046
2.		2.94 ± 0.000035	0.0118	99.80 ± 0.315	0.0031
* 3. *		3.05 ± 0.000044	0.0147	99.91 ± 0.472	0.0047

**Each value is the mean of three determinations.*

RECOVERY STUDIES (CNP- Method A & B)

Accuracy of the developed method was determined by the usual recovery studies. The recovery studies were performed by standard addition technique. It involves the addition of standard drug to the preanalysed sample. The resultant sample was then subjected to colorimetric reaction as discussed in method A & B separately. The results are shown in the table–24 & 25 for method A & B respectively. The results of recovery studies were found to lie between 98-102 % , for both methods which was within the limit. The results of the recovery studies indicate that the method developed is devoid of any interference by the sample matrix or excipients in the formulation.

Table 24: Results of Recovery studies (CNP-EAA)

Expected % Recovery	Amount of drug added (mg)		Total amount assayed (mg)*	Amount recovered (mg)	Assessed % Recovery	% Recovered ± S.D	RSD
	Sample	Standard					
27 %	3mg	0.8	3.8	0.8	27.62	102.17 ± 0.725	0.0260
50 %		1.5	4.44	1.50	48.91	98.15 ± 0.515	0.0105
100 %		3.0	5.91	2.90	100.95	98.11± 0.389	0.0039

*****Each value is the mean of three determinations*

Table 25 : Results of Recovery studies (CNP-AAC)

Expected % Recovery	Amount of drug added (mg)		Total amount assayed (mg)*	Amount recovered (mg)	Assessed % Recovery	% Recovered ± S.D	RSD
	Sample	Standard					
20%	3mg	0.6	3.57	0.59	19.82	98.50 ± 0.380	0.0191
40 %		1.2	4.17	1.19	39.80	99.38 ± 0.900	0.0225
100 %		3.0	5.97	2.99	99.76	100.07± 1.69	0.0170

**Each value is the mean of three determinations.*

PANTOPRAZOLE (Basavaiah K., et al., 2007; Adnan A., et al., 2002)

The visible or colorimetric determination of pantoprazole is based on the oxidation of PNP to its sulphone derivative by by *insitu* generated bromine followed by determination of unreacted bromine by two different reaction schemes. In method A, the residual bromine is treated with excess of iron (II), and the resulting iron (III) is complexed with thiocyanate and measured at 470 nm. Method B involves treating the unreacted bromine with a measured excess of iron (II) and remaining iron (II) is complexed with 1,10-phenanthroline at a raised pH, and measured at 510 nm. In both methods, the amount of bromine reacted corresponds to the amount of PNP.

METHOD A- USING 1, 10- PHENANTHROLINE

The colorimetric estimation of PNP is based on the oxidation of PNP by the *insitu* liberation of bromine during the reaction between excess bromate-bromide and hydrochloric acid. Pantoprazole is oxidized to form the sulphone analogue. The unreacted bromine left after oxidation of PNP is determined indirectly by the oxidation of large excess FAS to ferric ammonium sulphate. The unreacted FAS then treated with 1, 10-phenanthroline form a blood red complex of FAS - 1, 10-phenanthroline (ferroin) which is a very well known complexation reaction used for the estimation of iron in the ferrous state. The complex shows λmax at 510 nm. When a known excess amount of bromate-bromide mixture is allowed to react with increasing amount of PNP, there occurs decrease in the amount of bromine for oxidation of FAS to ferric ammonium sulphate. Thus, an increase in the

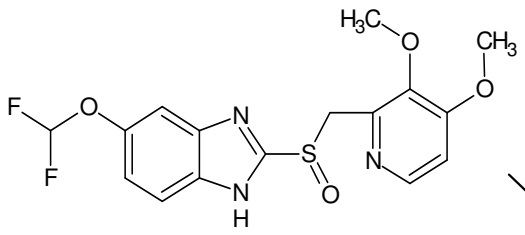
concentration of the FAS, available for complexation with 1, 10-phenanthroline is observed from the increase in the absorbance, as shown by the slope in the calibration chart (fig-20). Since the complex is formed and is stable only in alkaline media, ammonia solution is used.

REACTION MECHANISM:

I. Oxidation

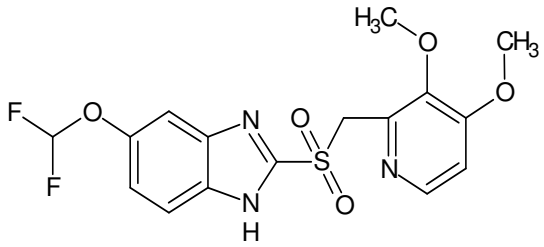
$KBrO_3 + 5KBr + 6HCl \longrightarrow 6\,KCl + 3Br_{2(g)} + 3H_2O$

(potassium bromate-bromide) ***bromine***



Pantoprazole

Br_2 (o)

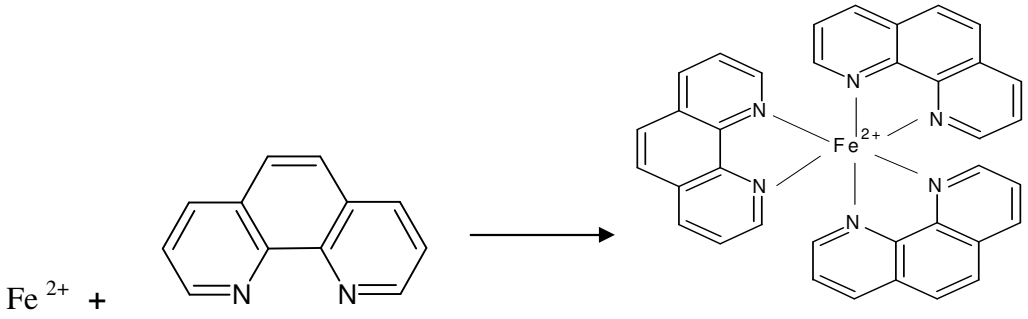


Oxidised form of panatoprazole

II. **Complexation** (Vogels., 2007)

Fe^{2+} + Br_2 (unreacted) —(o)→ Fe^{3+} + 2 Br^-

(ferrous) ***(ferric)***



Fe^{2+} +

(ferrous) 1,10 Phenanthroline $Fe[(C_{12}H_8N_2)_3]^{2+}$

Red chromogen (ferroin)

OPTIMISATION OF THE REAGENTS

The strength and volume of hydrochloric acid and bromate-bromide mixture was optimized. Any change in the volume of the reagent resulted in either insufficient liberation or large excess of bromine which did not give any good response. Thus 1ml of 5M hydrochloric acid and 2ml of bromate –bromide mixture and 2ml of FAS was selected for the complete reduction of the unreacted bromine. Moreover, 2mL of 1, 10- phenanthroline was required for the complete complexation of the excess, unreacted FAS. The complex is formed only in an alkaline medium which is provided by the addition of ammonia solution. About 2 ml of ammonia solution was sufficient to neutralize and slightly raise the pH of the solution at which the complexation is complete. Any more increase in volume of the ammonia solution,

drastically increases the pH of the solution resulting in the instability of the complex. The chromogen was red in colour which showed a maximum absorbance at 510 nm. Higher concentration of the analyte, required very high concentration and large excess of the bromate-bromide mixture. So the linearity of response with respect concentration of standard PNP was established using a low concentration range of PNP. Thus, beer's range was determined to be 1.2-5.4µg/mL. (table-26 & fig 21) The estimation of PNP by the above method was also performed within the Beer's range. The amount of the reagents, the condition of the reagents and the order of the addition of reagents were maintained throughout the analysis of the sample. The overlain spectrum is shown in fig-19.

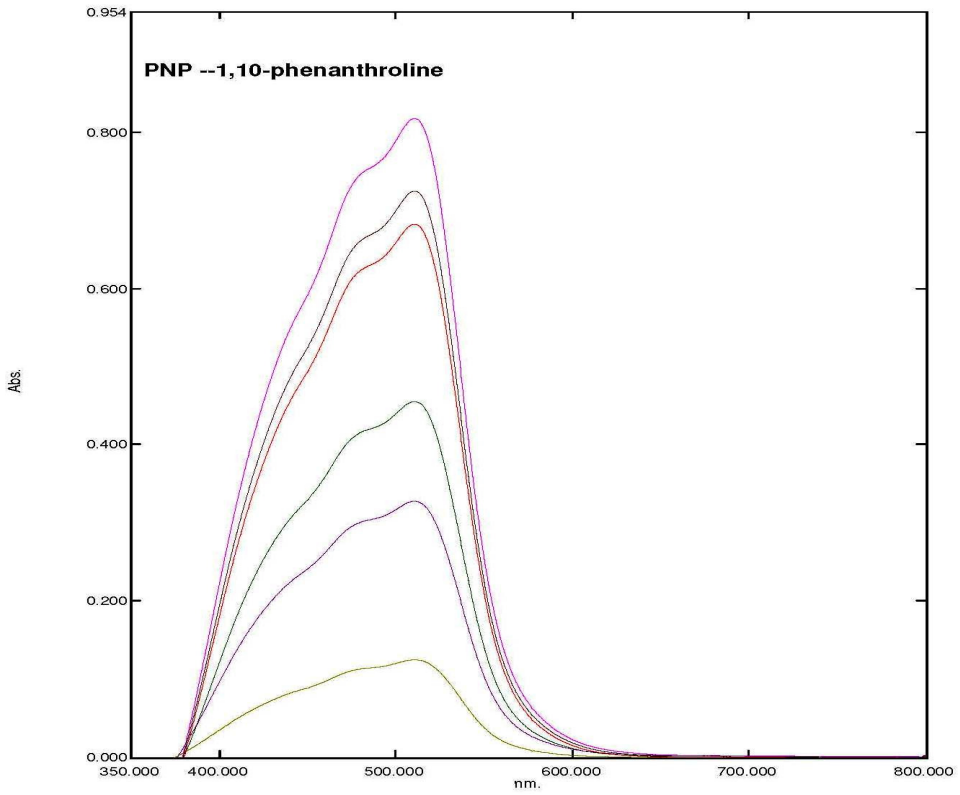


Fig 19 : Overlain spectra PNP using 1,10- phenanthroline

Table 26 : Absorbance of PNP using 1,10-phenathroline

S.No.	Concentration (in µg/mL)	Absorbance*
1	1.2	0.160
2	2.4	0.318
3	3.6	0.466
4	4.8	0.622
5	5.4	0.703

**Each value is the mean of three determinations*

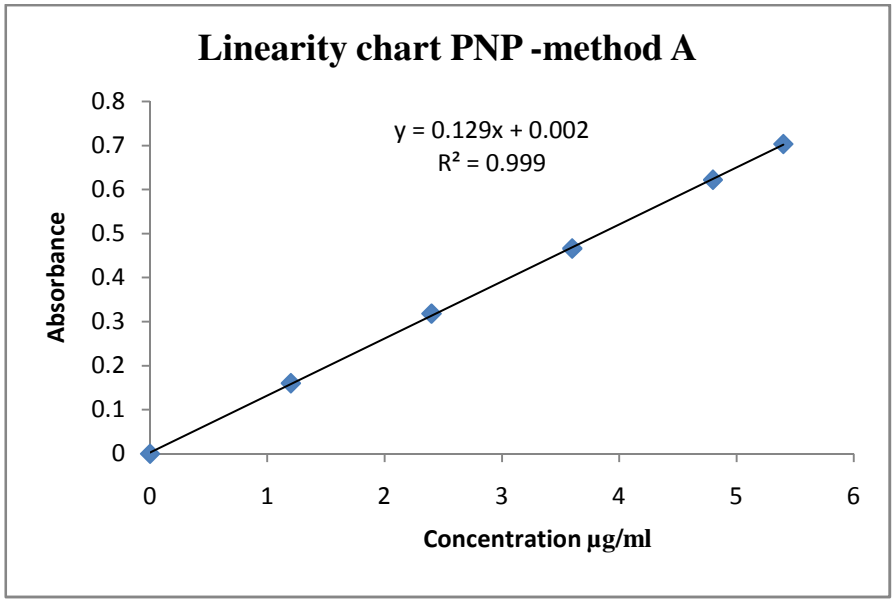


Fig 20: Calibration chart of PNP using 1,10-phenanthroline

ANALYSIS OF FORMULATION

The concentration of the sample was prepared such that it lies within the linearity range. The optimized reagents and conditions were applied for the sample analysis. The results of the sample analysis are presented in table–27. The percentage purity was determined and found to lie between 98.62 and 99.75%. The results were reproducible, when the assay was performed thrice in triplicate. So the method is said to be precise.

Table 27: Results of assay PNP– 1, 10-phenanthroline

S.No.	Label claim	Amount present (mg) ± SD*	RSD	% Purity ± SD	RSD
1.	40mg	39.5 ± 0.0002	0.9898	98.62 ± 0.731	0.9899
2.		39.5 ± 0.00028	0.9949	98.70 ± 0.710	0.9941
3.		39.9 ± 0.00026	0.9943	99.71 ± 0.665	0.9943

*****_Each value is the mean of three determinations._

METHOD B – USING AMMONIUM THIO CYANATE (ATC)

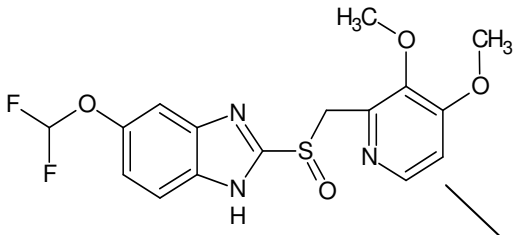
The colorimetric estimation of PNP is based on the oxidation of PNP by the *insitu* liberation of bromine during the reaction between excess bromate-bromide and hydrochloric acid. The unreacted bromine left after oxidation of PNP is determined indirectly by the oxidation of FAS to ferric ammonium sulphate. This in turn is complexed with ammonium thiocyanate to form a blood red complex of ferric thio cyanate which is a very well known reaction. The complex shows λmax at 477nm.

REACTION MECHANISM:

I. Oxidation

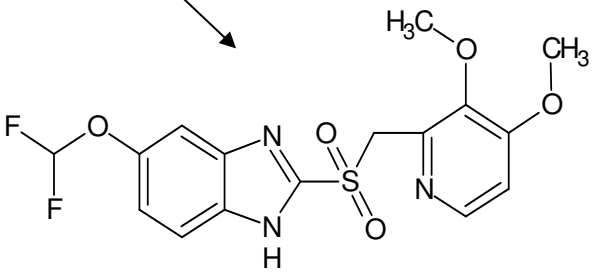
$KBrO_3 + 5KBr + 6HCl \longrightarrow 6\ KCl + 3Br_2 + 3H_2O$

(potassium bromate-bromide + hydrochloric acid) ***potassium chloride + bromine***



Pantoprazole

Br_2 (O)



Oxidised form of panatoprazole

II. Complexation (Vogels., 2007)

Fe^{2+} **+** Br_2 (Excess) $\xrightarrow{(o)}$ Fe^{3+} **+2 Br** $^-$

(ferrous) (ferric)

Fe^{3+} **+ 3**SCN^- $\longrightarrow$ $Fe(SCN)_3$

Red chromogen (ferric thiocyanate)

When a known excess amount of bromate-bromide mixture is allowed to react with increasing amount of PNP, there occurs decrease in the amount of bromine for oxidation of FAS to ferric ammonium sulphate. Thus, the decrease in the concentration of the complex is observed from the decreasing absorbance, resulting in a negative slope in the calibration chart fig-22. The overlain spectrum is shown (fig-21)

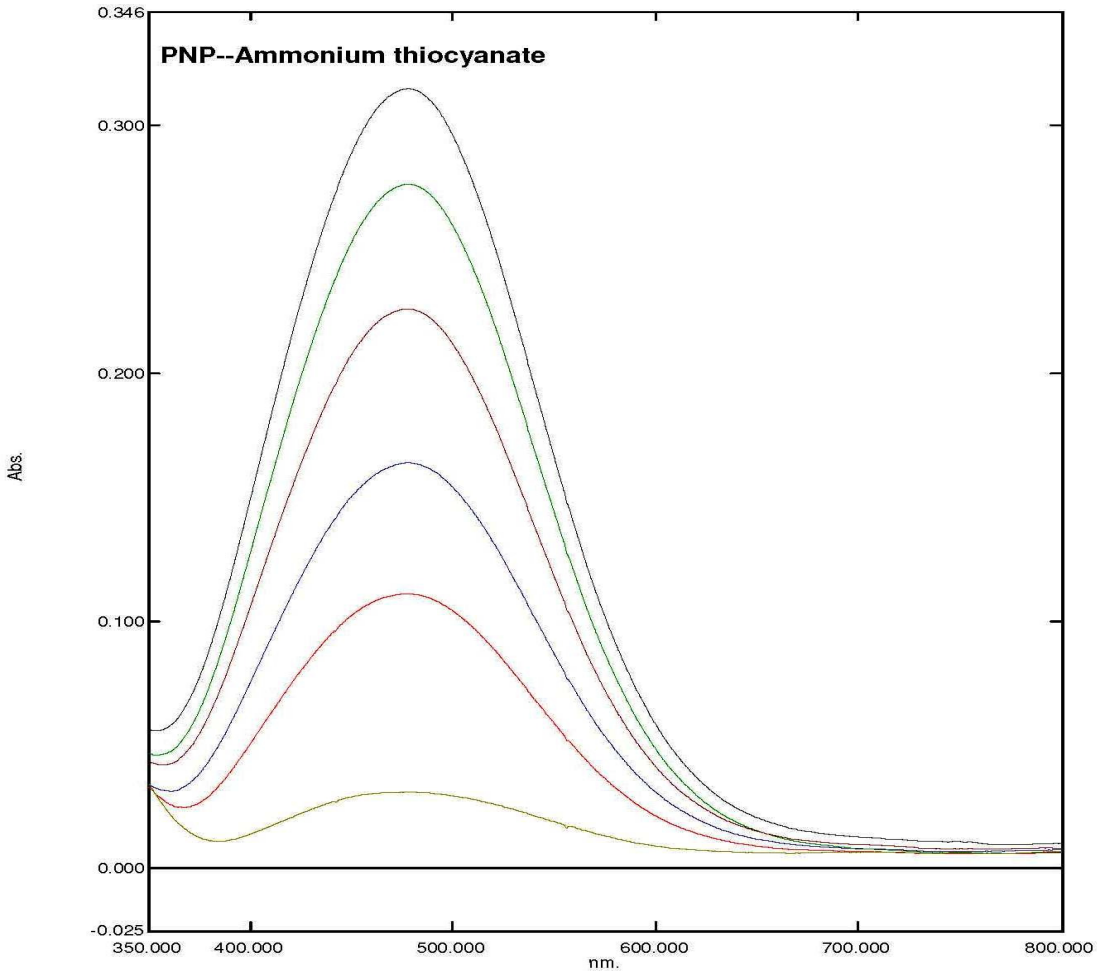


Fig 21 : Overlain spectra PNP – ATC at 477nm

OPTIMISATION OF THE REAGENTS

The strength and volume of the redox reagents viz; hydrochloric acid, bromated-bromide mixture, FAS was the same as that for method A.Thus 1ml of 5M hydrochloric acid and 2ml of bromate –bromide mixture and 2ml of FAS was selected for the completion of the redox reaction. Moreover, 1mL of 3 $molL^{-1}$ ammonium thio cyanate was required for the complete complexation of oxidised ferric ammonium sulphate to ferric thio cyanate. The ferric thio cyanate complex is blood red in colour and was found to be stable for 3 hrs. It showed maximum absorbance at 470 nm. Higher concentration of the analyte, required very high concentration and large excess of the bromate-bromide mixture. The above developed method obeyed Beer's law at the concentration of 0.4-2.4 µg/ml of PNP which showed a gradual decrease in the concentration of the bromine by the linear negative slope.(table-28)

Table 28: Absorbance of PNP – ATC

S.No.	Concentration (in µg/mL)	Absorbance*
1	0.4	0.315
2	0.8	0.276
3	1.2	0.216
4	1.6	0.164
5	2.0	0.111
6	2.4	0.061

*****_Each value is the mean of three determinations._

This is also evidenced by the negative slope of the calibration curve. (fig- 22)

The correlation coefficient was within the limit as 0.998.

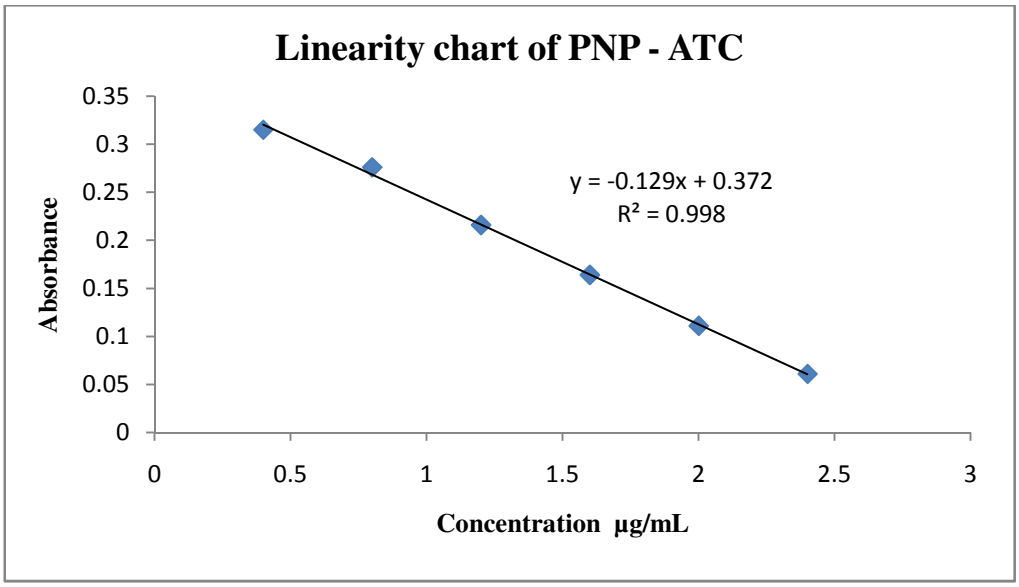


Fig 22: Calibration chart of PNP- ATC

ANALYSIS OF FORMULATION

The sample concentration was so selected that it lies in the linearity range. It was analysed similar to that of the standard drug. The results of the assay and percentage purity are shown in the table-29. The percentage purity was determined to be between 98 to 100%. The precision of the developed method has been analysed by performing the assay thrice in triplicate. The results of the repeatability studies show that the method is precise.

Table 29: Results of assay of PNP using ATC

S.No.	Label claim	Amount present(mg) ± SD*	RSD	% Purity ± SD	RSD
1.	40mg	40.2 ± 0.00059	0.983	100.40 ± 1.49	0.9834
2.		39.6 ± 0.00029	1.009	98.07 ± 0.746	1.0099
3.		39.4 ± 0.00023	0.9927	98.47 ± 0.593	0.9927

**Each value is the mean of three determinations*

RECOVERY STUDIES (PNP- Method A & B)

Accuracy of the developed method was determined by the usual recovery studies. The recovery studies were performed by standard addition technique. It involves the addition of standard drug to the preanalysed sample. The resultant sample was then subjected to colorimetric reaction as discussed in method A & B separately. The results are shown in the table-30 & 31 for method A & B respectively. The results of recovery studies were found to lie between 98-102 %, for method- A and for method–B, 99-102%, which was within the limit. The results of the recovery studies indicate that the method developed shows no interference by the sample matrix or excipients in the formulation.

The optical parameters of CNP and PNP BY colorimetric methods are presented in table-32

Table 30: Results of recovery studies PNP – 1,10 - phenanthroline

Expected % recovery	Amount of drug added in mg		Total amount assayed (mg)*	Amount recovered (mg)	Assessed % Recovery	% Recovered ± S.D	RSD
	Sample	Standard					
20%	40 mg	8	47.52	8.07	20.17	100.84 ± 0.152	0.0075
40%		16	59.09	19.6	49.09	98.18± 1.14	0.0234
100%		80	80.25	40.8	101.99	101.99± 0.79	0.0077

**Each value is the mean of three determinations.*

Table 31: Results of recovery studies for PNP-ATC

Expected % recovery	Amount of drug added in mg		Total amount assayed (mg)*	Amount recovered (mg)	Assessed % Recovery	% Recovered ± S.D	RSD
	Sample	Standard					
25%	40 mg	10	50.44	11.0	27.62	102.12± 1.180	0.1225
50%		20	58.45	19.1	47.66	99.5± 1.180	0.0524
100%		40	80.50	41.1	102.78	102.78± 1.180	0.0101

**Each value is the mean of three determinations.*

Table 32: Optical parameters of CNP and PNP by colorimetry

S.No	Optical Parameter	Cinitapride		Pantoprazole	
		Method A (EAA)	**Method B (AAC)**	**Method A (1,10-PHT)**	**Method B (ATC)**
1.	Wavelength λmax	392.5 nm	399nm	510nm	477 nm
2.	Molar absorptivity	8837.83	6571.97	5022.02	95581.60
3.	Beer's law limit (µg/mL)	10-60	10-50	1.2-5.4	0.4 -2.4
4.	Regression equation	y=0.021x+ 0.006	y=0.015x + 0.011	y=0.129X + 0.002	y=-0.1297X + 0.372
5.	Slope	0.021	0.015	0.129	-0.1297
6.	Intercept	0.006	0.011	0.002	0.372
7.	Correlation coefficient	0.999		0.999	0.998
8.	Sandell's Sensitivity	0.046	0.061	0.008	0.13
9.	LOD	4.40	5.18	0.234	18.9
10.	LOQ	13.33	15.68	0.707	57.4

III. RP- HPLC METHOD

An effort has been made to develop simple, precise, cost- effective and accurate methods for the estimation of Cinitapride and Pantoprazole in bulk and tablet dosage form.

Detector wavelength

A solution of 10 µg/ml of CNP standard drug in mobile phase was scanned in the UV region to determine the detector wavelength. It was observed that CNP showed λmax at 262 nm. Hence this was selected as the detecting wavelength for the estimation of CNP by HPLC. Similarly, 10 µg/ml solution of PNP in mobile phase showed λmax at 290 nm. So, 290 nm was selected for the estimation of PNP by HPLC.

Reverse phase-HPLC

The analytes are more polar in nature RP-HPLC method was selected.

Optimization of the chromatographic condition

The chromatographic condition was optimized using the standard drugs CNP and PNP. The optimization procedure involves the optimization of mobile phase in relation to its compatibility to the system suitability parameters as per ICH guidelines.

By trial and error method the mobile phase containing acetonitrile, methanol and phosphate buffer (pH-7) in the ratio of 40:20:40 % v/v was selected for the

study.The mobile phase of pH-7 and flow rate of 2 ml/minute was selected for the study of CNP and PNP. Thus it was observed that the optimized chromatographic condition was common for both CNP and PNP which is the main advantage of the developed method.

System suitability parameters

The system suitability parameters for both the drugs CNP and PNP in the optimized chromatographic condition were calaculated and present in the table-33. All the values were compared with the standard values given by ICH guidelines and found to be compatible. The number of theoretical plates for CNP and PNP was found to be 6497 and 5091 respectively which confirms the good efficiency of the column for the drugs and nature of mobile phase.

Table 33: System suitability parameters for CNP and PNP

S.no	Parameter	Cinitapride	Pantoprazole
1.	Theoretical Plates	6497	5091
2.	Tailing factor	0.93	1.32
3.	Capacity factor	0	0
4.	Temperature of the column	ambient	ambient
5.	Retention time	6.5 minutes	3.5 minutes
6.	Correlation coefficient (r^2)	1	1
7.	Correlation coefficient (r^2)	1	1
8.	Limit of Detection (LOD) (µg/ml)	1.5	1.9
8.	Limit of Quantitation (LOQ) (µg/ml)	5.3	5.8

The optimized chromatogram of CNP and PNP is shown in fig 23 & 24 respectively. The chromatograms shows the retention time (Rt) of CNP to be 6.5 minutes and that of PNP to be 3.5 minutes.

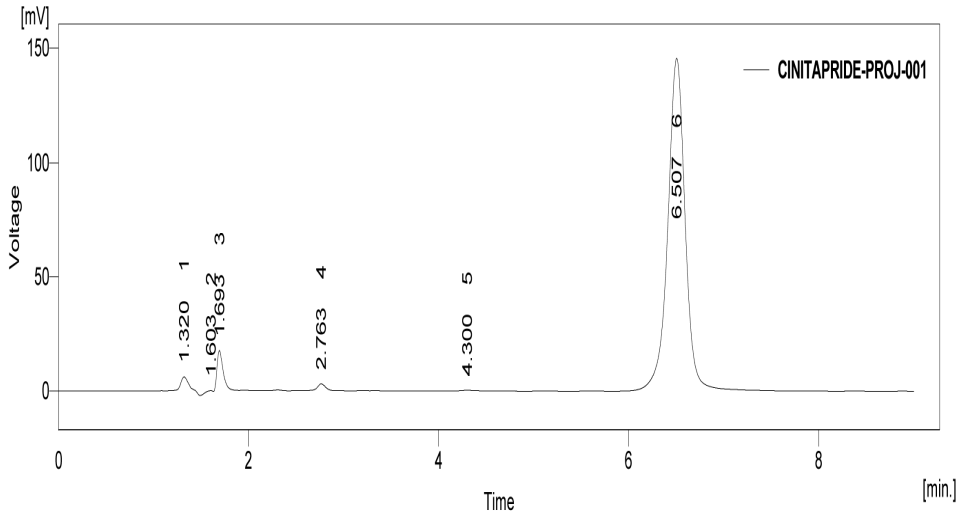


Fig 23: Chromatogram of CNP (Rt - 6.5 minutes)

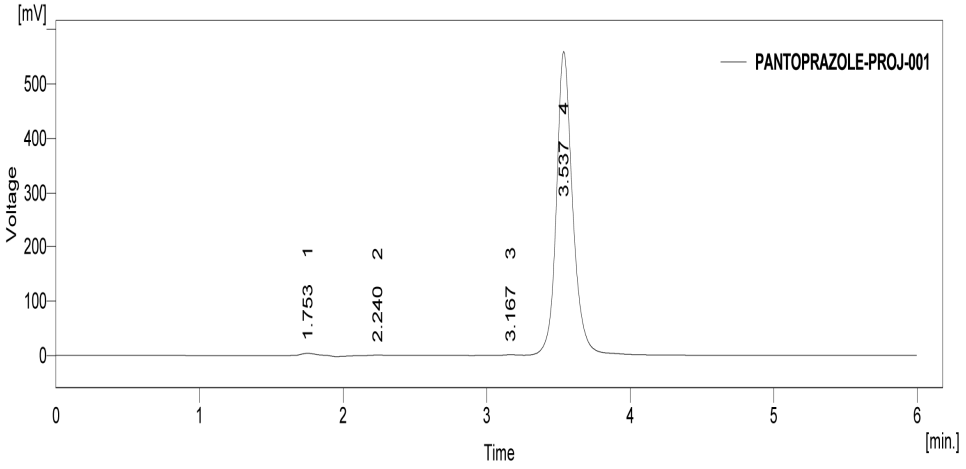


Fig 24: Chromatogram of PNP (Rt- 3.5 minutes)

ESTABLISHMENT OF LINEARITY

Linearity of the drug's concentration with respect to the instrumental response (peak area) was established for both CNP and PNP. The peak areas with respect to concentration are presented in the table-34 and 35. A calibration chart was plotted using concentration along X –axis and peak area along Y-axis. The linearity of response under the optimized chromatographic condition was analysed using regression analysis. CNP obeyed Beer's law at 84-132µg/ml and PNP at 140-220µg/ml. The correlation coefficient was found to be equal to 1 for both CNP and PNP which is shown in the respective linearity chart.(fig-25and 26).

LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION:

The LOD and LOQ were determined from the average of the three determinations of the slope and standard errors of estimate of the respective linearity charts. The LOD was found to be 1.5 µg/ml for CNP and 1.9µg/ml for PNP; LOQ as 5.3 µg/ml and 5.8µg/ml for CNP and PNP respectively.

Table 34: Peak area of CNP

S.No.	Concentration µg/mL	Peak area
1.	84	1670.30
2.	96	1863.70
3.	108	2110.90
4.	120	2325.40
5.	132	2571.90

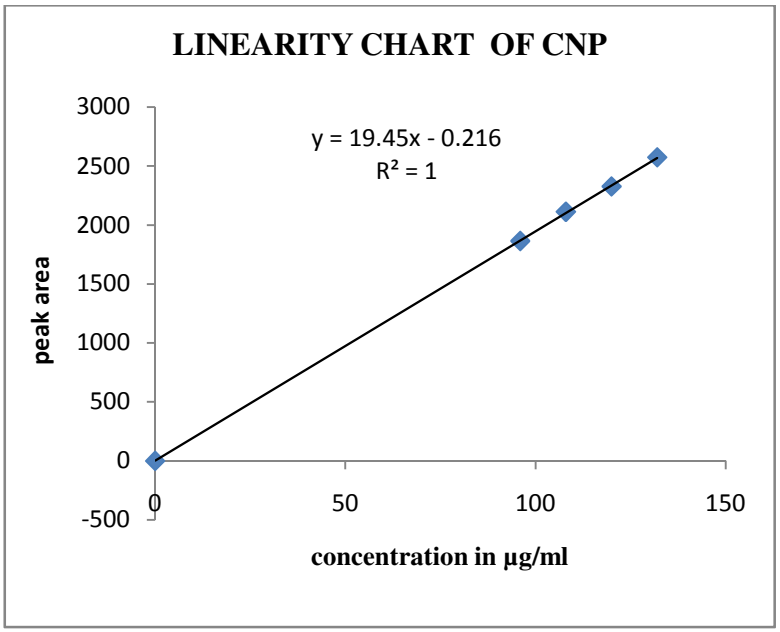


Fig 25 : Calibration chart of CNP - HPLC

Table 35: Peak area of PNP

S.No.	Concentration (µg/mL)	Peak area
1.	140	3964.82
2.	160	4442.76
3.	180	4966.63
4.	200	5542.18
5.	220	6082.93

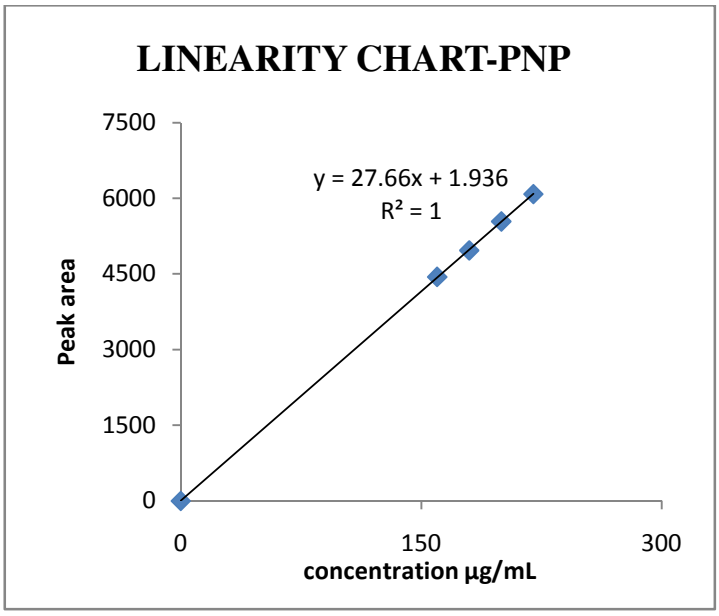


Fig 26 : Calibration chart of CNP- HPLC

ANALYSIS OF FORMULATION

The formulation of CNP and PNP was quantified using the optimized chromatographic condition. As the combined dosage form contains CNP and PNP as individual tablets, they are analysed separately.

The concentrations of the dosage forms in mobile phase was so selected that it lies in the respective linearity range. 20 µl of the samples were injected separately, CNP was detected at 262 nm and PNP at 290 nm; the chromatograms were recorded (figure-27 and 28). The sample was analysed thrice. The percentage purity of the CNP was determined to be 99% to 101% which is represented in table-36. Similarly the percentage purity of the sample PNP was determined to be 1005 to 101% which is represented in table-37

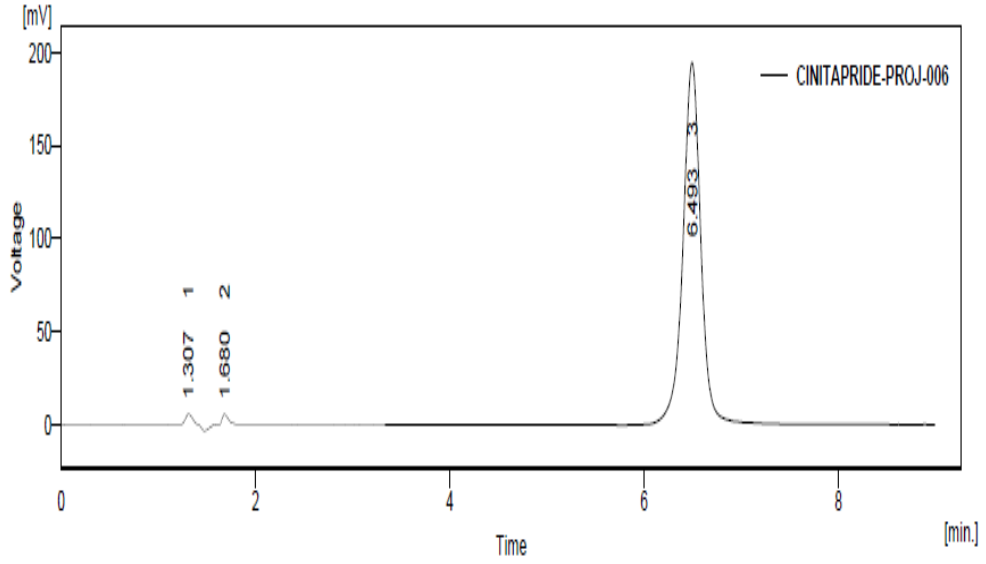


Fig 27: Chromatogram of Sample CNP

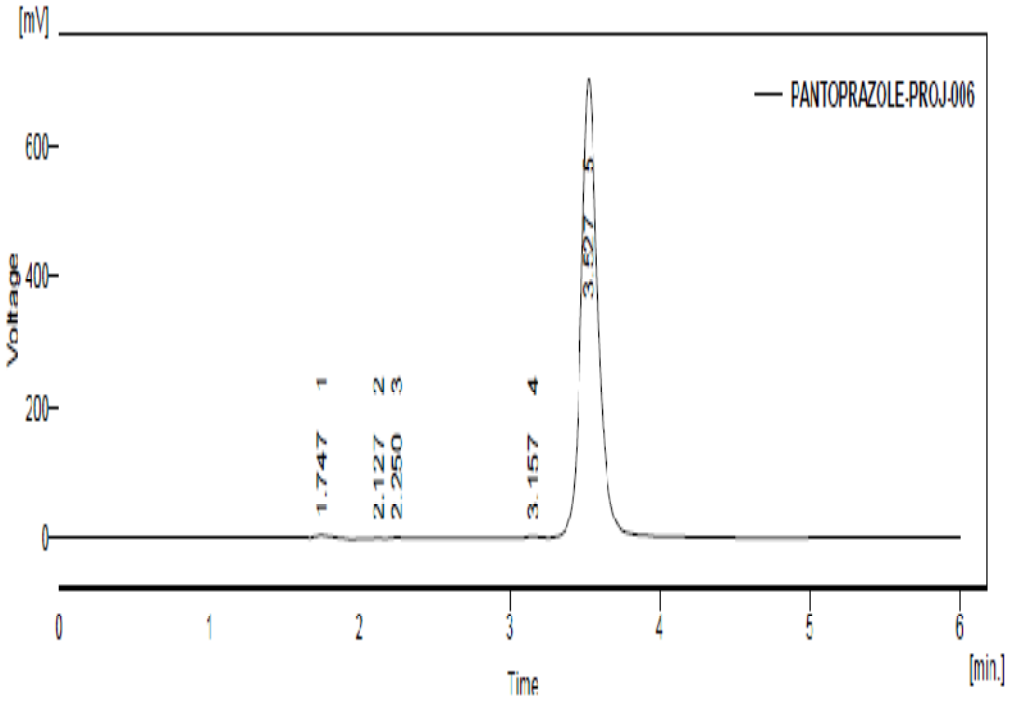


Fig 28: Chromatogram of Sample PNP

Table 36: Results of Assay of CNP

S.No.	Label claim	Amount present (mg)	%purity	SD	RSD
1.	3mg	2.97	99.16	± 0.7856	0.0078
2.		2.97	99.16		
3.		3.02	100.83		

Table 37 : Results of Assay of PNP

S.No.	Label claim	Amount present (mg)	%purity	SD	RSD
1.	40mg	40.0	100.01	± 0.4714	0.0046
2.		40.0	100.00		
3.		40.4	101.00		

VALIDATION OF THE PROPOSED RP –HPLC METHOD

ACCURACY: (Mousumi Kar., et al., 2009)

The accuracy of the developed method was determined by performing recovery studies using the standard addition technique. A known amount of the standard drugs were added to the respective samples and the chromatogram was

recorded for the same. The recovery studies were performed on 50% spiked samples and injected in duplicate. The analysis was performed thrice. The results of the recovery are shown in table-38 and 39. The percentage recovery was found lie between 98.89 and 100.00 % and 99.50 and 101% for PNP. The results of the recovery analysis suggest that the developed method has no interference by the sample matrix.

Table 38: Results of recovery studies of CNP

Expected % Recovery	Amount added (mg)		Total amount assayed in mg	Amount Recovered in mg	Assessed % Recovery	% Recovery	SD	RSD
	Sample	Standard						
50%	3	1.5	4.45	1.41	47.10	98.89	±0.4545	0.0045
			4.50	1.46	48.76	100.00		
			4.47	1.43	47.93	99.44		

Table 39: Results of recovery studies of PNP

Expected % Recovery	Amount added (mg)		Total amount assayed in mg*	Amount Recovered in mg	Assessed % Recovery	% Recovery	SD	RSD
	Sample	Standard						
50 %	40	20	60.40	20.4	51	100.66	0.6937	0.0069
			60.69	20.7	51.73	101.15		
			59.70	19.7	49.24	99.50		

Specificity (Lokesh Singh., et al., (2011)

The specificity test of the proposed method demonstrated that the excipients from tablets do not interfere in the drug peak. Furthermore, well shaped peaks indicate the specificity of the method. Better resolution was found for the drug peak with no interference proved that the method was found to be selective to the drug.

Precision

The precision of the developed method was assessed by performing the analysis thrice which is tabulated in table-35 and 36

The LOD and LOQ for CNP and PNP were predicted based on the parameters of standard error of estimate and slope, calculated from linearity of the

SUMMARY AND CONCLUSION

Simple, Precise, rapid and accurate methods were developed for the estimation of Cinitapride and Pantoprazole in bulk and in dosage form. The methods are

1. UV Spectroscopic method

2. Visible Spectroscopic method

3. RP-HPLC method.

The UV spectrophotometric method makes use the solubility of CNP and PNP in methanol. The λmax of CNP being 262nm and that of PNP is 290nm in methanol. The bulk drug CNP obeyed Beer's law at 4-20µg/mL and PNP at 5-30µg/mL. The correlation coefficient was found to be 1 for both the drugs. The dosage form of the drugs was quantified by the following three methods.

Method A involves standard absorbance method. The absorbance of both standard and sample were used to calculate the amount of the analyte. The recovery studies were performed by the standard addition technique at three spiked levels. The RSD proves the reproducibility of the method and thus the precision of the developed method.

Method B involves determination of AUC for both standard and sample spectra obtained in Method A between two selected wavelength.The correlation coefficient was found to be 0.9990 for both CNP andPNP. The sample concentration and the percentage recovery concentration were arrived by interpolation on calibration chart of AUC vs Concentration for both CNP and PNP. The percentage

purity was determined to be 99 to 101% and RSD <1 which was within the limit. Thus the method is said to be precise. The recovery percentage was found to be 98 to 102% which proves no interference by the sample matrix.

Method C involves the derivatisation of the zero order spectra to second order spectra. The amplitude of the negative maxima at 262nm for CNP and 290nm for PNP were used to determine the amount of analytes present. The recovery spectra were also derivatized and used.

The visible spectrophotometric method involve two methods each for CNP and PNP.Initially for CNP, both the methods involves diazotization of the primary aromatic amino group and coupling of the diazotized CNP with ethyl aceto acetate (EAA) coupling with acetyl acetone (AAC) to form a diazo couple or orange red colored dye. The order of addition of the reagents, strength and volume of the reagents were optimized. Method A follows Beer's law at 10-60µg/mL and method B at 10-50µg/mL.

For PNP both the methods initially involve oxidation to its sulfone analogue by the *insitu* liberation of excess bromine (Potassium bromate-bromide with hydrochloric acid). The excess bromine oxidizes ferrous ammonium sulphate to ferric ammonium sulphate. Method A involves complexation of the excess FAS to ferroin using 1,10 phenanthroline which has a λmax at 510nm. Method B involves complexation of the excess ferrous ammonium sulphate to ferric thiocyanate by ammonium thiocyanate which shows λmax at 479nm. Method A obeys Beer's law at 1.2 to 5.4 µg/mL and method B at 0.4 to 2.4 µg/mL. The reagents and the conditions of the reaction were optimized for both the methods. The respective optimized

procedure was adopted for the assay, precision studies and recovery studies for both CNP and PNP. The precision result showed RSD<1 for all the methods. Thus the method developed are said to be precise. The results of the recovery studies reveal that the developed method showed no interference with sample matrix. Thus the above devised visible methods are accurate.

RP-HPLC method uses mobile phase containing acetonitrile: methanol: phosphate buffer in the ratio of 40:20:40%v/v. The chromatographic conditions were optimized and were found common for both CNP and PNP. CNP showed Rt at 6.5 minutes and PNP at 3.5 minutes. The linearity was assessed by the correlation coefficient equal to 1. The assay, precision and recovery studies were performed. The assay percentage results were subjected to repeatability studies for thrice and found to be precise. The results of the recovery studies showed that the method was devoid of interference by the sample matrix.

All the methods have shown good linearity, precision and accuracy. The low % RSD values in recovery studies for all the above methods indicate that there is no interference due to excipients used in the formulations. **Hence it is concluded that the developed UV – Visible and RP-HPLC methods were found to be simple, precise, accurate and rapid methods for the analysis of Cinitapride and Pantoprazole in its pure form and in its pharmaceutical dosage formulation. Thus, all the above adopted methods can be effectively used for the routine analysis of Cinitapride and Pantoprazole in pharmaceutical dosage form.**

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