DEVELOPMENT OF ANALYTICAL METHODS FOR THE DETERMINATION OF NITAZOXANIDE IN BULK DRUG AND ITS PHARMACEUTICAL FORMULATION

Dissertation submitted in partial fulfillment of the requirement for the award of the degree of

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CERTIFICATE

This is to certify that the dissertation entitled **"DEVELOPMENT OF ANALYTICAL METHODS FOR THE DETERMINATION OF NITAZOXANIDE IN BULK DRUG AND ITS PHARMACEUTICAL FORMULATION"** submitted by **Mrs.Swathy Lakshmi.N**, to The Tamilnadu Dr.M.G.R Medical university, Chennai**,** in partial fulfillment of the requirement for the award of **Master of Pharmacy in Pharmaceutical Chemistry**, at K.M.College of Pharmacy, Uthangudi, Madurai.It is a bonafide work carried out by her under my guidance and supervision during the academic year of 2011 – 2012 This dissertation partially or fully has not been submitted for any other degree or diploma of this universityor other universities.

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I INTRODUCTION[1]

Chemical analysis holds an important position in our modern society. Knowledge of analytical procedures has become essential in practically all arts and science as well as technical separation of industry. The discovery of new medicines, new fabrics, and new alloys would not be possible without the services of analytical chemist. Analytical chemistry is such an important part of our industrial structure and advancing science that without it our present day economy couldn't exist.

In early days of chemistry, analysis was most synonymous with chemistry itself. Robert Boyle (1626-1691) was first introduced the word "analysis" to apply to the identification of individual substance in presence of each other. Berzelius (1779-1848) developed many new experimental methods which include the separation of the metal constituents from a number of complex minerals.

The last 50years have witnessed an expanding interest in the problems and methods of chemical analysis. There has been a vast increase in the number of analysis that is needed in daily problems of engineering metallurgy, manufacturing and many other fields. Agriculture has been aided with increased yields and enhanced soil fertility through accurate chemical analysis. Advances in medicine and public health would not be possible without chemical analysis.Eg-analysis of blood, urine.

SCOPE OF QUANTITATIVE ANALYSIS

Quantitative analysis is concerned with the identification and separation of chemical substances and chemical principles on which such procedures are based on. Quantitative analysis deals with the determination of the amount of a chemical substance represent either alone or in a simple or complex mixture of other substances.

There are two types of quantitative analysis.

Group 1:-Methods in which the final measurement of the substance sought is made by direct or indirect measurement of volume and weight after proper treatment of a measured portion of the material to be analysed.They are of 2 types.

a) Gravimetric
b) Titrimetric.

a) Gravimetric:- Analysis is carried out by a series of weighing operation . The most usual method is the isolation of a substance sought by direct precipitation of substance, or a suitable compound of it with subsequent purification. E.g. Detection of chlorides, sulphates in water soluble samples in the absence of interfering substances.

b) Titrimetric:-In this the substances sought is determined by a careful measurement of volume of a solution of known concentration required to react with the solution to be analysed. They are of 2 types.

1. Neutralization reaction. E.g.:-Determination of acids and bases
2. Oxidation-reduction reaction.

Group 2:-This involves methods in which the final measurement is made upon the system as a whole. Eg:-Measurement of specific gravity.

- Thermal measurement-Melting point, Boiling point
- Electrical measurement-Dielectric constant
- Optical measurement

ANALYTICAL CHEMISTRY[2]

Analytical chemistry deals with the solving of quantitative problems. In quantitative analysis the goal is to determine what are the constituents present in the sample while in qualitative analysis the goal is to determine how much of each constituent in sample. An important part of analytical chemist's task is choosing the optimum pathway, a choice which is simplified only through the assimilation of knowledge and experience. Thus in solving analytical problems the analytical chemist is often required to design or repair electronic systems, design instruments, interpret spectra and instrumental data, perform chemical analysis with simple chemicals and solutions, develop and evaluate new procedures or modify old ones, separation of simple, complex mixtures, purify samples and write computer programmes.

Historically chemistry could be easily divided into 5 areas.1.Analytical, 2.Biochemical, 3.Inorganic, 4.Organic, 5.Physical. The importance of analytical chemistry in related scientific areas can be illustrated by considering its impact on clinical analysis, in pharmaceutical research, QC and in environmental analysis.

The measurement of properties such as absorption or emission of energy, optical rotation, refractive index, equilibrium constant is more complex. The reliability, utility, accuracy, interpretation and specificity of these measurements are the responsibilities of an analytical chemist. Another important responsibility of an analyst is to make the measure which is usually based on a tried and tested procedure in a routine manner.

Analytical chemistry deals with the methods for determining the composition of samples or matter. A qualitative method yields information about the identity of atomic or molecular species or the functional groups in the sample. A quantitative method, in contrast, provides numerical information as to the relative amount of one or more of these components.

TYPES OF ANALYSIS[3]

1. Proximate analysis:-The amount of each element in a sample is determined with no concern as to the actual compounds present.
2. Partial analysis:-This deals with the determination of selected constituents in the sample.
3. Trace constituent analysis:-The determination of specified components present in very minute quantity.
4. Complete analysis:-In this the proportion of each component of sample is determined.

On the basis of sample size, analyses are classified into three. They are:-

a. Macro methods;-Determination of quantities of 0.1gm or more.
b. Semi micro methods:-Deals with quantities ranging from 0.01gm -0.1gm.
c. Micro methods:-Quantities not exceeding 0.001
   [Major constituents-One present in excess of 1%, Minor constituents-Constituents from 0.01-1% of sample, Trace constituents-One present to an extent of less than 0.01% of sample]

ANALYTICAL INSTRUMENTATION[4]

Analytical instrumentation plays an important role in the production and valuation of new products and the protection of consumers and the environment. Most analytical techniques fit into one of the three principal areas: spectroscopy, electrochemistry and chromatography. The first task is to define an analytical problem. The analyst should determine the nature of sample, the end use of the analytical results, the species to be analyzed and the information required. Once the problem has been defined the next task is to select the appropriate method.

Classification of analytical methods:-[5]

Various new analytical techniques which are being used can be categorized as follows:-

- GENERAL CLASSIFICATION
- SCIENTIFIC CLASSIFICATION
- TRADITIONAL CLASSIFICATION
- MODERN CLASSIFICATION

GENERAL CLASSIFICATION:-

This classification includes:-

a) Spectral methods: These are based on light absorption or emission characteristics of drugs.
   E.g.:-UV, Visible, NMR Spectroscopy, Fluorimetry, Colorimetric etc.
b) Chromatographic methods: These are based on the affinity or partition coefficient differences between drugs.
   E.g.:-Thin layer chromatography (TLC), High Performance Liquid Chromatography (HPLC), Paper chromatography etc.
c) Electro analytical method: These are based on the electrochemical property of the drug.
   E.g.:-Potentiometry, Conductometry, Polarography, Amperometry.
d) Biological &microbiological methods: In these methods animals (or) microorganisms are used and the activity is determined.

E.g.:-Biological assay of some vitamins, microbiological assay of antibiotics &vitamins.

e) Radioactive methods: In these methods the radioactivity is measured for analyzing the drugs.

E.g.:-Radio Immune Assay (RIA)

f) Physical methods: In these methods some physical characteristics of drugs are measured.

E.g.:-Differential Thermal Analysis (DTA), Differential Scanning Calorimetry (DSC).

g) Miscellaneous technique methods: Conventional titrimetric methods,polarimetric methods etc.

SCIENTIFIC CLASSIFICATION:-

This classification includes:-

a) Instrumental methods: A physical property of a substance is measured to determine its chemical composition.

E.g.:-Electro-chemical method- change in the electrical properties of the system.

Electrogravimetry, Potentiometry, Conductometry etc.

b) Chemical methods: In these methods chemical reaction or chemical extraction is involved. These are volumetric and gravimetric method.

TRADITIONAL CLASSIFICATION:-

This classification includes:-

a) Gravimetric method: This method involves measurement of weight. The substance to be determined is converted into an insoluble precipitate which is collected and weighed or in electro gravimetry, electrolysis is carried out and the material deposited on one of the electrodes is weighed. It may be carried out by:-

- Precipitation
- Electrode position
- Volatilization

b) Volumetric method: These are the methods in which measurements are made in volume. In this technique volume of titrant is measured and these are of four types depending on the type of reaction involved in it.
   - Acid-base titration
   - Oxidation-reduction titration
   - Precipitation titration
   - Complexometric titration

c) Optical method: These methods are based on how the sample acts towards electromagnetic radiation.
   - Absorption or emission of radiant energy
   - Bending of radiant energy
   - Scattering of radiant energy

d) Electrical method: It involves the detection of amount of concentration of a component in terms of
   - Component upon an electrode
   - Upon an impressed voltage
   - Sample in changing the chemical state of component

e) Separation method: Based on the principle of affinity of the component to be separated (or) analyzed.

MODEREN CLASSIFICATION:-

This classification includes the modern & latest methods of analysis.

a) Chemical methods:

i. Acidimetric and alkalimetric methods
ii. Non-aqueous titration methods
iii. Oxidation-reduction methods
iv. Argentrimetric methods
v. Complexometric methods
vi. Gravimetric methods
vii. Miscellaneous types of methods

b) Physical methods:

i. Refractometry
ii. Polarimetry
iii. Optical rotatory dispersion

c) Electrochemical methods:

i. Electro analytical methods
ii. Polarography

d) Spectroscopic methods:

i. Visible spectrophotometry
ii. Ultraviolet spectrophotometry
iii. Flame and atomic absorption spectrophotometry
iv. Fluorimetry
v. Infrared spectrophotometry
vi. Nuclear magnetic resonance spectrophotometry
vii. Mass spectrophotometry

e) Separation methods:

i. Chromatographic methods
ii. Electrophoresis

f) Radiochemical methods

g) Miscellaneous types:

i. Particle size analysis
ii. Differential Scanning Calorimetry (DSC)
iii Differential Thermal Analysis (DTA)

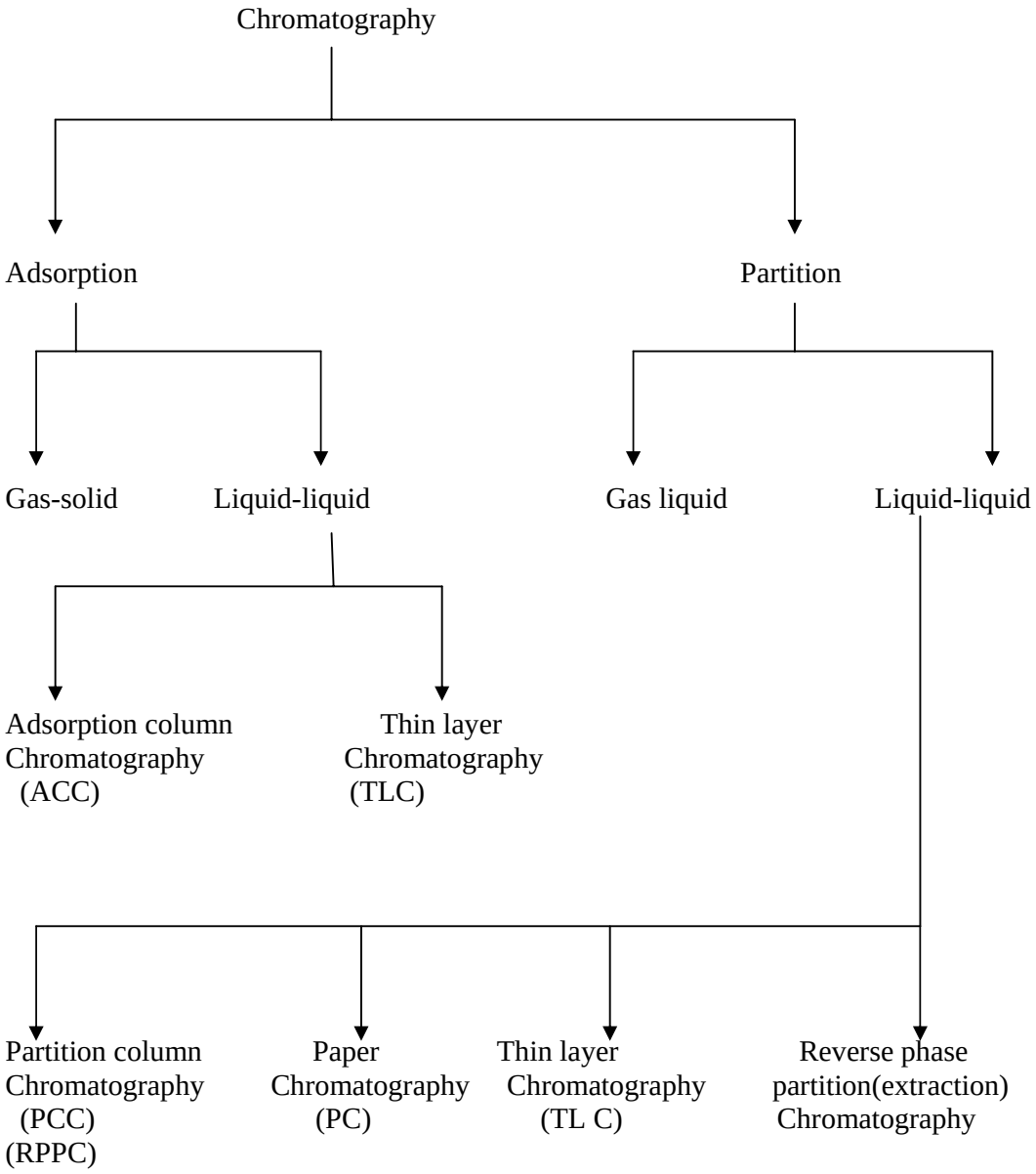
CHROMATOGRAPHY

According to USP[51] chromatography can be defined as a procedure by which solutes are separated by a differential migration process in a system consisting of two or more mobile phases, one of which the individual substance exhibit different mobilities by reason of difference in adsorption, partition, solubility, vapour phase, molecular size or ionic charge density.

Chromatography[52] is a new separation technique, discovered by Dr.Michael Tswett (1906) for the separation of complex mixtures by the process of adsorption. According to him chromatography is defined as a process used in separating substance by filtering the solution through a column of finely powdered adsorbent and then developing the column with a solvent.

Classification of chromatographic methods

- In all chromatographic techniques[53], difference in affinity involves the process of either adsorption or partition. In adsorption, the binding of a compound, to the surface of solid phase takes place where as in partition the relative solubility of a compound in two phases, results in partition of compound between two phases.
- Components of a mixture are carried through the stationary phase by the flow of a mobile phase and the separation is based on difference in migration rates among the mobile phase components.
- Elution: - It is a process in which solutes are washed through a stationary phase by the movement of a mobile phase. The mobile phase that exits the column is termed as eluate.
- Chromatogram: - If a detector that responds to solute concentration is placed at the end of the column during elution and its signal is plotted as a function of time, a series of peaks are obtained called chromatogram.


Chromatography
Adsorption
Partition
Gas-solid
Liquid-liquid
Gas liquid
Liquid-liquid
Adsorption column Chromatography (ACC)
Thin layer Chromatography (TLC)
Partition column Chromatography (PCC) (RPPC)
Paper Chromatography (PC)
Thin layer Chromatography (TL C)
Reverse phase partition(extraction) Chromatography

High Performance Liquid Chromatography (HPLC) [54]

The technique of HPLC was discovered by Csaba Horvath (1964), Kirkland & Huber (1969). The first mixture to be separated was nucleic acid components associated with thyroid function.

All forms of LC are differential migration processes where sample components are selectively retained by a stationary phase. The different techniques are LSC (adsorption chromatography), LL (Partition chromatography). LLC is divided, based on relative polarities of stationary phase and mobile phase i.e. Reverse Phase chromatography and Ion Pair chromatography. In addition to LSC and LLC. LC comprises bonded phase chromatography, gel permeation chromatography, ion exchange & affinity chromatography.

In HPLC, eluents from the solvent reservoir is filtered, pressurized and pumped through the column. A mixture of solutes injected at the top of the column is separated into components. Individual solutes are monitored by the detector and recorded automatically.

The principal advantages of HPLC compared to classical column chromatography are:-

- Improved resolution of separated substances.
- Faster separation time.
- Increased accuracy, precision, sensitivity.

Types of chromatographic techniques[55]

I. Based on modes of chromatography

- Normal phase- Stationary phase is polar
  Mobile phase is non-polar
- Reverse phase- Stationary phase is non polar
  Mobile phase is polar

II. Based on principle of separation

- Adsorption (Liquid chromatography) - Analyte species are adsorbed into the surface of a polar packing.

- ❖ Partition (Liquid-liquid chromatography) –Based upon the difference in partition coefficient of the individual components of a mixture between a liquid stationary phase and a gaseous or liquid mobile phase.
- ❖ Ion-exchange (Ion pair chromatography)
- ❖ Size exclusion (Gel permeation/gel filtration) – In this the fractionation is based on molecular weight.
- ❖ Affinity – In this a covalent bonding between the reagents called "affinity ligand" and solid supports takes place.
- ❖ Chiral phase- In this a "Chiral resolving agent or Chiral stationary phase" preferentially complexes one of the enantiomers.

III. Based on elution technique

- ❖ Isocratic- In this the composition of solvent remains constant.
- ❖ Gradient -In this the composition is changed continuously or in a series of steps.

IV. Based on scale of operation

- ❖ Analytical HPLC
- ❖ Preparative HPLC

II DRUG PROFILE[6]

INTRODUCTION

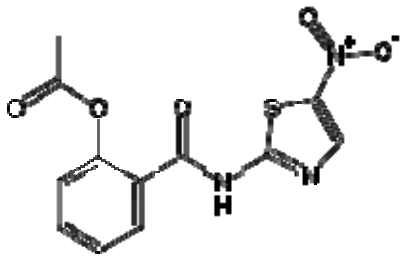
Nitazoxanide[49] was originally discovered in the 1980s by Jean- François Rossignol at the Pasteur Institute. Initial studies demonstrated activity versus tapeworms. In vitro studies demonstrated much broader activity. Dr. Rossignol co-founded Romark Laboratories, with the goal of bringing nitazoxanide to market as an anti-parasitic drug. Initial studies in the USA were conducted in collaboration with Unimed Pharmaceuticals, and focused on development of the drug for treatment of cryptosporidiosisin AIDS. Controlled trials began shortly after the advent of effective anti-retroviral therapies.

Romark launched an impressive series of controlled trials. No other agent has proven efficacy in the treatment of cryptosporidiosis. However, a placebo-controlled study of nitazoxanide in cryptosporidiosis demonstrated significant clinical improvement in adults and children with mild illness. Among malnourished children in Zambia with chronic cryptosporidiosis, a three-day course of therapy not only led to clinical and parasitological improvement, but also improved survival. In treatment of giardiasis, nitazoxanide was superior to placebo and comparable to metronidazole. Nitazoxanide was successful in the treatment of metronidazole-resistant giardiasis. Studies have suggested efficacy in the treatment of cyclosporiasis, isosporiasisand ameobiasis.

DESCRIPTION[7]

Nitazoxanide is a synthetic nitro thiazolyl-salicylamide derivative approved for the treatment of infectious diarrhoea caused by Cryptosporidium parvum and Giardia lamblia. Following oral administration it is rapidly hydrolyzed to its active metabolite, tizoxanide, which is observed 1-4 hours after administration. It is excreted in the urine, bile and faeces.

CHEMICAL STRUCTURE[8]



IUPAC NAME[9]

2-[(5-nitro-1, 3-thiazol-2-yl) carbamoyl] phenyl acetate

MOLECULAR FORMULA[10]

$C_{12}H_9N_3O_5S$

MOLECULAR WEIGHT[11]

307.283g/mol

SYNONYMS[12]

- 2-(Acetolyloxy)-N-(5-nitro-2-thiazolyl)benzamide
- 2-Acetyloxy-N-[(5-nitro-2-thiazolyl)]benzamide
- Nitazoxanid
- Nitazoxanida[INN-Spanish]
- Nitazoxanide
- Nitazoxanidum[INN-Latin]
- NTZ

BRAND NAMES

- Alinia
- Fental
- Phavic-1

CATEGORY

Anti parasitic agents

PROPERTIES

1	Physical state	Solid, white or yellowish, crystalline powder	
2	Melting point	202 ^{0}C	
3	Experimental properties	**Property**	**Value**
		log P	1.2
4	Predicted properties	Water solubility	7.55e-03g/l
		log P	2.14
		Log S	-4.61
		Hydrogen acceptor count	5
		Hydrogen donor count	1
		Polar surface area	114.11
		Rotatable bond count	5
		Refractivity	73.89
		Polarizability	27.54
5	Solubility	Soluble in *N, N*-dimethyl acetamide; very slightly soluble in alcohol; practically insoluble in water.	

PHARMACOLOGY[13]

Mechanism of action: The antiprotozoal activity of nitazoxanide is believed to be due to interference with the pyrvate: ferredoxinoxido reductase (PFOR) enzyme-dependent electron transfer reaction which is essential for anaerobic energy metabolism. Studies have shown that PFOR enzyme from Giardia lamblia directly reduces nitazoxanide by transfer of electrons in the absence of ferredoxin. The DNA –derived PFOR protein sequence of Cryptosporidium parvum appears to be similar to that of the Giardia lamblia. Interference with the PFOR enzyme-dependent electron

transfer reaction may not be the only pathway by which nitazoxanide exhibits antiprotozoal activity.

Pharmacokinetics[14]:

1. Absorption: The relative bioavailability of the suspension compared to the tablet was 70%.
2. Volume of distribution: Not available.
3. Metabolism: Rapidly hydrolyzed to an active metabolite, tizoxanide (desacetyl-nitazoxanide)
4. Excretion: Tizoxanide is excreted in urine

Protein binding: Very high (greater than 99%), bound to proteins. Binding is not affected by degree of renal impairment.

Clearance: Not available.

Half life: 3.5 hours in patients with normal renal function.

Toxicity: In acute studies in rodents and dogs, the oral LD_{50} was higher than 10,000mg/kg. Single oral dose administered to healthy adult volunteers without significant adverse effects.

Storage and stability: [15]Store the tablets, unsuspended powder, and the reconstituted oral suspension at 25^0C ($77\ ^0$F)

Precautions:

i. General: Nitazoxanide must be administered with caution to patients with hepatic and biliary disease, to patients with renal disease and to patients with combined renal and hepatic disease.
ii. Information for patients: Alinia tablets and Alinia suspension should be taken with food. Diabetic patients and caregivers should be aware that the oral suspension contains 1.48gm of sucrose per ml.
iii. Carcinogenicity, mutagenicity, impairment of fertility, no direct effect reported.

iv. Pregnancy: Teratogenic effect.

v. Nursing mothers: Caution must be exercised during administration.

ADVERSE DRUG REACTIONS:-

Alinia tablets:-:

- ✓ Body as a whole-asthenia, fever, pain, allergic reaction, pelvic pain, back pain, chills.
- ✓ Nervous system- dizziness, somnolence, insomnia, tremor, hypesthesia.
- ✓ Digestive system-vomiting, dyspepsia, anorexia, flatulence, constipation, dry mouth, thirsts.
- ✓ Urogenital system-discoloured urine, dysuria, kidney pain
- ✓ Metabolic and nutrition-increased SGPT
- ✓ Hemic and Lymphatic system- Anemia, leukocytosis.
- ✓ Skin- rash, pruritis.
- ✓ Respiratory system- epitaxis, pharyngitis.
- ✓ Cardiovascular system- tachycardia, hypertension.
- ✓ Muscular system-myalgia, leg cramps.

Alinia oral suspension:-

- ✓ Digestive system-nausea, anorexia, flatulence
- ✓ Body as whole- fever, infection, malaise.
- ✓ Metabolic and nutrition-increased creatinine, increased SGPT
- ✓ Skin-pruritis, sweat
- ✓ Respiratory system-rhinitis
- ✓ Nervous system-dizziness
- ✓ Urogenital system-discolored urine

DRUG INTERACTION:-

Since it is highly bound to plasma proteins, caution should be taken while administering nitazoxanide with other highly plasma-bound drugs with narrow therapeutic indices. It is expected that no interaction would occur when nitazoxanide is co-administered with drugs that either metabolized or inhibited by cytochrome P450enzyme.

INDICATIONS: - [16]

1. Antiprotozoal agent

(1) The first-line choice for the treatment of illness caused by Cryptosporidium parvum or Giardia lamblia infection in immunocompetent adults and children, and is an option to be considered in the treatment of illness caused by other protozoa and/or helminthes.

(2) Treatment of infectious diarrhea caused by Cryptosporidium parvum and Giardia lamblia in pediatric patients 1 year through 11 years of age, which has been approved by FDA, USA

(3) Metronidazole-resistant giardiasis.

2. Anti-virus

(1) Phase II clinical trials for the treatment of hepatitis C, in combination with pig interferon α - 2α and ribavirin.

(2) Chronic hepatitis B

(3) Reduced the duration of rotavirus disease in hospitalized pediatric patients.

(4) Activity against influenza A virus

1. Treatment of equine protozoal myeloencephalitis (EPM) caused by sarcocystisneurona in horse
2. Grass and flowers in western countries.

CONTRAINDICATIONS: - [17]

Alinia tablets and Alinia suspensions are contraindicated in patients with a prior hypersensitivity to nitazoxanide or any other ingredients in the formulations.

DOSAGE&ADMINISTRATION:-

Indication	Age	Dosage	Duration
Treatment of diarrhea caused by Giardia lamblia or Cryptosporidium parvum	1-3 years	5ml of alinia for oral suspension (100mg NTZ) every 12hrs with food.	3days
	4-11years	10ml of alinia for oralsuspension (200mg NTZ) every 12hrs with food.	
	≥12years	1Alinia tablet (500mg NTZ) every 123hrs with food or 25ml of Aliniaoral suspension (500mg NTZ) every 12hrs with food.	

OVERDOSAGE:-

In the event of over dosage, gastric lavage may be given soon after oral administration. Patients should be carefully observed and given symptomatic and supportive treatment.

DOSAGE FORMS:-

- Oral suspension
- Tablet

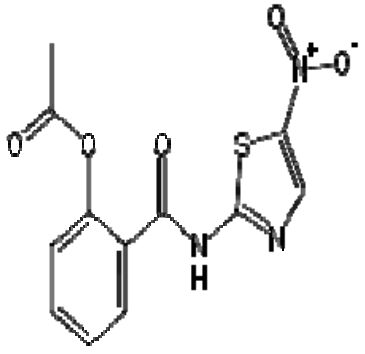
III LITERATURE REVIEW

The following methods of analysis have been reported for the determination of nitazoxanide and its formulations.

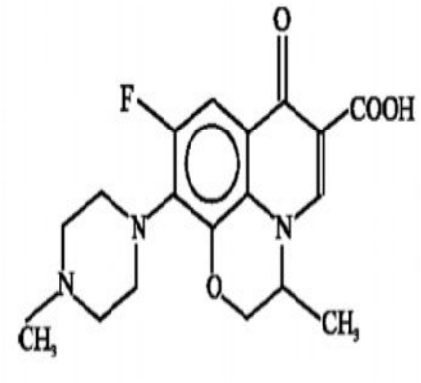
1. ***Spectrophotometric estimation of nitazoxanide in pharmaceutical dosage form.***[18]

Prabhakar. G, et al; developed three simple and sensitive spectrophotometric methods(1, 2, 3). These methods are based on the reaction of reduced nitazoxanide with p-dimethyl amino benzaldehyde, p-dimethyl amino cinnamaldehyde and vanillin in acidic conditions to form pink, orange red and orange yellow colored chromogens with absorption maxima at 559nm,534.5nm and 475nm respectively. The reduction of nitazoxanide was carried out with Zn granules and 5N HCl at room temperature in methanol. Beer's law is obeyed in the concentration range of 5-25mg/ml, 5-25mg/ml, and 10-50mg/ml respectively. The results of analysis have been validated precise, rapid and economic. The results were compared with those obtained with visible spectrophotometric methodsin methanol at 402nm.

2. ***Spectrophotometric methods for simultaneous determination of nitazoxanide and ofloxacin in combined bulk and pharmaceutical formulations***[19]**.**



NITAZOXANIDE



OFLOXACIN

Singh H.P, et al; developed the simultaneous determination of nitazoxanide & ofloxacin using three simple, rapid, economical, accurate & reproducible spectrophotometric methods, namely vierodt's method, Q-analysis, dual wavelength method. 1st method, is based on formation and solving of simultaneous equation at 346.361nm (nitazoxanide) and 296.496nm (ofloxacin). Second method is based on absorbance ratio at two selected wavelength 307.520nm & 346.361nm. In third method two wavelengths were selected for each drug in a way so that the difference in absorbance were measured for the determination of ofloxacin, similarly difference in absorbance at 302.4nm and 289.2nm were measured for determination of nitazoxanide. The results of analysis were validated statistically. Recovery studies gave satisfactory results indicating that none of common additives & recipients interfere the assay method.

3. ***Colorimetric estimation of Nitazoxanide and Ofloxacin in Pharmaceutical dosage form.***[20]

Vidya Sagar, et al; developed the simultaneous estimation of nitazoxanide & ofloxacin in tablet dosage form i.e. they launched a combined therapy of both drugs. The colorimetric estimation where the wavelengths selectedfor quantitation was486nm (ofloxacin) and 747nm (nitazoxanide). The method obeys Beer's law in concentration range from 5-50mcg/ml of OFL and 10-100mcg/ml for NTZ. The percentage label claim and percentage label claim and percentage recoveries estimated were close to 99.55% and 98.75% with low value of standard deviation.

4. ***Development of a simple, rapid and validated spectrophotometric method for nitazoxanide in pharmaceutical formulations and compressed with HPLC***[21].

Marcelo Donald Malesuik, et al; developed a rapid, economical, reproducible and a simple direct spectrophotometric method was developed for the assay of nitazoxanide in pharmaceutical formulations. Nitazoxanide concentration was estimated in water at 345 nm and pH 4.5. The method was suitable and validated for specificity, linearity, precision, and accuracy. There was no interference of the excipients in the determination of the active pharmaceutical ingredient. The proposed method was successfully applied in the determination of nitazoxanide

in coated tablets and in powders for oral suspension. This method was compared to a previously developed and validated method for liquid chromatography to the same drug. There was no significant difference between these methods for nitazoxanide quantitation.

5. ***Estimation of Nitazoxanide by U.V Spectrophotometric method***[22].

   Vanitha J.A, et al; developed an accurate, precise, specific, linear, rapid and cost effective spectroscopic methods in UV region for the determination of nitazoxanide in bulk and tablet dosage forms. Nitazoxanide is a new anti-protozoal drug, which showed maximum absorbance at 346nm with apparent molar absorptivity of 23783.92 Lmol-1cm-1. Beers law obeyed in the concentration range of 2-14μg/ml. According to ICH guidelines, results of the analysis were validated statistically and by recovery studies.

6. ***Simultaneous UV Spectrophotometric Method for the Estimation of Nitazoxanide and Ofloxacin in Combined Dosage Form***[23].

   Senthilraja M, developed a simple, sensitive, rapid, accurate and precise simultaneous UV-spectrophotometric method for the estimation of nitazoxanide and ofloxacin dosage form. Ratio of absorbance at two selected wavelengths, one of which an isoabsorbance point and being the λ max of the one of the two components. Ofloxacin has absorbance maxima at 300nm and nitazoxanide has absorbance maxima at 344nm in Ethanol. The isoabsorbance point of ofloxacin and nitazoxanide was found to be 347nm. Linearity was obtained in the concentration range of 2-25μg/ml for each nitazoxanide and ofloxacin. Results of analysis have been validated statically and by recovery studies.

7. ***Simultaneous determination of nitazoxanide and ofloxacin in tablet by ultraviolet spectrophotometry (dual wavelength method)*** [24].

   Shailendra Bindaiya, et al; devoloped a simple, accurate, and precise dual wavelength spectrophotometric method for the simultaneous determination of nitazoxanide and ofloxacin in combined pharmaceutical dosage forms. The principle for dual wavelength method is “the absorbance difference between two points on the mixture spectra is directly proportional to the concentration of the component of interest”. The wavelengths selected for determination of

nitazoxanide were 271.5nm & 359.5nm, whereas, the wavelengths selected for determination of ofloxacin were 300.5nm and 365.5nm. A mixture of dichloromethane and n-Hexane (60:40) was taken as a solvent. Regression analysis of beers plots showed good correlation in the concentration range of 5-25µg/ml for nitazoxanide and 2-10 µg/ml for ofloxacin. Accuracy of method was found between 98.5-101.5%. The precision (intra-day, inter-day and analyst to analyst) of method was found within limits (%CV<2). The proposed method was successfully applied to determination of these drugs in laboratory-prepared mixtures and commercial tablets.

8. ***Development and Validation of Spectrophotometric and Colorimetric Method for the determination of Nitazoxanide in its Bulk and Pharmaceutical Dosage Form(Tablets)***[25]

   Lopamudra Adhikari, et al; developed two simple and sensitive spectroscopic methods in UV region and visible region for the estimation of Nitazoxanide in its pharmaceutical dosage forms. Method A is based on Nitazoxanide showing its absorption maxima at 238.3nm in acetonitrile and water (9:1). The method B is based on the reaction of Nitazoxanide with 1ml, 1% ferric chloride and 2ml, 0.1%MBTH to produce a greenish color, characteristic light absorption at 732nm. These method obey Beer- Lambart's law at a concentration range of 5-40mcg/ml and 50-250mcg/ml respectively. The % recoveries were found out to be 98.75 – 100.45. The results obtained with the proposed methods are in good agreement with the labeled amounts when tablet dosage forms were analyzed.

9. ***Simultaneous High-Performance Liquid Chromatographic Determination of Nitazoxanide and Ofloxacin in Tablet Formulation***

   Vipul P. Ranea, et al; developed a simple, rapid, and precise method for the quantitative simultaneous determination of nitazoxanide and ofloxacin in new tablet formulation. Chromatographic separation of the two drugs were achieved on an Ymc pack-AM C18, 25-cm analytical column using mobile phase consisting of 10 mmol L-1 dipotassium hydrogen phosphate: acetonitrile (65:35, v/v) finally the pH of the mobile phase was adjusted to 7.0 using o- phosphoric acid. The instrumental settings are flow rate of 1 mL min-1, column temperature at 30^0C, and detector wavelength of 254 nm. The internal standard method was

used for the quantification. Caffeine was used as an internal standard. The method validated for linearity, accuracy, precision, limit of detection, limit of quantification and robustness. The calibration curve shows excellent linearity over the concentration ranges of ofloxacin and nitazoxanide in the range of 20-200µg mL-1 and 8-80 µg mL-1, respectively. The separation was completed less than 6 minutes. The proposed method can be used for the quality control of formulation products.

10. ***Simultaneous RP-HPLC Estimation of Nitazoxanide and Ofloxacin in Tablet DosageForms***[27].

Siva Kumar R, et al; developed a Reverse Phase HPLC method for the determination of nitazoxanide and ofloxacin in bulk and tablet formulations. The determination was carried out by using Phenomenex C18 column with 0.24% sodium lauryl sulphate: acetonitrile: acetic acid(pH-4.0) (58:40:02) as the mobile phase. The flow rate was 1.5 ml/ min. and the eluents were monitored at 295 nm. The Retention time of nitazoxanide and ofloxacin were 2.2 and 5.4 respectively. Linearity for the nitazoxanide and ofloxacin were found in the range of 400-600 µg/ml and 160 - 240 µg/ml respectively. The method was reproducible, with good resolution between nitazoxanide and ofloxacin and can be use for routine analysis.

11. ***A Validated RP-HPLC Method for Simultaneous Estimation of Nitazoxanide and Ofloxacin in Pharmaceutical Formulation***[28].

Premanand D.C, et al; devoloped a validated reverse phase high performance liquid chromatography method for the simultaneous determination of Nitazoxanide and Ofloxacin in combined dosage form. Chromatography was carried out on a Cosmosil 5 C18 (4.6 mm × 250 mm, 5 µm) using Acetonitrile: 0.005 M triethylamine Buffer in the ratio of 55:45 (v/v) as the mobile phase at a flow rate of 1.0 mL/min and eluents were monitored at 240 nm. The calibration curves were linear over the range of 160-240 µg/mL for Nitazoxanide and 400-600 µg/mL for Ofloxacin. The average retention time of Dexrabeprazole and Domperidone were found to be 6.051 min and2.106 min respectively. The results of the analysis have been validated statistically and by recovery studies.

12. ***RP-HPLC method for simultaneous estimation of nitazoxanide and ofloxacin in tablets.***[29]

Sharma S, et al; developed a reverse phase high performance liquid chromatography method and for the simultaneous estimation of nitazoxanide and ofloxacin in tablet formulation. The separation and quantification was achieved by HiqSil C18 V Size 4.6mm 250mm column in isocratic mode, with mobile phase consisting of acetonitrile-methanol 0.4mm citric acid, (60:30:10,v/v/v). Citric acid is used to mobilize nitazoxanide and ofloxacin in mobile phase. The mobile phase was pumped at a rate of 0.6ml/mt and the detection was carried out at 304nm. The retention time of nitazoxanideand ofloxacin was found to be 3.122 and 5.902min, respectively. The method was validated for linearity,accuracy and precision. Linearity for ofloxacin and nitazoxanide were in range 2-36µg/ml and 5-90µg/ml, respectively. The developed method was found to be accurate, precise and selective for simultaneous estimation of ofloxacin and nitazoxanide in tablets.

13. ***Rapid determination of nitazoxanide in tablets using reversed-phase ultra-performance liquid chromatography (UPLC) and high-performance liquid chromatography***[30]**.**

Sakamoto T, et al; developed a simple and rapid determination method for nitazoxanide (NTZ), using reverse-phase HPLC and Ultra Performance Liquid Chromatography (UPLC). Only six minutes gradient condition for NTZ analysis using UPLC was achieved. The mobile phase consisted of a mixture of phosphate buffer (pH 6.0) and acetonitrile. The repeatability (relative standard deviation (RSD), n = 6) and the correlation coefficient from linearity (the range from 80% to 120% of amount) were 0.25% and 0.9963 for UPLC and 0.15% and 0.9988 for HPLC, respectively. The quantitative values of NTZ in tablets were 103.2% for HPLC and 98.7% for UPLC. The RSDs of quantitative values of sample solution were calculated to be 4.06% to 4.64% for HPLC and 0.15% to 0.36% for UPLC.

14. ***Simultaneous RPHPLC determination of nitazoxanide and ofloxacin in combined tablet dosage form. (Short Communication)(reverse phase high performance liquid chromatography)(Clinical report)*** [31].

Kalta R, et al; devoloped a simple, precise, accurate, rapid and reproducible reverse phase high performance liquid chromatographic procedure for simultaneous determination of nitazoxanide and ofloxacin in tablet dosage form at a single wavelength. The mobile phase used was a combination of acetonitrile: 0.25M potassium dihydrogen phosphate buffer (80:20) with 0.5%v/v of triethylamine and the pH was adjusted to 2.5 by adding orthophosphoric acid. The detection of the combined dosage form was carried out at 320 nm and flow rate was set to 1ml/min. Linearity was obtained in the concentration range of 5 to 25 mg/ml of nitazoxanide and ofloxacin with correlation coefficients of 0.9987 and 0.9995, respectively. The results of the analysis were validated statistically and recovery studies confirmed the accuracy of the proposed method.

15. ***Development and validation of spectrophotometric methods for the estimation of nitazoxanide in tablet dosage form*** [32].

Lakshminarayana K.V, et al; developed two simple and sensitive visible spectrophotometric methods (A and B) for the quantitative estimation of nitazoxanide, in bulk drug and pharmaceutical dosage forms. Methods were based on the formation of reddish yellow coloured and green colouredchromogens, which were measured at 544 nm and 715 nm, respectively. The results obtained with the proposed methods are in good agreement with the labeled amounts when tablet dosage forms were analyzed.

16. ***A validated stability indicating HPTLC method for the determination of nitazoxanide*** [33].

Gopu C.L, et al; developed and validated a simple, precise, selective and stability indicating HPTLC method for the determination of nitazoxanide in bilk drug and its formulations. Method employed TLC aluminium plates precoated with silica gel 60F 254 as the stationary phase. Solvent system consisted of ethyl acetate-toluene methanol (4:6:1v/v/v). Nitazoxanide was subjected to hydrolysis, oxidation, photolysis and thermal decomposition to establish a validated stability indicating HPTLC method. Extensive degradation occurred in alkaline medium

and in oxidative stress condition and degradation product was well separated from pure drug. Densitometric analysis of nitazoxanide was carried out in absorbance mode at 350nm. Linear regression analysis data for mean calibration plots showed good linear relationship with r^2=0.9997 in 400-1600ng/spot. Method was validated with respect to linearity, precision, accuracy, specificity, robustness. The limits of detection and quantization were 15 and 50ng/spot, respectively. HPTLC method could effectively separate drug from its degradation product and can be employed as a stability-indicating method.

17. ***Development of a Validated Stability-Indicating LC Method for Nitazoxanide in Pharmaceutical Formulations***[34]

Marcelo Donadel Malesuik, et al; devoloped a reversed-phase liquid chromatographic (LC) method was developed for the assay of nitazoxanide (NTZ) in solid dosage formulations. An isocratic LC separation was performed on a Phenomenex Synergi Fusion C18 column (250 mm 4.6 mm, i.d., 4 lm particle size) using a mobile phase of 0.1% o-phosphoric acid solution, pH 6.0: acetonitrile (45:55, v/v) at a flow rate of 1.0 mL min-1. Detection was achieved with a photodiode array detector at 240 nm. The detector response for NTZ was linear over the concentration range from 2 to 100 μg mL -1 (r = 0.9999). The specificity and stability-indicating capability of the method were proved using stress conditions. The RSD values for intra-day precision were less than 1.0% for tablets and powder for oral suspension. The RSD values for inter-day precision were 0.6 and 0.7% for tablets and powder for oral suspension. The accuracy was 100.4% (RSD = 1.8%) for tablets and 100.9% (RSD = 0.3%) for powder for oral suspension. The limits of quantization and detection were 0.4 and 0.1 μg mL -1. There was no interference of the excipients on the determination of the active pharmaceutical ingredient. The proposed method was precise, accurate, specific, and sensitive. It can be applied to the quantitative determination of drug in tablets and powder for oral suspension.

18. ***Evaluation of Nitazoxanide and Oxfendazole Efficacy against Cystic Echinococcosis in Naturally Infected Sheep***[35].

    Cesar M. Gavidia, et al; developed a method to determine the efficacy of nitazoxanide (NTZ) and oxfendazole (OXF) against CE in naturally infected sheep. A total of 151 ewes were assigned to the following groups: 15 mg/kg of NTZ weekly for five weeks (NTZ5); two rounds of 15 mg/ kg of NTZ a day for five days (NTZ5×2) two weeks apart; 30 mg/kg of OXF a week for 11 weeks (OXF11); 30 mg/kg of OXF plus 15 mg/kg of NTZ a week for 11 weeks (OXF/NTZ); and the control group. OXF11 and OXF/NTZ decreased the number of fertile cysts, increased the number of degenerated cysts, and were more efficacious (49.6–61.2%) against lung cysts and liver cysts (91.8–100%) than any other treatment group. OXF might be an additional strategy for control programs and an optional treatment of human CE after it is licensed.

19. ***High dose prolonged treatment with nitazoxanide is not effective for cryptosporidiosis in HIV positive Zambian children: a randomized controlled trial***[36].

    Beatrice Amadi, et al; carried out a double-blind, randomized, placebo controlled trial in paediatric patients which was shown to be positive for both HIV and cryptosporidiosis, in the children's diarrhoea/malnutrition ward of the University Teaching Hospital, Lusaka. Children were included if between 1 and 11 years of age, if positive in at least 2 of 3 stool samples for Cryptosporidium spp. (oocysts identified using auramine phenol staining), if they had diarrhoea with 3 or more unformed stools daily, and if HIV seropositive as confirmed by the Capillus Rapid Test (Trinity Biotech, Ireland). If Cryptosporidium spp. oocysts were present in the initial screening but not in the baseline samples, the patient was excluded unless they tested positive for oocysts within one week from this first sample. Children were excluded if they had a bacterial cause for diarrhoea or if positive for Entamoebahistolytica or Giardia lamblia by the Triage Parasitic Panel (Biosite Diagnostics, San Diego, CA). Other exclusion criteria were a history of investigational drug therapy within one month prior to enrolment, or other recognised anti-protozoal therapy within two weeks prior to enrolment. Children who were moribund were not randomised and gravely ill were observed for a period of 1-2 weeks prior to randomisation.

20. ***Stability indicating CZE method and stress release degradation studies of Nitazoxanide[37].***

    Marcelo DonadelMalesuika, et al; devoloped and validated a new, simple, and effective stability-indicating CZE method for the determination of nitazoxanide in pharmaceutical formulations, using nimesulide as an internal standard. The optimum separation was carried out on a fused silica capillary (48.5 cm × 75 μmi.d., effective length 40 cm) maintained at 25°C, and a running electrolyte containing sodium acetate buffer (pH 5.2; 30 mM)-acetonitrile (80:20, v/v). The injections of the samples were performed using the pressure mode at 50 mm bar for 5 s, with detection at 360 nm using a photo-diode array detector. The method was suitably validated for specificity, linearity, precision, and accuracy, limit of detection and quantitation, and robustness. The high sensitivity of the method was proven with the limit of detection (0.05 μg mL−1) and quantitation (0.2 μg mL−1). The stability-indicating capability of the method was proven using stress conditions (acid and basic hydrolysis, oxidation, and photolysis). The proposed method was successfully applied for the determination of nitazoxanide in coated tablets and oral suspension powder.

21. ***Liquid chromatography–tandem mass spectrometry analysis of nitazoxanide and its major metabolites in goat[38]***.

    Zhanzhong Zhao, et al; devoloped a rapid, sensitive and specific liquid chromatography–electrospray ionization (ESI) tandem mass spectrometry (LC–MS–MS) method for the identification of nitazoxanide metabolites in goat plasma and urine. The purified samples were separated using an XTerra MS C8 column with the mobile phase consisted of acetonitrile and 10-mM ammonium acetate buffer (pH 2.5) followed a linear gradient elution, and detected by MS–MS. Identification and structural elucidation of the metabolites were performed by comparing their retention-times, full scan, product ion scan, precursor ion scan and neutral loss scan MS–MS spectra with those of the parent drug or other available standard. Four metabolites (tizoxanide, tizoxanideglucuronide, tizoxanide sulfate and hydroxylatedtizoxanide sulfate) were found and identified in goat after single oral administration of 200 mg/kg dose of nitazoxanide. In addition, the possible metabolic pathway was proposed for the first time. The results proved that the established method was simple, reliable and sensitive,

revealing that it could be used to rapid screen and identify the structures of active metabolites responsible for pharmacological effects of nitazoxanide and to better understand its in vivo metabolism.

22. ***Nitazoxanide for the treatment of Giardia Duodenalis infection: A comparative trial with Secnidazole***[39].

    Maria Elena Gonzalezi, et al; carried out a randomized controlled open-label trial at the Cuban Institute of Gastroenterology in adults with confirmed Giardia duodenalis mono-infection. 125 patients were randomly assigned to receive either NTZ [500 mg two times daily for three days (n= 62)] or SNZ [2 g single dose (n= 63)]. The evaluation of the efficacy was based on parasitological response. All patients were asked to provide three faecal samples on days 3, 5, and 10 after treatment completion. Patients were considered to be cured if no Giardia trophozoites or cysts were found in any of the three post-treatment faecal specimens evaluated by direct wet mounts and/or after Ritchie concentration techniques.

23. ***Development of Visible Spectrophotometric Methods for the Estimation of Nitazoxanide in Bulk and Pharmaceutical Formulation Using Ferric Chloride***[40].

    Sharma S, et al; developed a simple, accurate, precise and economical procedure for UV, simultaneous estimation of first and second derivative of Nitazoxanide single component tablet dosage form has been developed utilizing concept of standard addition. The method is based upon determination of Nitazoxanide at 218.5 nm absolute ethanol. Nitazoxanide at their respective 8 max 274 nm and 297 nm shows linearity in the concentration range of 5-30µg/ml. For the second method, first derivative Spectrophotometry, the response (dA/d8) of standard solutions was measured at 277 nm. Calibration curve was constructed by plotting dA/d8 values against concentrations, 5-40 µgmlG1 of Nitazoxanide standards in ethanol. For second derivative spectrophotometry, the response (d A/d8) of standard solutions was measured at 314 nm. The method was validated statistically and recovery study was performed to confirm the accuracy of the method.

24. ***Development and Validation of a Dissolution method with Isocratic High-Performance Liquid Chromatographic Determination of Nitazoxanide and Ofloxacin in Pharmaceutical Dosage form***[41].

Sharma M.C, et al., devoloped a simple, specific, accurate and precise Isocratic reverse phase high pressure liquid chromatographic method for the simultaneous determination of Nitazoxanide and Ofloxacin from combined dosage form by reverse phase separation on columns containing different stationary phases, the final choice giving satisfactory resolution and run time was the 25 cm × 4.6 mm i.d, 5-µm particle; Phenomenex Luna C18 reversed-phase column. 2.0gm sodium dihydrogen phosphate and 5M of triethylamine are mixed into 500mL Milli Q water and pH was adjusted to 4.5 by orthophosphoric acid and diluted to 1000mL as a mobile phase at a flow rate of 1.2ml/min and detection at 246nm. The average retention times for amoxicillin (Internal standard), Nitazoxanide and Ofloxacin was found to be 3.11, 5.28 and 7.31 min, respectively and recoveries from combined dosage form were between 98 and 102%. Quantification and linearity was achieved at 276 nm over the concentration range of 100-400 µg mLG1 for Nitazoxanide and 10-150 µg mLG1 for Ofloxacin. The method was validated for specificity, linearity, accuracy, precision, LOD, LOQ and robustness. The proposed method was optimized and validated as per the ICH guidelines. The method can be used for estimation of combination of these drugs in combined dosage form.

25. ***A Validated Stability Indicating RP-LC Method for Nitazoxanide, a New Ant parasitic Compound***[42].

Ashok S. Jadhav, et al; devoloped a method in which the drug substance was subjected to stress conditions of hydrolysis, photolysis and thermal degradation. The considerable degradation of nitazoxanide was observed under base and peroxide hydrolysis. The drug was found to be stable in other stress conditions attempted. The chromatographic separation of the drug was achieved on reversed-phase C-18 column. Eluents were monitored on photo-diode array detector at a wavelength of 240 nm. The mobile phase was aqueous 0.005 M tetra butyl ammonium hydrogen sulphate and acetonitrile (45:55, v/v). In the developed HPLC method, resolution between nitazoxanide and its potential impurities, namely Imp-A (5-nitro-1,3-thiazol-2-amine), Imp-B (N-(5-nitro-1,3-

thiazol-2-yl) acetamide) and Imp-C (2-{[(5-nitro-1,3-thiazol-2-yl) amino] carbonyl} phenyl 2-(acetyloxy) benzoate) was found greater than three. The developed RP-HPLC method was validated with respect to response function, accuracy, precision, specificity, stability of analytical solutions and robustness. Also to determine related substances and assay determination of nitazoxanide that can be used to evaluate the quality of regular production samples. The developed method can also be conveniently used for the assay determination of nitazoxanide in pharmaceutical formulations.

26. ***Method Development and Validation for Assay of Nitazoxanide in Tablet Using RP-HPLC[43].***

    Valarmathy J, developed a simple, selective, rapid, precise and accurate reverse phase high pressure liquid chromatographic method for the assay of nitazoxanide (NTZ) in tablets. An isocratic LC separation was performed on a inertsil C18 (250mm×4.6mm) Column with mobile phase consisting of 0.005M tetra butyl ammonium hydrogen sulphate (45): acetonitrile (55): at a flow rate at 1.0 (ml/min). Detection was carried out at 345 nm. The retention time of nitazoxanide was 7.468 min respectively. The developed method was validated for linearity, accuracy, precision, solution stability, robustness, ruggedness. There was no interference of excipients on the determination of active pharmaceutical ingredients. It can be applied to the quantitative determination of drug in tablets and powder for oral suspension.

27. ***RP-HPLC Determination of Nitazoxanide in Bulk and Different Tablet Formulations[44].***

    Vijay Y. Jadhav, et al; devoloped a simple, accurate and precise validated RP-HPLC method for the determination of Nitazoxanide. Analysis was carried out on Jasco HPLC system with HiQ-sil C18 column (250 x 4.6 mm i.d.) using Acetonitrile: 0.005 mol.L–1 Tetrabutyl ammonium hydrogen sulphatein ratio of 55:45 v/v as mobile phase and Satranidazole as an internal standard. The detection was carried out using UV detector set at 240 nm. For this method, Beer's law is obeyed in the concentration range of 5.0 to 30.0g mL–1 of Nitazoxanide. The developed method has been successfully applied for the analysis of drug in bulk and pharmaceutical formulations.

The mean percent recoveries were found to be 100.19. 0.584 for Brand 1 and 100.26. 1.1341 for Brand 2. The method was validated with respect to linearity, precision and accuracy as per the International Conference on Harmonisation (ICH) guidelines.

28. ***Development and validation of nitazoxanide in tablet dosage form.***[45]

Sonal Bhale, et al; put forwarded a simple, precise, accurate, rapid and reproductive RPHPLC procedure for the determination of Nitazoxanide (NTZ) in tablet dosage form at a single wavelength. The mobile phase used was a combination of methanol: water: acetonitrile (30:40:30% v/v/v). The detection of NTZ was carried out at 330nm & flow rate was set to 1ml/min. Linearity was obtained in the concentration range of 5-25ug/ml of NTZ with correlation coefficients of 0.9948. The results of the analysis were tested and validated statistically for various parameters according to ICH guidelines and recovery studies confirmed the accuracy of the proposed method.

29. ***RP-HPLC methods for estimation of Nitazoxanide single and simultaneous estimation of Nitazoxanide with Ofloxacin in pharmaceutical dosage forms***[46].

Hemendra P. Singh, et al; devoloped two simple, selective, rapid, precise and economical reverse phase high-pressure liquid chromatography methods. First method for estimation of nitazoxanide and second method for simultaneous estimation of nitazoxanide & ofloxacin from pharmaceutical dosage forms. The first method carried out on a Hypersil BDS C8 column (5µ particles size) (250 mm X 4.6 mm), with mobile phase consisting of acetonitrile: 0.2M potassium dihydrogen phosphate in ratio 70:30 (pH 3.0 adjusted with orthophosphoric acid) at a flow rate of 1.0ml/min. Detection was carried out at 319nm. The retention time of nitazoxanide was 4.51 min. First developed method cannot easily apply for the simultaneous estimation of nitazoxanide and ofloxacin. So second method carried out on same column but different mobile phase whichconsisting the acetonitrile: 0.2M potassium dihydrogen phosphate: methanol in ratio 70:10:20 (pH 3.5 adjusted with orthophosphoric acid) at a flow rate of 1.0ml/min. Detection was carried out at 302nm. The retention time of nitazoxanide and ofloxacin were 6.93 min and 9.32 min, respectively. The result

of analysis shows that the amounts of drugs were in good agreement with the labeled claim of the formulations. The method validation parameters checked as per the ICH guidelines. Thus the methods are specific and sensitive.

30. ***Mixed Hydrotropy in Spectrophotometric Analysis of Nitazoxanide***[47]

Sherje AP, et al; investigated and illustrated the application of mixed hydrotropy. There was significant synergistic effect on enhancement in solubility of a poorly water soluble drug by mixing two hydrotropic agents. The enhancement in solubility of nitazoxanide was more than 10 and 12 folds in 1M sodium benzoate solution (SB) and 1M sodium salicylate (SS) solution, respectively as compared to its solubility in distilled water. The enhancement in the solubility of nitazoxanide in a mixed hydrotropic solutions (SB-SS) containing 1M sodium benzoate and 1M sodium salicylate was more than 17 folds. Thus, a mixed hydrotropic solution of sodium benzoate and sodium salicylate was employed to carry out spectrophotometric analysis precluding use of organic solvents. The tablets containing nitazoxanide were analyzed successfully. Recovery studies and statistical data proved accuracy, reproducibility and the precision of the proposed method. The presence of hydrotropic agents did not interfere in the analysis.

31. ***Stripping Voltammetric Methods for Determination of the Ant parasitic Drug Nitazoxanide in Bulk Form, Pharmaceutical Formulation and Human Serum***[48.]

Hanaa S. El-Desoky, et al; recorded the cyclic voltammograms of nitazoxanide at the hanging mercury drop electrode in the Britton-Robinson universal buffer of pH values 2 to 11 containing 20% (v/v) ethanol exhibited a single 4-electron irreversible cathodic peak corresponding to the reduction of its NO_2 group to the hydroxylamine stage. Nitazoxanide was found to adsorb onto surface of the mercury electrode in a monolayer surface coverage of 3.16×10-10 mol cm-2 in which each adsorbed molecule occupies an area of 0.525 nm2. Based on its adsorption behavior onto the mercury electrode surface, validated linear sweep (LS), differential pulse (DP) and square wave (SW) adsorptive cathodic stripping voltammetric methods were described for determination of bulk

nitazoxanide. Limits of detection of 1.5×10-10, 2.4×10-10 and 3.0×10-11 mol L-1 and limits of quantification of 5.0×10-10, 8.0×10-10 and 1.0×10-10 mol L-1 nitazoxanide in the bulk form were achieved by means of the described LS, DP and SW adsorptive cathodic stripping voltammetric methods, respectively. The described methods were successfully applied for determination of nitazoxanide in its pharmaceutical formulation (Cryptonaz powder) and in spiked human serum without the necessity for sample pretreatment, time consuming extraction steps or formation of colored chromogens prior to the analysis. Besides, nitazoxanide was successfully determined without interference from its acid or base-induced degradation products indicating the stability-indicating power of the described voltammetric methods.

IV AIM OF PRESENT STUDY

No official methods have been mentioned in IP, BP for the estimation of nitazoxanide in raw materials and in pharmaceutical dosage forms. From the literature review it was revealed that there were only few HPLC and spectrophotometric methods reported.

So there is a need to develop simple, rapid, reproducible and economic spectrophotometric methods for the estimation of nitazoxanide.

The aim of present study is to develop good analytical methods, having good accuracy and cost effectiveness for the determination of nitazoxanide in bulk drug and its pharmaceutical formulations.

V PLAN OF WORK

The present work deals with the determination of three spectrophotometric methods.

i. First method is based on the formation of Schiff's base.
ii. Second method is based on diazotization coupling method.
iii. Third method is based on a simple spectrophotometric method.
iv. Fourth method is Reverse-Phase High Performance Liquid Chromatography.

IV INSTRUMENTS AND MATERIALS USED

1. Perkin Elmer EZ 301-UV-Visible Double beam spectrophotometer
2. Shimadzu - Analytical balance-0.1mg
3. Shimadzu,Japan - Electronic balance AY220
4. Micro pipette (1ml,0.02ml) - Huawei
5. Accu pipette (0.2ml) - Huawei
6. Zinc powder (AR)
7. Conc. HCl (AR)
8. 4-Hydroxy benzaldehyde (AR)
9. Sodium nitrite (AR)
10. Ammonium sulphamate (AR)
11. Phluroglucinol (AR)
12. Methanol (AR)
13. Distilled water
14. HPLC (Shimadzu)
15. Dipottasium hydrogen phosphate:acetonitrile

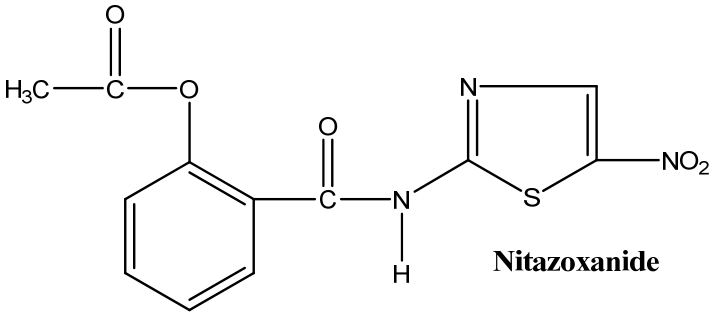
VII INTRODUCTION TO PRESENT STUDY

Nitazoxanide[49] is used for the treatment of diarrhea caused by Giardia lambia /intestinalis or Cryptosporidium parvum. This novel agent has a broad spectrum of activity against many other gastrointestinal pathogens, including bacteria, round worms, flat worms and flukes. Nitazoxanide is used in many areas of the world, especially in Central and South America, as broad-spectrum parasiticidal agents in adults and children. Chemically nitazoxanide is [2-[(5-nitro-1, 3-thiazol-2-yl) carbamoyl] phenyl] ethanoate.

Absorption spectroscopy is considered as a versatile technique frequently used in pharmaceutical analysis. Using this technique many pharmaceutical substances can be determined by UV-visible spectrophotometry, HPLC with great precision and accuracy.

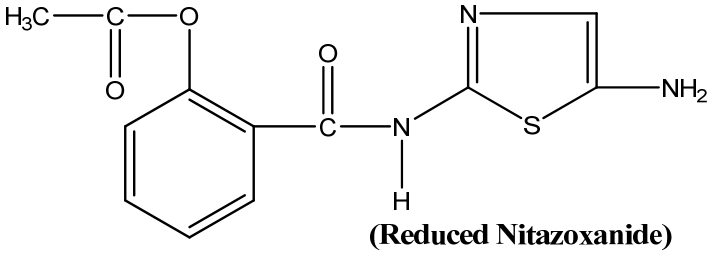
1st Method:-It is based on the formation of Schiff's base (Aromatic aldehydes react with aliphatic /aromatic amines to form a derivative known as Schiff's base)

In this method nitazoxanide was reduced by Zn dust and 5N HCl. The reduced nitazoxanide was condensed with 4-hydroxy benzaldehyde to produce light orange colored compound (Schiff's base).The absorption of resultant light orange coloured product was measured at 460nm.



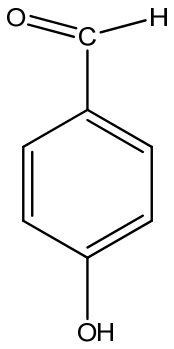
Nitazoxanide

Zn / HCl

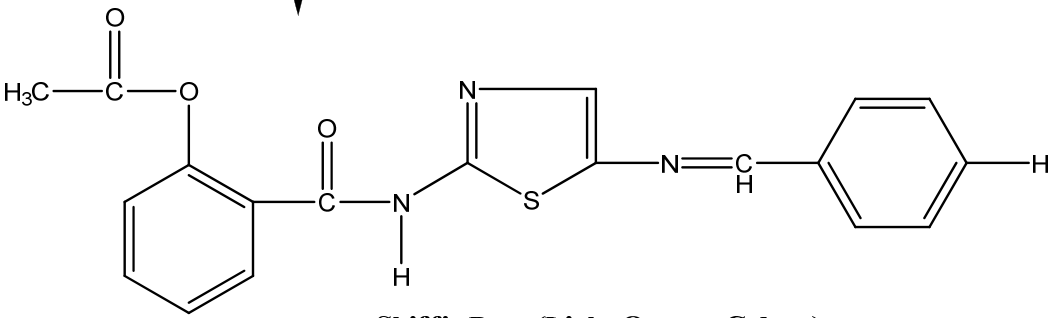


(Reduced Nitazoxanide)

+

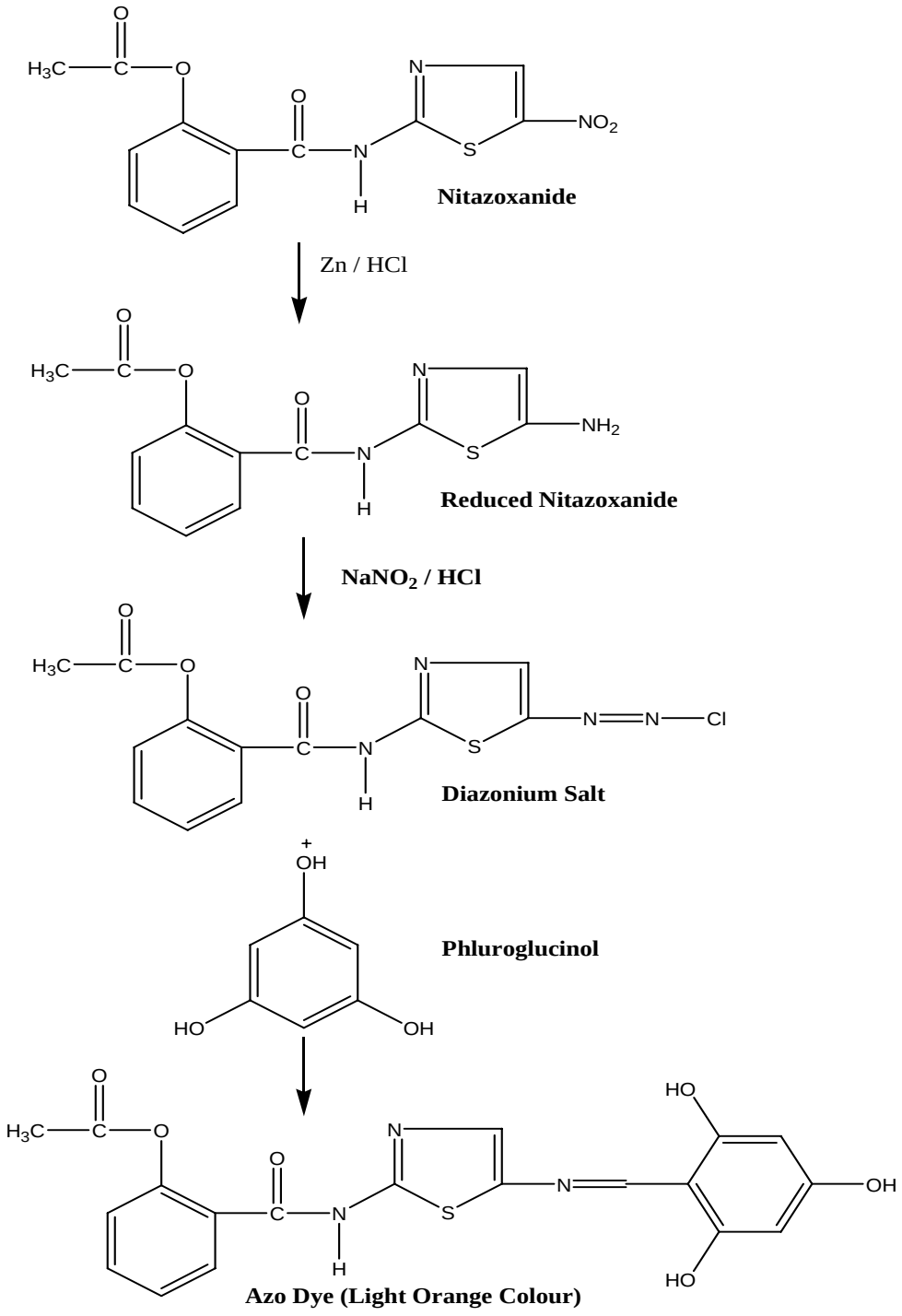


4 Hydroxy benzaldehyde



Shiff's Base (Light Orange Colour)

2nd Method: -It is based on diazotization & coupling method. In this method nitazoxanide was reduced by Zn dust and 5N HCl, and then it is diazotized with HNO_2 and excess of HNO_2 was destructed by ammonium sulphamate. The diazotized salt was coupled with phluroglucinol to form a light orange coloured dye having maximum absorbance at 450nm.



3rd Method:-It is a simple spectrophotometric method using methanol as solvent. It shows maximum absorbance at 340nm.

4th method:-A rapid, precise and accurate high performance liquid chromatographic method for the determination of nitazoxanide in bulk drug and pharmaceutical formulation. The devoloped method was validated in terms of different parameters.

VIII METHODOLOGY -I

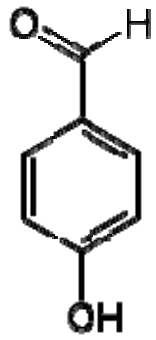
SPECTROPHOTOMETRIC DETERMINATION-I

Spectrophotometric determination of nitazoxanide by Schiff's base method using 4-hydroxybenzaldehyde[56]

Chemicals used:-

1. 4-Hydroxy benzaldehyde (AR)
2. Methanol (AR)
3. Conc.HCl (AR)
4. Zn powder (AR)

Chemical Structure:



Chemical Name: - p-hydroxyl benzaldehyde

Molecular Formula: - $C_7H_8O_2$

Molecular Weight: - 122.12g/mol

Solubility:-

Slightly soluble in water and soluble in methanol

Preparation of 4-hydroxy benzaldehyde (2%)

It was prepared by dissolving 2g of 4-hydroxy benzaldehyde in 100ml methanol.

Preparation of standard stock solution

25mg of nitazoxanide was dissolved in 5ml of methanol and was treated with 2.5ml 5N HCl and 200mg Zn powder with continuous stirring for 20mts at room temperature. It was then filtered and the residue was washed with methanol. Then the volume was made upto25ml with methanol (Stock solution I, 1000µg/ml)

Absorption spectra of coloured species

5ml of standard stock solution I was pipetted into 50ml volumetric flask and it was diluted to 50ml with methanol (Stock solution II, 100µg/ml). From this 3ml was pipetted out into 10ml volumetric flask and 1ml of 2% 4-hydroxy benzaldehyde, 1ml of Conc.HCl were added and kept aside for 5mts. The volume was made up to 10ml with methanol. The final volume concentration of the solution was 30µg/ml. The absorbance was measured between 340-480nm against reagent blank.

Readings were shown in table no:1 and plotted in graph no:1

Absorption spectra for the drug nitazoxanide by using 4-hydroxy benzaldehyde

Drug concentration-30µg/ml

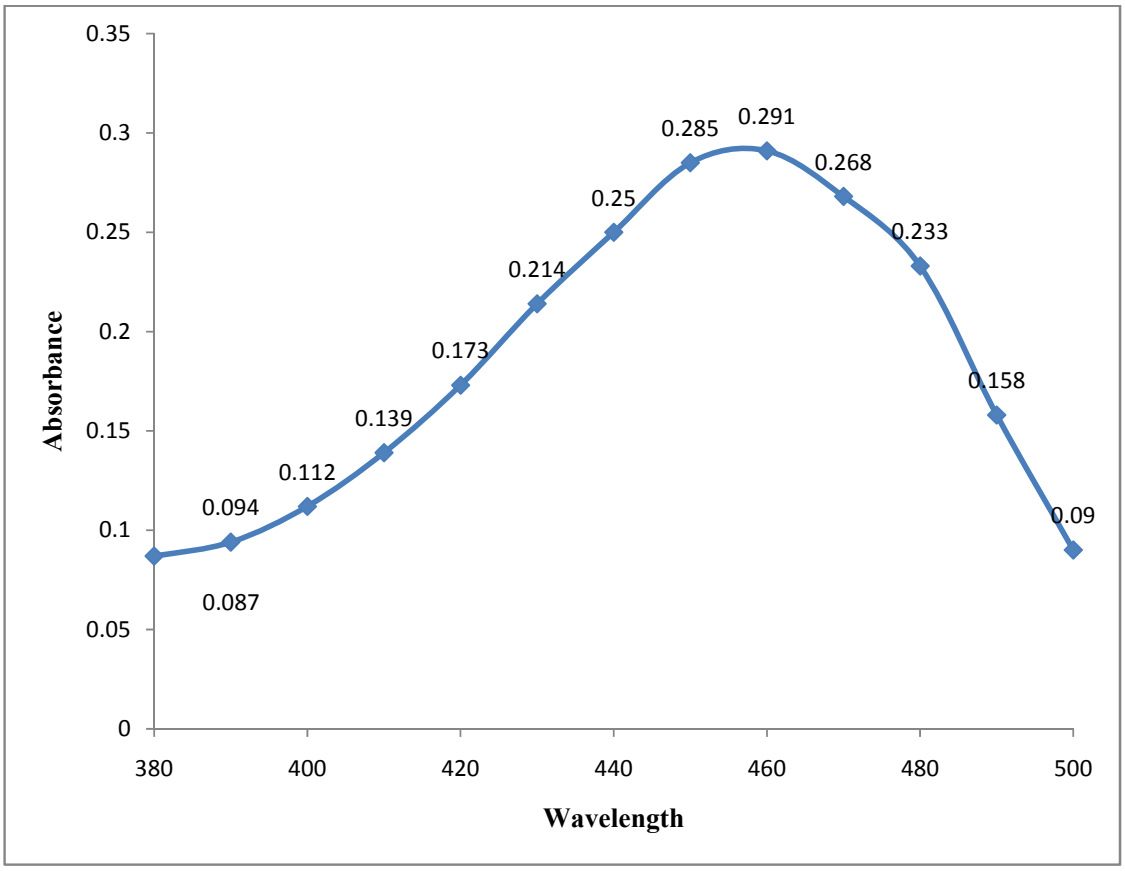
TABLE NO: 1

WAVELENGTH(nm)	ABSORBANCE
380	**0.087**
390	**0.094**
400	**0.112**
410	**0.139**
420	**0.173**
430	**0.214**
440	**0.250**
450	**0.285**
460*	**0.291**
470	**0.268**
480	**0.233**
490	**0.158**
500	**0.090**

The maximum absorbance was measured at 460nm.

Calibration curve for nitazoxanide using 4-hydroxy benzaldehyde

GRAPH NO: 1



Effect of reagent concentration

Choosing of correct reagent concentration was an important aspect in the colorimetric determination. The optimum concentration of reagent was chosen by adding 1ml of reagent of different concentration .ie 1%, 2%, 3% of 4-hydroxy benzaldehyde solution in methanol with series concentration of drug solution.

The calibration curve for each concentration was prepared by using drug in the concentration range of 10-50µg/ml and the absorbance was measured at 460nm against reagent blank. The readings were recorded in table no: 2 graphically plotted in graph number 2.

Data for calibration curve plot with different reagent concentration

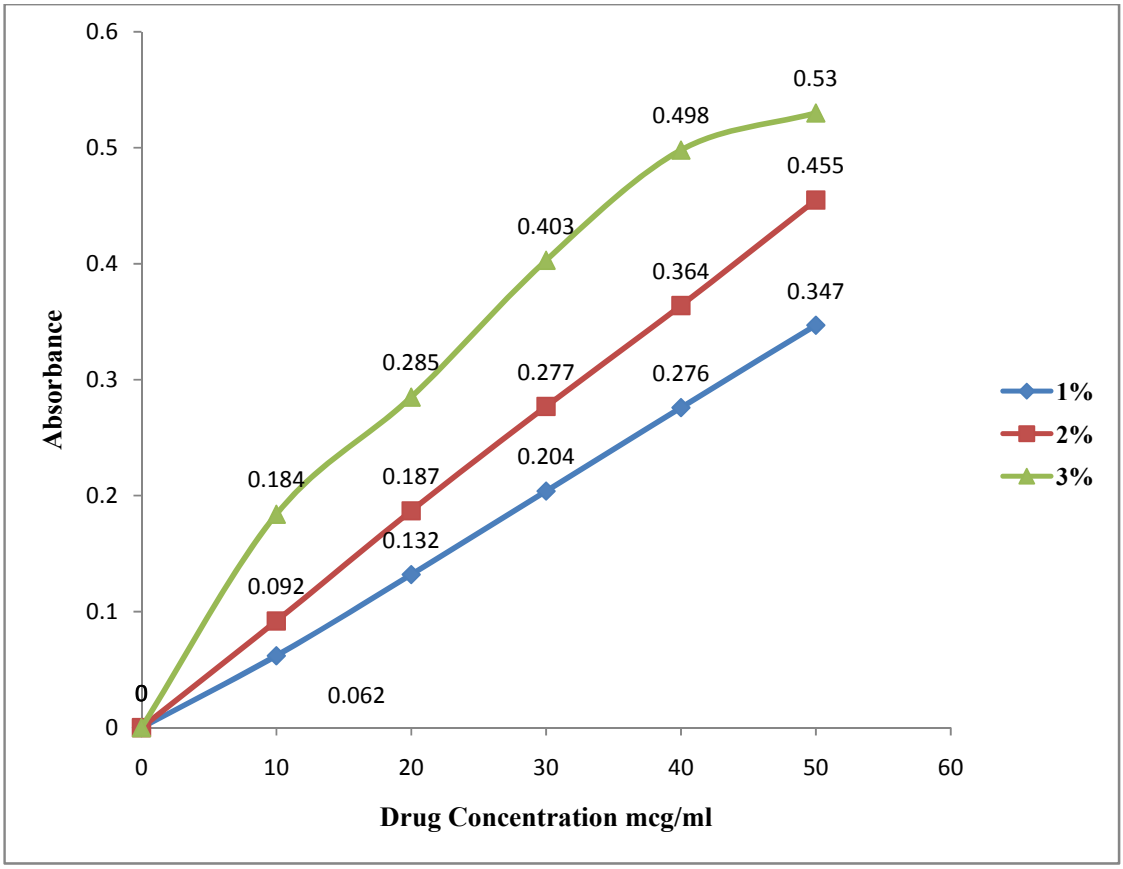
TABLE NO: 2

Drug concentration (µg/ml)	**Absorbance of reagent concentration**		
	1%	2%	3%
10	**0.062**	**0.092**	**0.184**
20	**0.132**	**0.187**	**0.285**
30	**0.204**	**0.277**	**0.403**
40	**0.276**	**0.364**	**0.498**
50	**0.347**	**0.455**	**0.530**

The optimum concentration was found to be 2%,since it exhibits linearity

Calibration curve plot for nitazoxanide with different reagent concentration

GRAPH NO: 2



Effect of reagent amount

Addition of correct amount of reagent was an important aspect in this experiment. The optimum amount of reagent was fixed by constructing calibration curve. The calibration curve was prepared by employing the drug in the concentration range of 10-50µg/ml with different amount of reagent.ie 0.5ml, 1ml, 1.5ml. The readings were recorded in table no: 3 and graphically plotted in graph no: 3

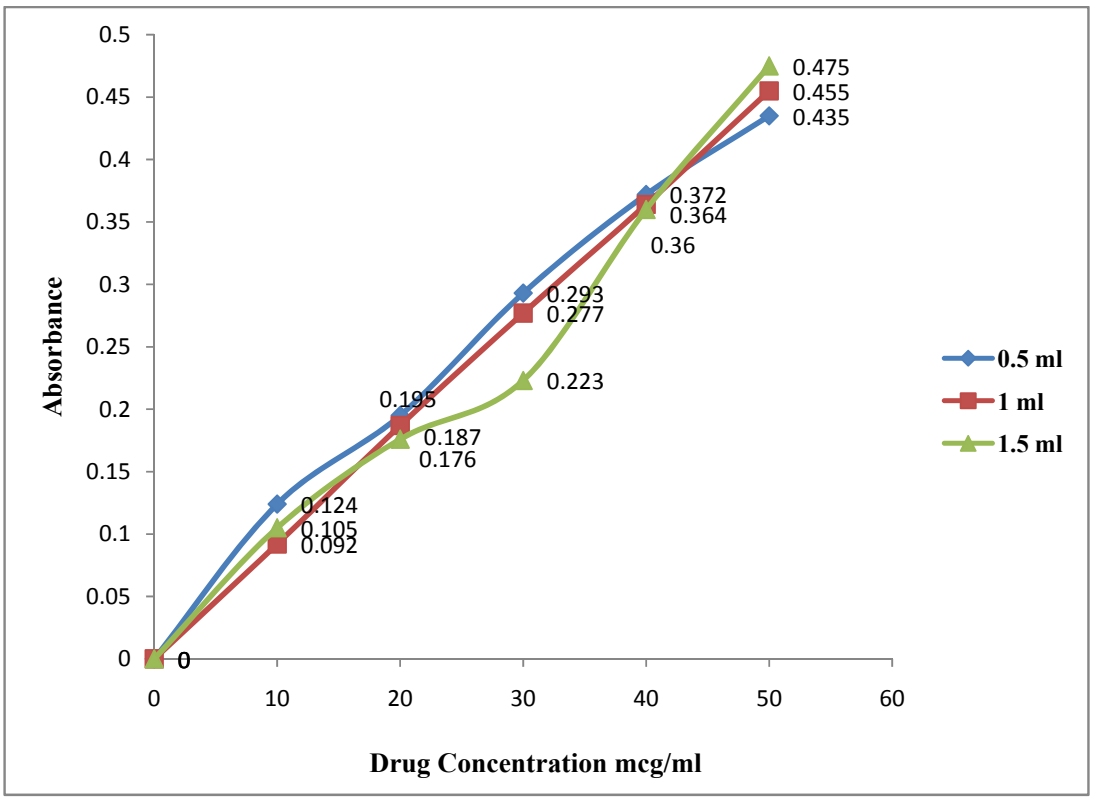
TABLE NO: 3

Drug concentration (µg/ml)	Absorbance at 460nm		
	0.5ml	1ml	1.5ml
10	0.124	0.092	0.105
20	0.195	0.187	0.176
30	0.293	0.277	0.223
40	0.372	0.364	0.360
50	0.435	0.455	0.475

The optimum amount of reagent was found to be 1ml.

Calibration curve plot for nitazoxanide using different amounts of 2%reagent concentration

GRAPH NO: 3



FIXATION OF VARIOUS PARAMETERS

1. **λ_{max}** (Wavelength maximum)

The absorption spectral data showed that the maximum absorbance was observed at 460nm when scanned between 380-500nm.

2. **Specific extinction coefficient**

The specific extinction coefficient can be calculated by using the formula:-

$$E^{1\%}_{1cm=A/IC}$$

where,

A-Absorbance

I-Light pathlength expressed in cm

C-Concentration of drug in g/100ml

BEERS'S LAW PLOT

Beer's law states that the fraction of the monochromatic radiant energy absorbed on passing through a solution is directly proportional to concentration of the absorbent.

$$Log_{10} I_0/I_t=K.C$$

where,

K-Proportionality constant

C-Concentration

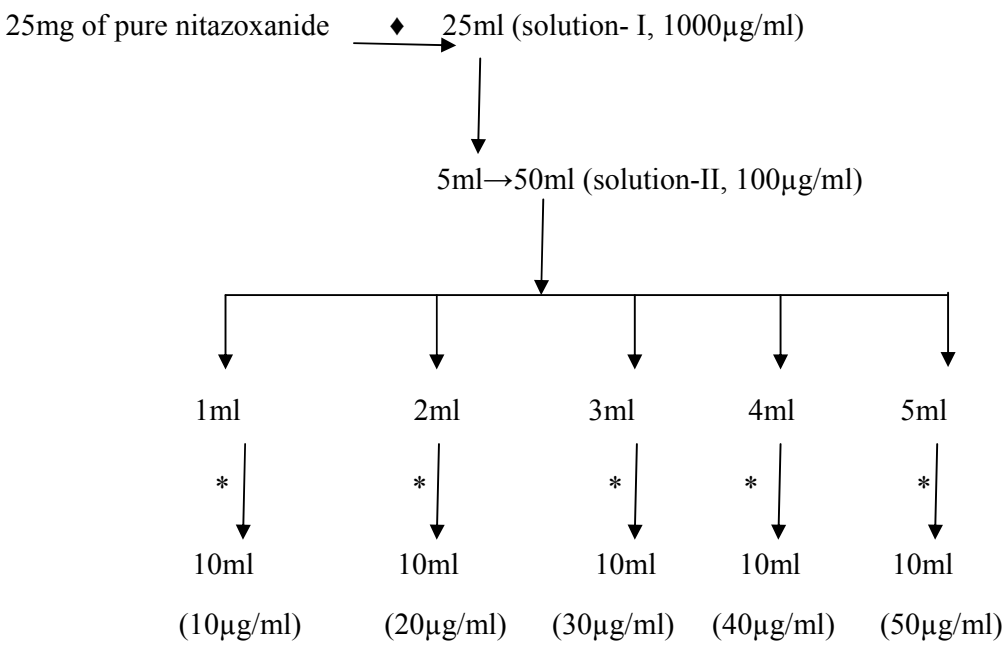
I_0-I intensity of incident light

I_t-Intensity of transmitted light

Having fixed other parameter, Beer's law plot was constructed by measuring the absorbance of various concentration of drug solution against reagent blank.

5ml of stock solution-1 was pipetted in 50ml volumetric flask and made upto 50ml with methanol. The concentration of solution was 100µg/ml (Stock solution-II). From stock solution II, various aliquots of 1ml, 2ml, 3ml, 4ml and 5ml were pipetted out into a 10ml volumetric flask followed by addition of 1ml, 2% 4-hydroxybenzaldehyde and 1ml conc. HCl and kept aside for 5mts.The volume was made upto 10ml with methanol to produce concentration in the range of 10-50µg/ml.Absorbance of each solution was observed at 460nm against reagent blank. The readings were recorded in table no: 4 and graphically plotted in graph no: 4.

SCHEMATIC REPRESENTATION OF BEER'S LAW PLOT



♦200mg Zn powder+2.5ml 5N HCl+5ml methanol

*1ml 2%4-hydroxy benzaldehyde+1ml Conc.HCl

DATA FOR BEER'S LAW PLOT

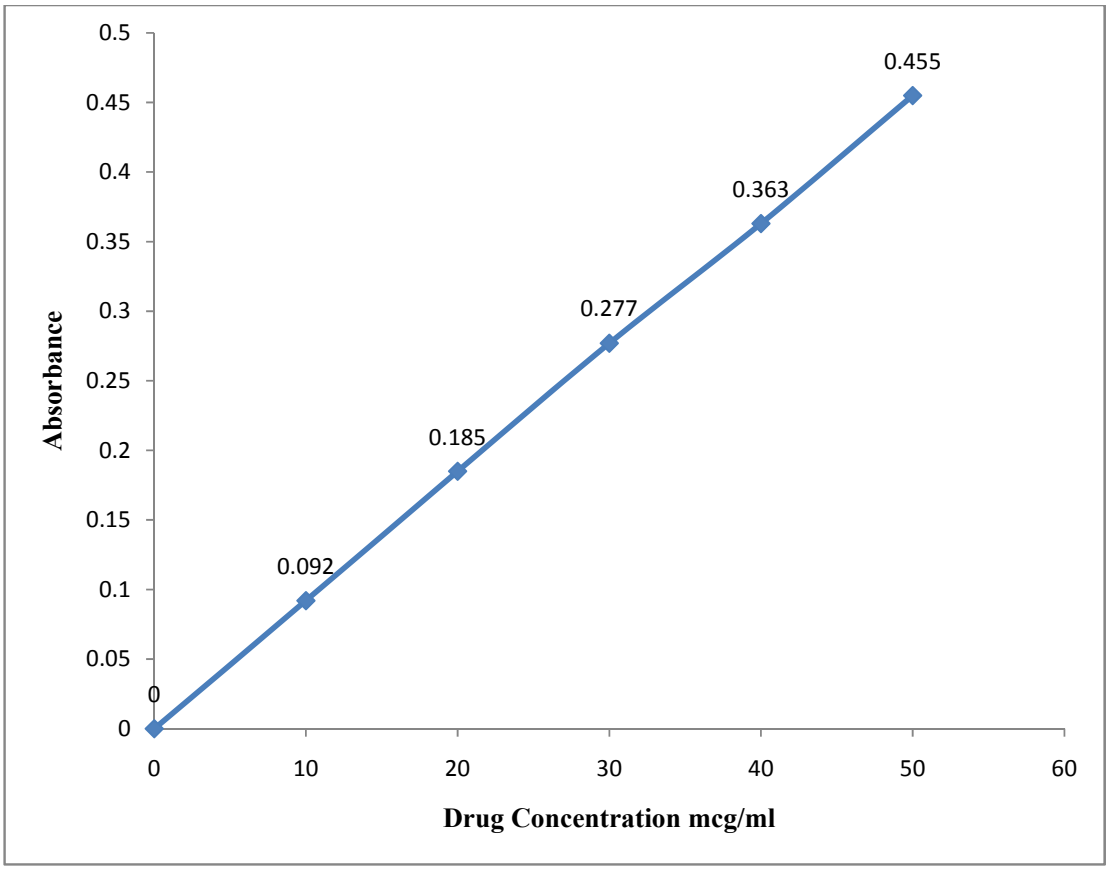
TABLE NO: 4

Drug concentration (µg/ml)	Absorbance
10	**0.092**
20	**0.185**
30	**0.277**
40	**0.363**
50	**0.455**

Beer's law was obeyed in the concentration range of 10-50µg/ml

Calibration curve for nitazoxanide using 4-hydroxy benzaldehyde

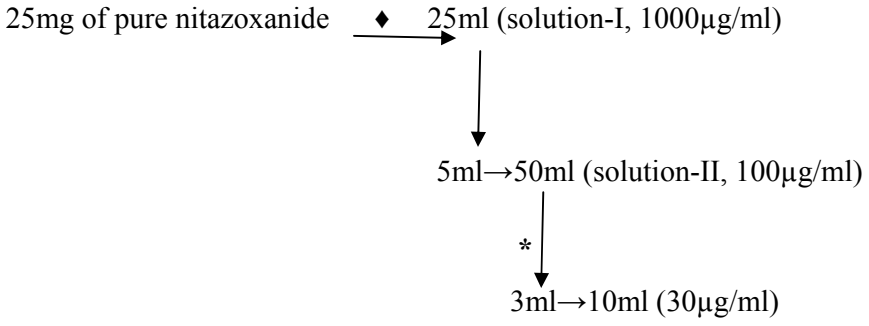
GRAPH NO: 4



ASSAY OF TABLET

10 tablets of nitazoxanide were taken for analysis. The average weights of tablets were calculated and were powdered in a glass mortar. Tablet powder equivalent to 25mg was accurately weighed and dissolved in 5ml of methanol and treated with Zn powder (200mg) and 2.5ml 5N HCl with continuous stirring for 1hr at room temperature. It was filtered and the residue was washed with methanol and then the volume was made up to 25ml with methanol (Solution-I). 5ml of stock solution-1 was pipetted into 50ml volumetric flask and the volume was made upto 50ml with methanol solution-II). From this 3ml was pipette out into 10ml volumetric flask followed by addition of 1ml 2%, 4-hydroxy benzaldehyde and 1ml concentrated HCl and kept aside for 5mts. The volume was made up with methanol to produce final drug concentration of 30µg/ml. The absorbance of this solution was measured at 460nm against reagent blank. The same procedure was repeated 5 times. In similar manner standard absorbance was measured with pure drug in same final concentration that of assay method. The data for assay of tablet were recorded in table no: 5.

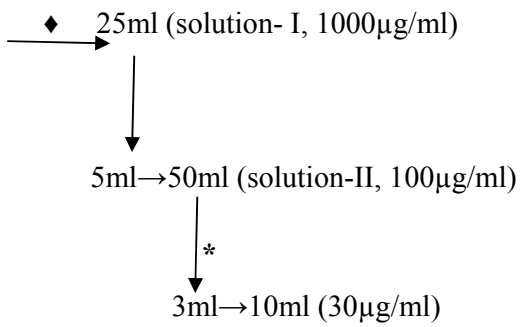
STANDARD DILUTION



♦200mg Zn powder+2.5ml 5N HCl+5ml methanol

*1ml, 2% 4-hydroxy benzaldehyde+1ml Conc.HCl

TEST DILUTION



♦200mg Zn powder+2.5ml 5N HCl+5ml methanol

*1ml ,2% 4-hydroxy benzaldehyde+1ml Conc. HCl

CALCULATIONS

Using the absorbance of the standard and sample solution, the content of tablet was calculated as follows:-

The content of nitazoxanide present in each tablet of average weight =

$$\frac{\text{Test absorbance}}{\text{Std absorbance}} \times \frac{\text{Wt of std drug(mg)}}{25} \times \frac{5}{10} \times \frac{25}{\text{wt of tablet powder (mg)}} \times \text{Avg wt oftablet (mg)}$$

DATA FOR ASSAY OF TABLET

(Label claim: 500mg)

TABLE NO: 5

Sl no:	Brand name	Avgwt of tablet (mg)	Wt. of std drug (mg)	Std absorbance	Wt. of Tablet Powder (mg)	Wt.of tab powder (mg)	Content of drug in tablet (mg)	Avg content (mg)
1	NIZONIDE -500mg	1261.96	25.9	0.295	62	0.280	500.37	498.73
2					63	0.285	501.28	
3					64	0.288	498.58	
4					65	0.292	497.72	
5					66	0.297	498.58	

INTERFERENCE STUDIES

The interference studies of additives used in the formation of tablet were done by distributing them individually in distilled water and set aside for 10mts before filtering. The filtrate was diluted and proceeded as per tablet assay and the absorbance was measured at 460nm against reagent blanks.

This procedure was repeated 5 times of each additive and the average value for each additive was given the following table no: 6

DATA FOR THE INTERFERENCE STUDIES

TABLE NO: 6

Sl no:	Name of the excipients	Absorbance at 460nm
1	Talc	**0.001**
2	Lactose	**0.003**
3	Starch	**0.002**
4	Magnesium state	**0.002**

The results show that the effect of interference studies in this spectroscopic method was found to be negligible.

RECOVERY STUDIES

In order to justify the reliability and suitability of the proposed method, recovery studies were carried out.

The recovery experiment was performed on nitazoxanide tablet. The powder equivalent to 25mg was accurately weighed and dissolved in 5ml methanol and was treated with 200mg Zn powder and 2.5ml 4N HCl with continuous stirring for 1hr at room temperature. It was filtered and washed with methanol. An aliquot of 5ml of

standard solution (1mg/ml) of pure sample of nitazoxanide was added to the flask. It was shaken well and the volume was made upto 25ml with methanol and the procedure for the assay of nitazoxanidewas followed. The experiment was repeated 5 times. The results were recorded in table no: 7. The percentage recovery was calculated by using the formula:-

$$\% \text{ of Recovery} = \frac{\text{Avg content from recovery} - \text{Avg content from assay}}{\text{Amount of std drug added}} \times 100$$

TABLE NO: 7

Sl no:	Brand name	Avgwt of tablet (mg)	Wt. of std drug (mg)	Std absorbance	Wt. of tablet powder (mg)	Pure drug added	Abs of recovered sample	%recovery
1	NIZONIDE -500mg	1261.96	25.7	0.302	63.3	5	0.298	104.93%
2					63	5	0.296	
3					64	5	0.300	
4					64.1	5	0.301	
5					63.6	5	0.299	

STANDARD DEVIATION

It is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimation of the standard deviation using the formula:-

$$\mathbf{SD} = \frac{\sqrt{\Sigma(X-x)2}}{N-1}$$

X-Observed value

x-mean/arithmetic average

N-Number of observations

The square root of standard deviation is called variance./The more accurate measure of precision is called Coefficiant of variation/%RSD

%RSD= (SD/*x*) x 100

PRECISION[57]

The precision of an analytical method is the degree of reproducibility among the individual test results when the procedure was applied repeatedly to multiple sampling of homogenous sample. The precision of an analytical method is usually expressed as standard deviation or percentage RSD of a series of measurements.

The precision study was done based on the data obtained from table no: 7 and the results were shown in table no: 8

TABLE NO: 8

Sl no:	Name of tablet	Standard Deviation (SD)	Coefficient of variation(%RSD)
1	NIZONIDE	1.3623	0.2735

ACCURACY[57]

Accuracy is the closeness of the test results obtained by the procedure to the true value. Accuracy of the proposed method was evaluated by comparing the average value obtained by the proposed method with that of reported method (standard method) and results were given table no: 9

TABLE NO: 9

Sl no:	Amount obtained by standard method	Amount obtained by proposed
1	499.05mg	498.73mg

STANDARD METHOD

Preparation of standard

10mg of pure nitazoxanide was accurately weighed and transferred into 25ml volumetric flask and made up to 10ml with DMF (stock solution-I). 1ml of solution-I was pipetted into 10ml volumetric flask and the volume was made up to 10ml with DMFsolution-II). From this 2ml was pipetted out into 10ml volumetric flask and made upto 10ml with DMF. From this again 2ml of the solution was pippeted out and made upto 10ml with DMF to produce final drug concentration of 4µg/ml. The absorbance of this solution was measured at 340nm against reagent blank

ASSAY OF TABLET

10 tablets of nitazoxanide were taken for analysis. The average weights of tablets were calculated and were powdered in a glass mortar. Tablet powder equivalent to 10mg was accurately weighed and dissolved in 5ml of DMF .It was filtered and the residue was washed with DMF and then the volume was made up to 10ml with DMF (Solution-I). 1ml of solution-1 was pipette into 10ml volumetric flask and the volume was made upto 10ml with DMFsolution-II). From this 2ml was pipette out into 10ml volumetric flask and made upto 10ml with DMF. From this 2ml of the solution was pippeted out and made upto 10ml with DMF to produce final drug concentration of 4µg/ml. The absorbance of this solution was measured at 340nm against reagent blank. The same procedure was repeated 5 times. In similar manner standard absorbance was measured with pure drug in same final concentration that of assay method. The data for assay of tablet were recorded in table no: 10.

STANDARD DILUTION

25mgof pure nitazoxanide → 25ml (Solution-I, 1000µg/ml)

↓

1ml→10ml (Solution-II, 100µg/ml)

↓

2ml→10ml (30µg/ml)

↓

2→10ml (4µg/ml)

TEST DILUTION

Tablet powder equivalent to 25mgnitazoxanide → 25ml(Solution-I, 1000µg/ml)

↓

1ml→10ml (Solution-II, 100µg/ml)

↓

2ml→10ml (30µg/ml)

↓

2→10ml (4µg/ml)

CALCULATIONS

Using the absorbance of the standard and sample solution, the content of tablet was calculated as follows:-

The content of nitazoxanide present in each tablet of average weight =

$$\frac{\text{Test absorbance}}{\text{Std absorbance}} \times \frac{\text{Wt of std drug(mg)}}{25} \times \frac{5}{10} \times \frac{25}{\text{wt of tablet powder (mg)}} \times \text{Avg wt oftablet (mg)}$$

DATA FOR ASSAY OF TABLET

(Label claim: 500mg)

TABLE NO: 10

Sl no:	Brand name	Avgwt of tablet (mg)	Wt. of std drug (mg)	Std absorbance	Wt. of tablet powder (mg)	Wt.of tablet powder	Content of drug in tab(mg)	Avg content (mg)
1	NIZONIDE -500mg	1261.96	25.5	0.243	63.4	0.239	499.21	499.05
2					63	0.237	498.18	
3					64	0.241	498.67	
4					63.5	0.240	500.51	
5					63.2	0.238	498.70	

IX METHODOLOGY II

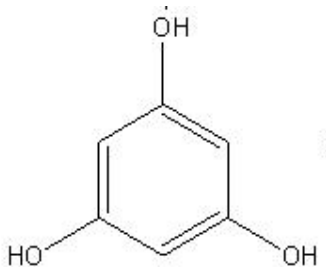
SPECTROPHOTOMETRIC DETERMINATION-II

Spectrophotometric determination of nitazoxanide by diazotization coupling method using phluroglucinol[56]

Chemical used

1. Zinc granules (AR)
2. Conc.HCl (AR)
3. Sodium nitrite (AR)
4. Ammonium sulphamate (AR)
5. Phluroglucinol (AR)

Chemical structure



Chemical name

1, 3, 5 trihydroxy benzene/benzene 1, 3, 5 triol

Molecular formula- C6H6O

Molecular weight- 126.1

Solubility-Freely soluble in alcohol and water

Preparation of 4N HCl

It was prepared by dissolving 34ml of conc.HCl in 100ml distilled water.

Preparation of 0.1%w/v sodium nitrite

It was prepared by dissolving 0.1g of sodium nitrite in 100ml distilled water.

Preparation of 0.5%w/v of ammonium sulphamate

It was prepared by dissolving 0.5g of ammonium sulphamate in 100ml distilled water.

Preparation of 0.5%w/v phluroglucinol

It was prepared by dissolving 0.5g of phluroglucinol in 100ml distilled water.

Preparation of standard stock solution

10mg of nitazoxanide was accurately weighed and dissolved in 5ml methanol. The methanolic solution of nitazoxanide was treated with 200mg of Zn powder and 2.5ml of 4N HCl and kept aside for 1hour at room temperature. The solution was filtered and the volume was made up to 10ml with methanol. (1000µg/ml).

Absorption spectra of coloured species

5ml of standard stock solution I was pipette into 50ml volumetric flask and made up with methanol. From this 3ml was pipetted out into 10ml volumetric flask followed by the addition of 1ml conc:HCl, 1ml of 0.1%sodium nitrite, 1ml of 0.5%w/v ammonium sulphamate and 1ml of 0.5%w/v phluroglucinol were added. Finally the volume was made upto 10ml with methanol. The final concentration of the solution was 30µg/ml. The absorbance was scanned between 400-500nm against reagent blank. Readings were shown in table no: 11 and plotted in graph no: 5

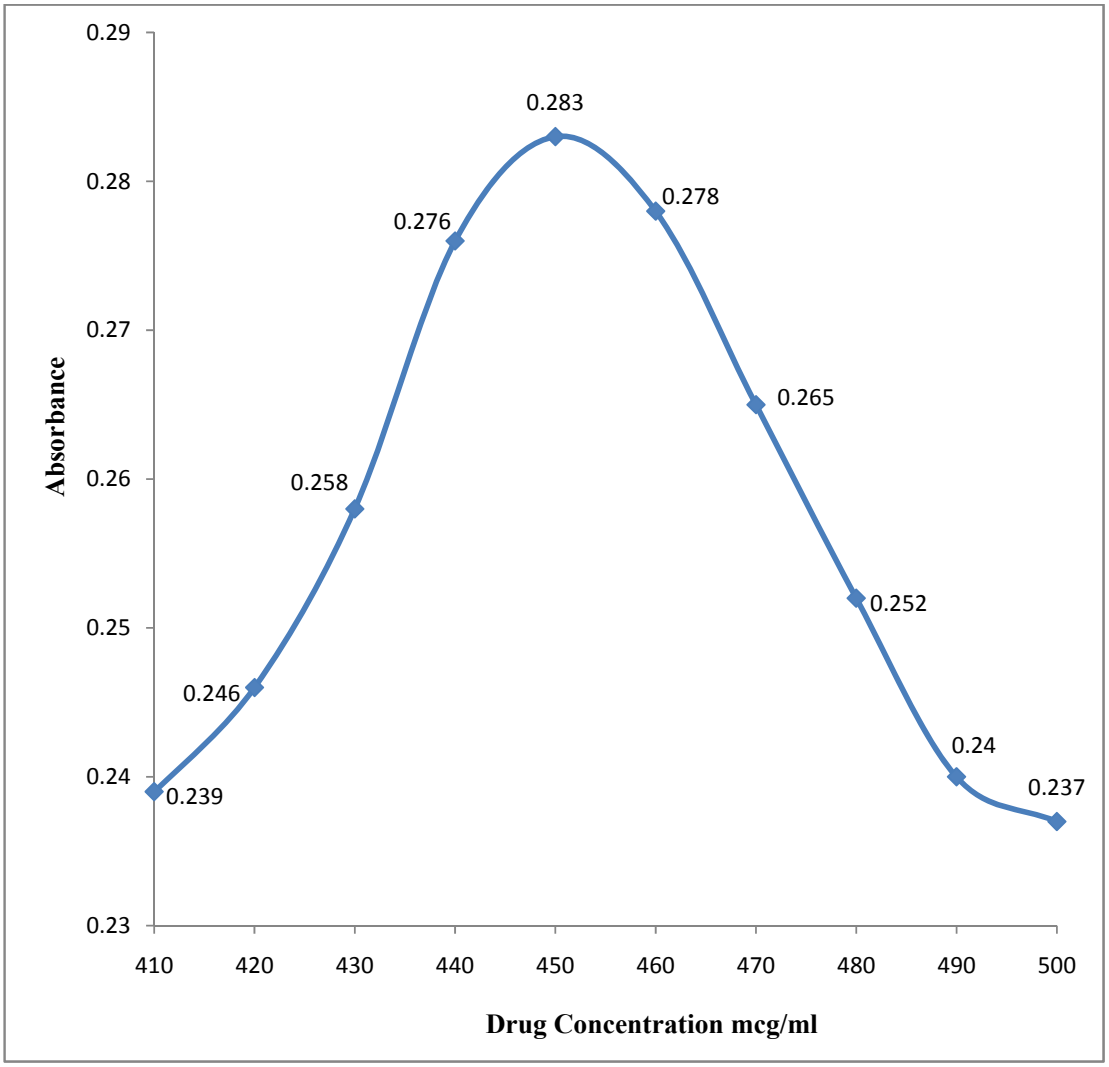
TABLE NO: 11

WAVELENGTH	ABSORBANCE
410	**0.239**
420	**0.246**
430	**0.258**
440	**0.276**
450*	**0.283**
460	**0.278**
470	**0.265**
480	**0.252**
490	**0.240**
500	**0.237**

The maximum absorbance was measured at 450nm.

Calibration curve for nitazoxanide using phluroglucinol

GRAPH NO: 5



Effect of reagent concentration

Choosing of correct reagent concentration was an important aspect in the spectrophotometric determination. The optimum concentration of reagent was chosen by adding 1ml of reagent of different concentration. i.e. 0.25%, 0.5%, 1% of phluroglucinol solution in methanol with series concentration of drug solution.

The calibration curve for each concentration was prepared by using drug in the concentration range of 10-50µg/ml and the absorbance was measured at 450nm against reagent blank. The readings were recorded in table no: 12 and graphically plotted in graph number: 6.

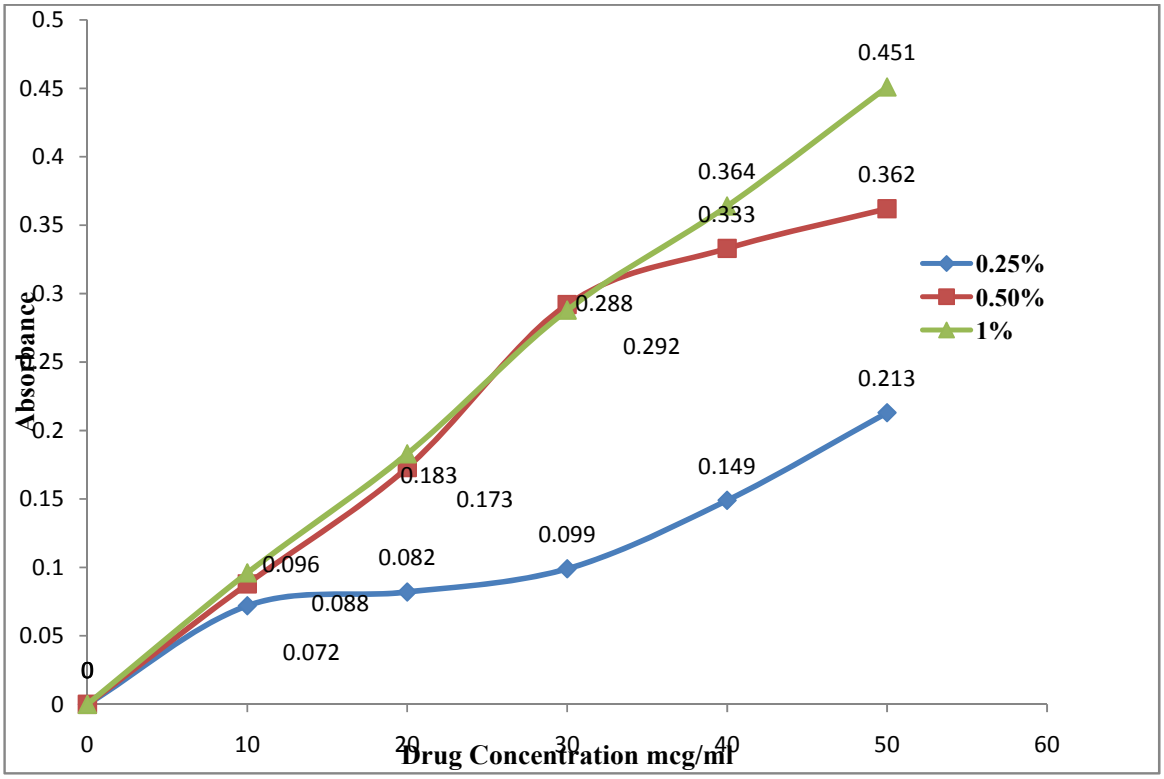
TABLE NO: 12

Drug concentration(µg/ml)	Effect of reagent concentration		
	0.25%	0.5%	1%
10	0.072	0.088	0.096
20	0.082	0.173	0.183
30	0.099	0.292	0.288
40	0.149	0.333	0.364
50	0.213	0.362	0.451

The optimum concentration was found to be 1%.Since it exhibits linearity.

Calibration curve of nitazoxanide with different concentration of phluroglucinol

GRAPH NO: 6



Effect of reagent amount

Addition of correct amount of reagent was an important aspect in this experiment. The optimum amount of reagent was fixed by constructing calibration curve. The calibration curve was prepared by employing the drug in the concentration range of 10-50µg/ml with different amount of reagent i.e.0.5ml, 1ml, 1.5ml.The readings were recorded in table no: 13 and graphically plotted in graph no: 7 \

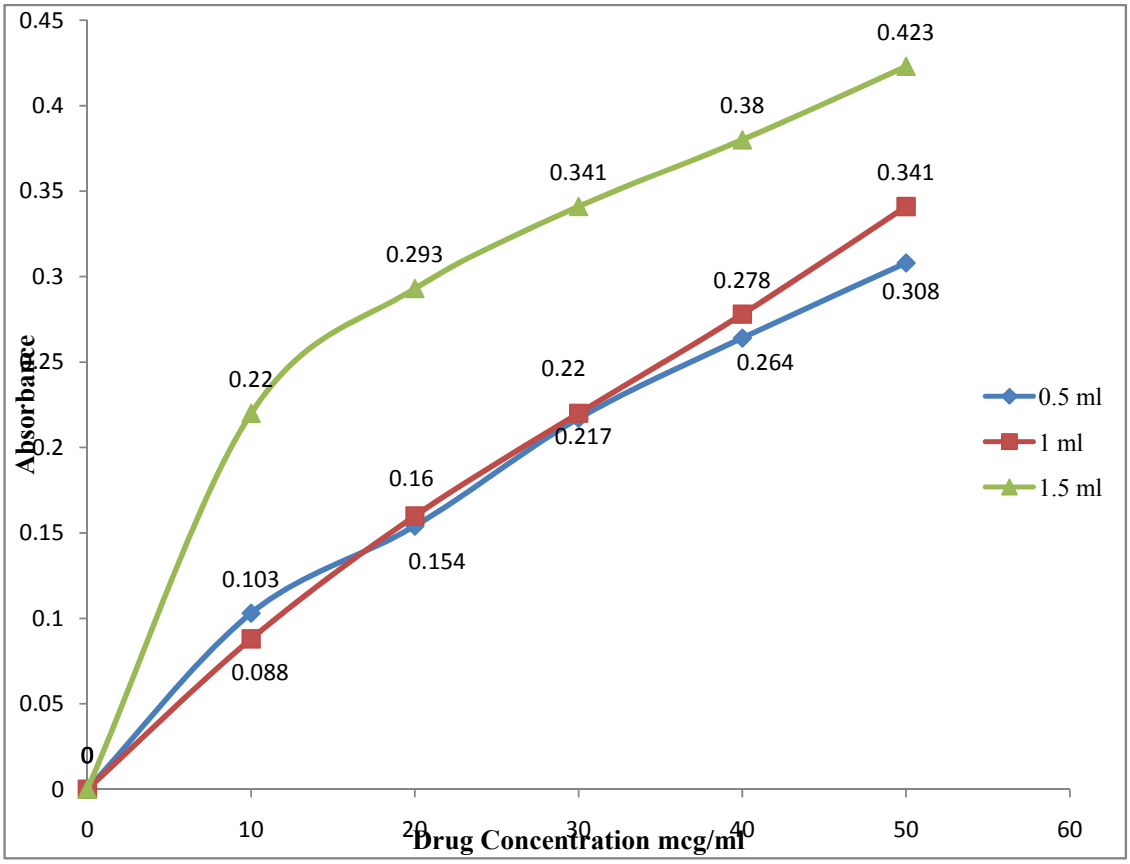
TABLE NO: 13

Drug concentration(µg/ml	Absorbance at 450nm		
	0.5ml	1ml	1.5ml
10	0.103	0.088	0.220
20	0.154	0.16	0.293
30	0.217	0.220	0.341
40	0.264	0.278	0.380
50	0.308	0.341	0.423

The optimum amount of reagent was found to be 1ml

Calibration curve plot for nitazoxanide using different amounts of 2%reagent concentration

GRAPH NO: 7



FIXATION OF VARIOUS PARAMETERS

1. λ_{max}(Wavelength maximum)

The absorption spectral data showed that the maximum absorbance was observed at 460nm when scanned between 380-500nm.

2. **Specific extinction coefficient**

The specific extinction coefficient can be calculated by using the formula.

$$E^{1\%}_{1cm=A/IC}$$

where,

A-Absorbance

I-Light path length expressed in cm

C-Concentration of drug in g/100ml

BEERS'S LAW PLOT

Beer's law states that the fraction of the monochromatic radiant energy absorbed on passing through a solution is directly proportional to concentration of the absorbent.

$$Log_{10} I_0/I_t=K.C$$

where,

K-Proportionality constant

C-Concentration

I_0-I intensity of incident light

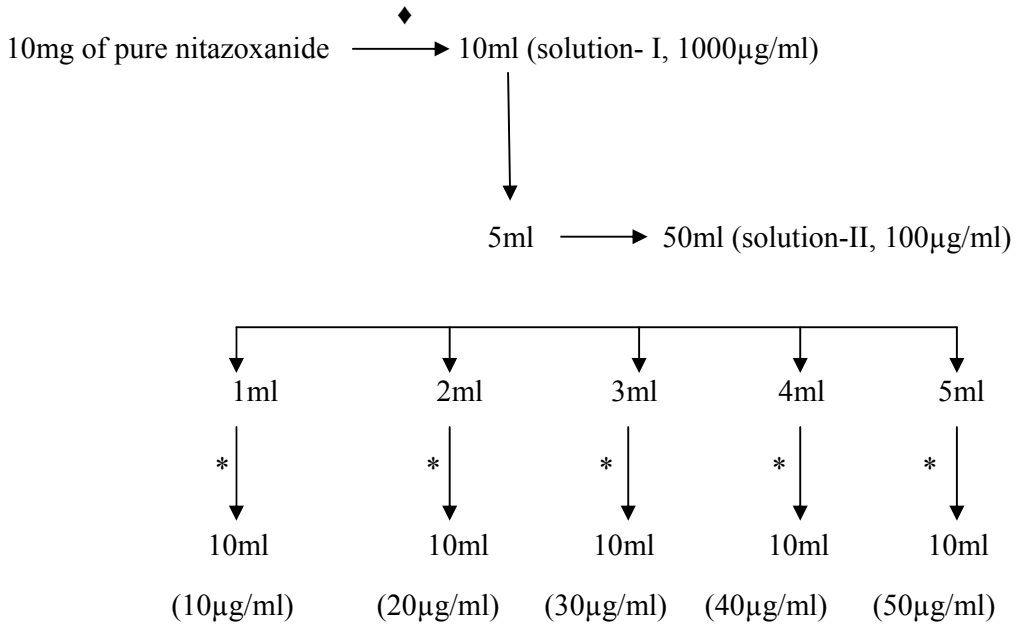
I_t-Intensity of transmitted light

Having fixed other parameter, Beer's law plot was constructed by measuring the absorbance of various concentration of drug solution against reagent blank.

5ml of stock solution-I was pipetted in 50ml volumetric flask and made upto 50ml with methanol. The concentration of solution was 100µg/ml (Stock solution-II). From stock solution II, various aliquots of 1ml, 2ml, 3ml, 4ml and 5ml were pipette out into a 10ml volumetric flask followed by addition of 1ml of 0.1% $NaNO_2$ and 1ml conc:HCl and kept aside for 5mts at room temperature. An aqueous solution of 1ml of 0.5% ammonium sulphamate and 0.5% phluroglucinol were added. The volume was made upto 10ml with methanol to produce concentrations in the range of 10-50µg/ml

respectively. Absorbance of each solution was observed at 450nm against reagent as blank. Readings were shown in table no: 14 and readings were shown graphically in graph no: 8

SCHEMATIC REPRESENTATION OF BEER'S LAW PLOT



♦200mg Zn powder + 2.5ml 5N HCl + 5ml methanol

*1ml conc: HCl + 1ml of 0.1% $NaNO_2$ + 1ml of 0.5% ammonium sulphamate + 1ml of 0.5 % phluroglucinol

DATA FOR BEER'S LAW PLOT

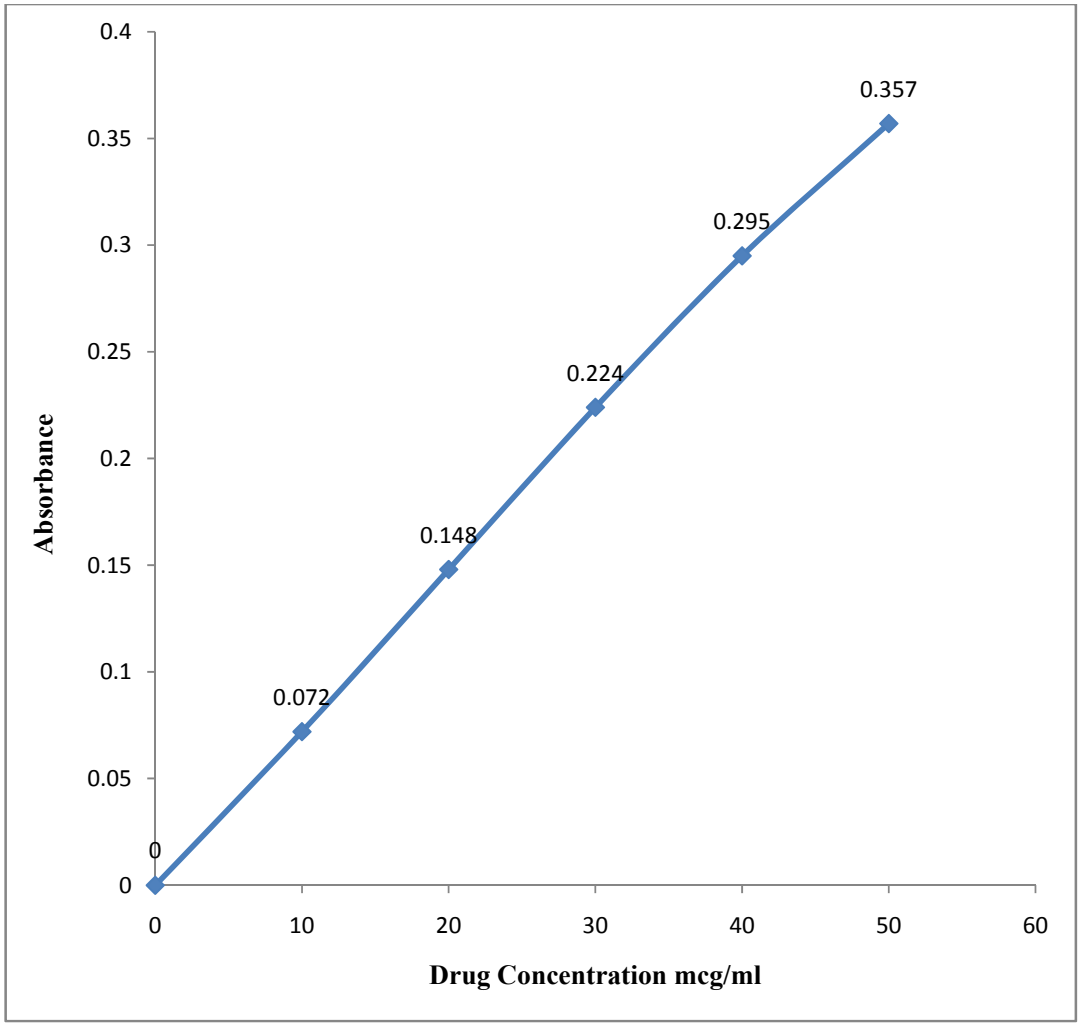
TABLE NO: 14

Drug concentration (µg/ml)	Absorbance
10	0.072
20	0.148
30	0.224
40	0.295
50	0.357

Beer's law was obeyed in the concentration range of 10-50µg/ml

Calibration curve for nitazoxanide using 4-hydroxy benzaldehyde

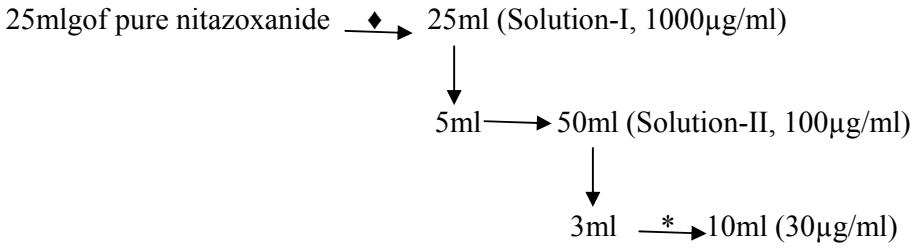
GRAPH NO:8



ASSAY OF TABLET

10 tablets of nitazoxanide were taken for analysis. The average weights of tablets were calculated and were powdered in a glass mortar. Tablet powder equivalent to 10mg was accurately weighed and dissolved in 5ml of methanol and treated with Zn powder (200mg) and 2.5ml 5N HCl with continuous stirring for 1hr at room temperature. It was filtered and the residue was washed with methanol and then the volume was made up to 10ml with methanol (Solution-I). 5ml of solution-I was pipette into 50ml volumetric flask and the volume was made upto 10ml with methanol (solution-II). From this 3ml was pipette out into 10ml volumetric flask followed by addition of 1ml of concentrated HCl, 1ml of 0.1% $NaNO_2$ and kept aside for 5mts. Then an aqueous solution of 1ml of 0.5% ammonium sulphamate and 1ml of 0.5% phluroglucinol were added. The volume was made up with methanol to produce final drug concentration of 30µg/ml. The absorbance of this solution was measured at 450nm against reagent blank. The same procedure was repeated 5 times. In similar manner standard absorbance was measured with pure drug in same final concentration that of assay method. The data for assay of tablet were recorded in table no: 15

STANDARD DILUTION



♦200mg Zn powder+2.5ml 5N HCl+5ml methanol

*1ml conc: HCl+1ml 0f 0.1% $NaNO_{2+}$ 1ml of 0.5% ammonium sulphamate+1ml of 0.5%phluroglucinol.

TEST DILUTION

Tablet powder equivalent to

25mg of pure nitazoxanide ♦→ 25ml (Solution-I, 1000µg/ml)

↓

5ml → 50ml (Solution-II, 100µg/ml)

↓

3ml *→ 10ml (30µg/ml)

♦200mg Zn powder+2.5ml 5N HCl+5ml methanol

*1ml conc:HCl+1ml 0f 0.1% $NaNO_{2+}$ 1ml of 0.5% ammonium sulphamate+1ml of 0.5%phluroglucinol.

CALCULATIONS

Using the absorbance of the standard and sample solution, the content of tablet was calculated as follows:-

The content of nitazoxanide present in each tablet of average weight =

$$\frac{\text{Test absorbance}}{\text{Std absorbance}} \times \frac{\text{Wt of std drug(mg)}}{25} \times \frac{5}{10} \times \frac{25}{\text{wt of tablet powder (mg)}} \times \text{Avg wt oftablet (mg)}$$

DATA FOR ASSAY OF TABLET

(Label claim: 500mg)

TABLE NO: 15

Sl no:	Brand name	Avgwt of tablet(mg)	Wt of std drug (mg)	Std absor bance	Wtof tab powder (mg)	Wt.of tablet powder	Content of drug in tab(mg)	Avg content (mg)
1	NIZONIDE -500mg	1261.96	10	0.214	25	0.211	497.70	498.097
2					24.8	0.209	496.96	
3					25.1	0.212	498.07	
4					25.1	0.213	500.42	
5					24.9	0.210	497.34	

INTERFERENCE STUDIES

The interference studies of additives used in the formation of tablet were done by distributing them individually in distilled water and set aside for 10mts before filtering. The filtrate was diluted and proceeded as per tablet assay and the absorbance was measured at 460nm against reagent blanks.

This procedure was repeated 5 times of each additive and the average value for each additive was given the following table: 16

DATA FOR THE INTERFERENCE STUDIES

TABLE NO: 16

	Name of the excipients	Absorbance at 460nm
1	Talc	**0.002**
2	Lactose	**0.001**
3	Starch	**0.003**
4	Magnesium sterate	**0.001**

The results show that the effect of interference studies in this spectroscopic method was found to be negligible.

RECOVERY STUDIES

In order to justify the reliability and suitability of the proposed method, recovery studies were carried out.

The recovery experiment was performed on nitazoxanide tablet. The powder equivalent to 25mg was accurately weighed and dissolved in 5ml methanol and was treated with 200mg Zn powder and 2.5ml 4N HCl with continuous stirring for 1hr at room temperature. It was filtered and washed with methanol. An aliquot of 5ml of standard solution (1mg/ml) of pure sample of nitazoxanide was added to the flask. It was shaken well and the volume was made upto 25ml with methanol and the procedure for the assay of nitazoxanidewas followed. The experiment was repeated 5 times. The results were recorded in table no: 17. The percentage recovery was calculated by using the formula:-

$$\% \text{ of Recovery} = \frac{\text{Avg content from recovery} - \text{Avg content from assay}}{\text{Amount of std drug added}} \times 100$$

DATA FOR THE RECOVERY STUDY OF NITAZOXANIDE TABLET

TABLE NO: 17

Sl no:	Brand name	Avgwt of tablet (mg)	Wt.ofstd drug (mg)	Std absorbance	Wt.of tablet powder (mg)	Pure drug added	Abs of recovered sample (mg)	%recovery
1	NIZONIDE -500mg	1261.96	25.7	0.302	63.3	5	0.298	104.93%
2					63	5	0.296	
3					64	5	0.300	
4					64.1	5	0.301	
5					63.6	5	0.299	

STANDARD DEVIATION

It is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimation of the standard deviation using the formula:-

$$\mathbf{SD} = \frac{\sqrt{\Sigma(X-x)2}}{N-1}$$

X-Observed value

x-mean/arithmetic average

N-Number of observations

The square root of standard deviation is called variance. The more accurate measure of precision is called Coefficient of variation/%RSD

%RSD= (SD/*x*) x100

PRECESION[57]

The precision of an analytical method is the degree of reproducibility among the individual test results when the procedure was applied repeatedly to multiple sampling of homogenous sample. The precision of an analytical method is usually expressed as standard deviation.

The precision study was done based on the data obtained from table no: 15 and the results were shown in table No: 18

TABLE NOS: 18

Sl no:	Name of tablet	Standard deviation (SD)	Coefficient of variation(%RSD)
1	NIZONIDE	0.9436	0.1891

ACCURACY[57]

Accuracy is the closeness of the test results obtained by the procedure to that true value. Accuracy of the proposed method was evaluated by comparing the average value obtained by the proposed method with that of reported method (standard method) and results were given table no: 19

TABLE NO: 19

Sl no:	Amount obtained by std method	Amount obtained by proposed
1	499.05mg	498.09mg

X METHODOLOGY – III

SPECTROPHOTOMETRIC DETERMINATION OF NITAZOXANIDE USING METHANOL AS SOLVENT[56]

Preparation of standard stock solution

10mg of nitazoxanide was accurately weighed and dissolved in 5ml methanol and was made up to 10ml with methanol. (1000µg/ml)

Absorption spectra

From that 1ml was pippeted out and made up to 10ml with methanol. Then pippeted out 2ml and again made up with 10ml.The final concentration was 4µg/ml.The absorbance was scanned between 300-400nm against reagent blank. Readings were plotted in table no: 20 and shown in graph no: 9

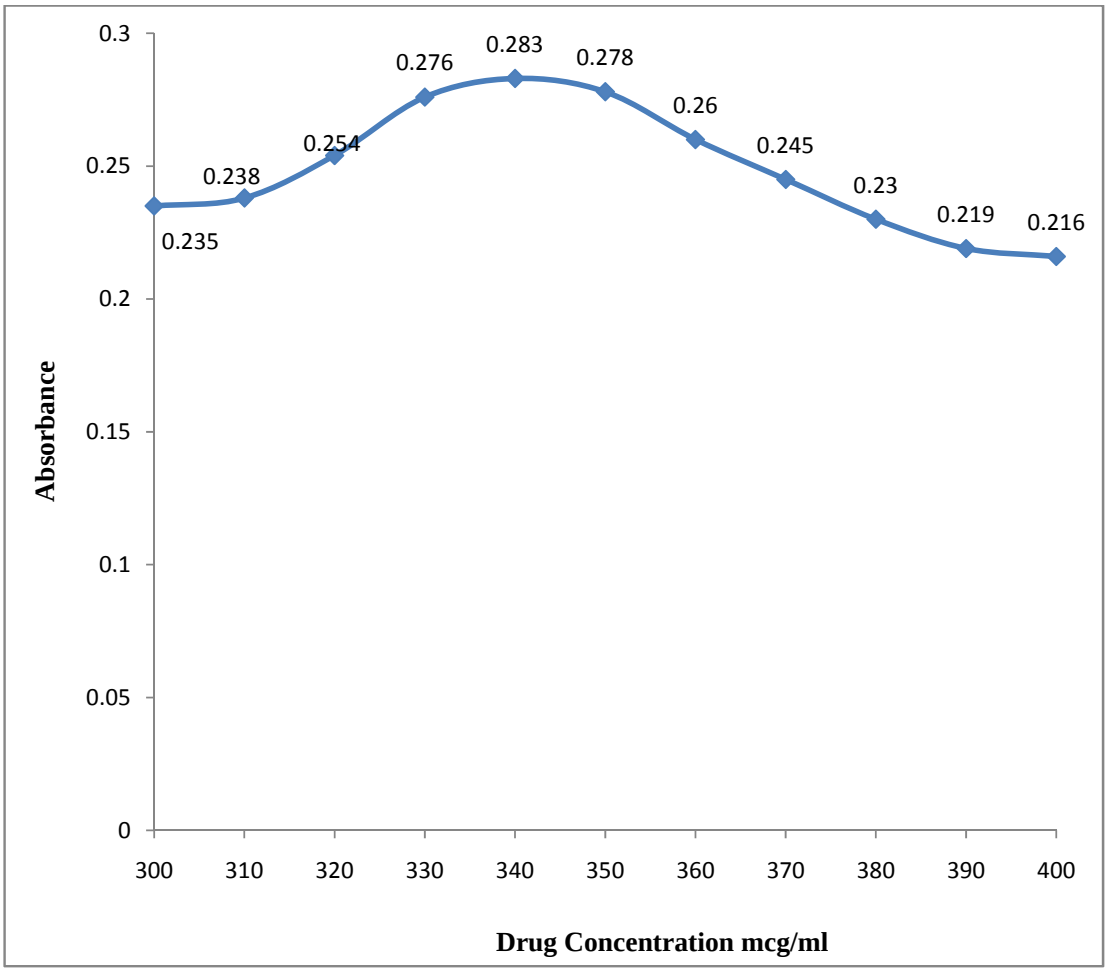
TABLE NO: 20

WAVELENGTH	ABSORBANCE
300	**0.235**
310	**0.238**
320	**0.254**
330	**0.276**
340*	**0.283**
350	**0.278**
360	**0.260**
370	**0.245**
380	**0.230**
390	**0.219**
400	**0.216**

The maximum absorbance was measured at 340nm

Calibration curve for nitazoxanide by using methanol as solvent

GRAPH NO: 9



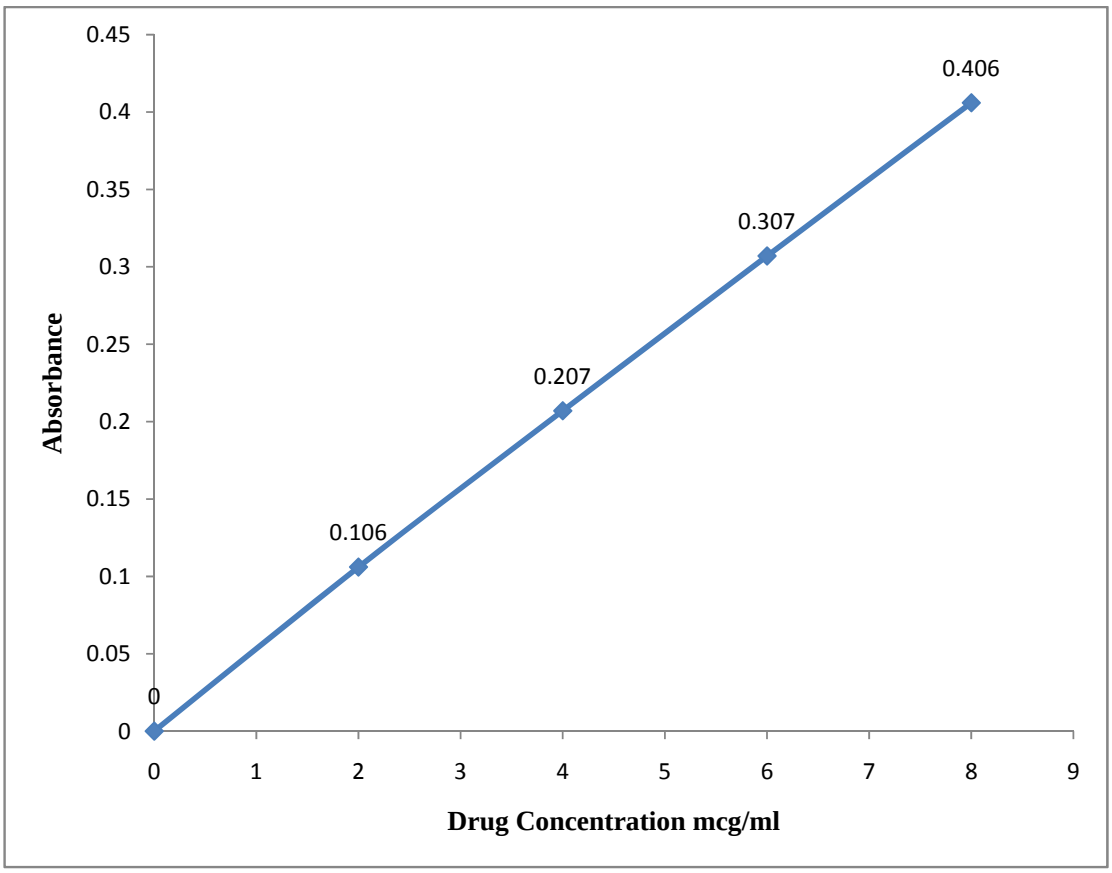
DATA FOR BEER'S PLOT

TABLE NO: 21

Drug concentration (µg/ml)	Absorbance
2	0.106
4	0.207
6	0.307
8	0.406

Calibration curve for nitazoxanide by using methanol as solvent

GRAPH NO:10



ASSAY OF TABLET

10 tablets of nitazoxanide were taken for analysis. The average weights of tablets were calculated and were powdered in a glass mortar. Tablet powder equivalent to 10mg was accurately weighed and dissolved in 5ml of methanol. It was filtered and the residue was washed with methanol and then the volume was made up to 10ml with methanol (Solution-I). 1ml of solution-I was pipetted into 10ml volumetric flask and the volume was made upto 10ml with methanol (solution-II). From this 2ml was pipette out into 10ml volumetric flask and made upto 10ml with methanol. From this 2ml of the solution was pippeted out and made upto 10ml with methanol to produce final drug concentration of 30µg/ml. The absorbance of this solution was measured at 340nm against reagent blank. The same procedure was repeated 5 times. In similar manner standard absorbance was measured with pure drug in same final concentration that of assay method. The data for assay of tablet were recorded in table no: 22

STANDARD DILUTION

25mg of pure nitazoxanide → 25ml (Solution-I, 1000µg/ml)

↓

1ml → 10ml (Solution-II, 100µg/ml)

↓

2ml → 10 ml (30µg/ml)

↓

2ml → 10ml (4µg/ml)

TEST DILUTION

Tablet powder equivalent to

25mg of pure nitazoxanide → 25ml (Solution-I, 1000µg/ml)

↓

1ml → 10ml (Solution-II, 100µg/ml)

↓

2ml → 10 ml (30µg/ml)

↓

2 ml → 10ml (4µg/ml)

CALCULATIONS

Using the absorbance of the standard and sample solution, the content of tablet was calculated as follows:-

The content of nitazoxanide present in each tablet of average weight =

$$\frac{\text{Test absorbance}}{\text{Std absorbance}} \times \frac{\text{Wt of std drug(mg)}}{25} \times \frac{5}{10} \times \frac{25}{\text{wt of tablet powder (mg)}} \times \text{Avg wt oftablet (mg)}$$

DATA FOR ASSAY OF TABLET

(Label claim: 500mg)

TABLE NO: 22

Sl no	Brand name	Avgwt of tablet(mg)	Wt of std drug (mg)	Std absorbance	Wtof tab powder (mg)	Wt.of tablet powder	Content of drug in tab(mg)	Avg content (mg)
1	NIZONIDE -500mg	1261.96	25.5	0.201	63.3	0.197	498.25	498.818
2					62.4	0.195	500.31	
3					63.5	0.198	499.20	
4					63	0.196	498.08	
5					63.3	0.197	498.25	

RECOVERY STUDIES

In order to justify the reliability and suitability of the proposed method, recovery studies were carried out.

The recovery experiment was performed on nitazoxanide tablet. The powder equivalent to 25mg was accurately weighed and dissolved in 5ml methanol and made up with 25ml methanol. An aliquot of 1ml of standard solution was pippeted out and made up with 10ml methanol and the volume was made upto 25ml with methanol and the procedure for the assay of nitazoxanide was followed. The experiment was repeated 5 times. The results were recorded in table no: 24. The percentage recovery was calculated by using the formula:-

$$\% \text{ of Recovery} = \frac{\text{Avg content from recovery} - \text{Avg content from assay}}{\text{Amount of std drug added}} \times 100$$

DATA FOR THE RECOVERY STUDY OF NITAZOXANIDE TABLET

TABLE NO: 24

Sl no:	Brand name	Avgwt of tablet (mg)	Wt of std drug (mg)	Std absorbance	Wt of tablet powder (mg)	Pure drug added	Abs of recovered sample	%recovery
1	NIZONIDE -500mg	1261.96	25.7	0.302	63.3	5	0.211	100.48%
2					63.8	5	0.213	
3					63	5	0.210	
4					63.3	5	0.212	
5					62.8	5	0.209	

STANDARD DEVIATION

It is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimation of the standard deviation using the formula:-

$$\mathbf{SD} = \frac{\sqrt{\Sigma(X-x)2}}{N-1}$$

X-Observed value

x-mean/arithmetic average

N-Number of observations

The square root of standard deviation is called variance. The more accurate measure of precision is called Coefficient of variation/%RSD

%RSD= (SD/*x*) x100

PRECESION[57]

The precision of an analytical method is the degree of reproducibility among the individual test results when the procedure was applied repeatedly to multiple sampling of homogenous sample.The precision of an analytical method is usually expressed as standard deviation.

The precision study was done based on the data obtained from table no: 22 and the results were shown in table No: 25

TABLE NO: 25

Sl no:	Name of tablet	Standard Deviation (SD)	Coefficient of variation(%RSD)
1	NIZONIDE	0.9484	0.1893

ACCURACY[57]

Accuracy is the closeness of the test results obtained by the procedure to that true value. Accuracy of the proposed method was evaluated by comparing the average value obtained by the proposed method with that of reported method (standard method) and results were given table no: 26

TABLE NO: 26

Sl no:	Amount obtained by std method	Amount obtained by proposed
1	499.05mg	498.818mg

XI METHODOLOGY IV

RP-HPLC DETERMINATION OF NITAZOXANIDE IN BULK AND TABLET FORMULATION.

Materials required

1. Balance-Electronic balance (Shimadzu)
2. HPLC Separation module (Shimadzu)
3. Detector-UV (254nm)
4. Chromatographic data software- Spin chrome CFR
5. Column- C_{18}

Reagents required

1. Acetonitrile (HPLC grade)
2. Dipottasium hydrogen phosphate

Reference standard used

Nitazoxanide (Lupin Pharmaceuticals, Pondicherry)

Tablet brand used

NIZONIDE-500mg

Chromatographic conditions

A chromatographic system (Shimadzu, Japan) consisting of a solvent delivery pump, a degasser, an injector, an RP column, UV detector. An ODS (octadecylsilane) packed C_{18} column was used for separation. The instrumental settings were at the flow rate of 1.2ml/mt. The injection volume was 20µl. The peak purity was checked with the UV detector (SPD 20A). Detection was performed at 254nm. Software used is Spin chrome.

Selection of wavelength

From the UV spectrum of the compound, the λ_{max} of nitazoxanide was found to be 254nm and that wavelength is suitable for detection. So the appreciable absorbance was found at 254nm.

Preparation of Mobile phase

The mobile phase consisted of dipottasium hydrogen phosphate buffer and acetonitrile in the ratio (70:30). The mobile phase was premixed and filtered through a nylon filter and degassed.

Preparation of buffer

Dipottasium hydrogen phosphate was prepared as per IP and P^H was adjusted to 6.8.

Preparation of Standard solution

Standard solution was prepared by dissolving 100mg of nitazoxanide in methanol and it was made up to 100ml with methanol (1000µg/ml)

Preparation of test solution

Tablet powder equivalent to100mg of pure nitazoxanide was accurately weighed and transferred into a volumetric flask, dissolved in small volumes of diluents and volume was made up with methanol.

Method development

A rapid HPLC method was devoloped and validated for the estimation of nitazoxanide. A C_{18} column with mobile phase containing mixureof buffer and acetonitrile was used. Mobile phase was pumped at the flow rate of 1.2ml/mt and the eluents were monitored at 254nm with 20µl loop injector. The selected chromatographic condition were found to effectively separate nitazoxanide. The method validated in the terms of no: of theoretical plates, tailing factor, linearity, correlation coefficient, limit of detection (LOD) limit of quantitation (LOQ) for nitazoxanide.

Limit of Quantitation (LOQ)[58, 59]

It is a characteristic of quantitative assays for low level of compounds in sample matrices, such as impurities in bulk drug and degradation products in finished pharmaceuticals. It is the lowest level of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The quantitation limits is expressed as concentration of analyte (e.g.: percentage, parts per billion) in sample.

Determination: For instrumental and non-instrumental methods, LOQ is determined by analysis of sample with known concentration of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision.

$$LOQ = 3.3 \times \frac{\text{Standard deviation}}{\text{Slope}}$$

Limit of Detection (LOD)[58, 59]

It is a characteristic of limit tests. It is the lowest amount of the analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The detection limit is expressed as concentration of analyte (e.g.: percentage, parts per billion).

Determination: It is determined by assaying a sufficient no: of aliquots of homogenous sample to be able to calculate statistically valid estimates of standard deviation or % RSD.

$$LOD = 10 \times \frac{\text{Standard deviation}}{\text{Slope}}$$

Linearity[58, 59]

It is the ability to elicit test results that are directly, or by a well defined mathematical transformation, proportional to concentration of analyte in samples which in a given range. If linearity is not attainable, a non-linear model may be used, however, the goal is to have a model, whether linear/non-linear that describes closely the concentration-response relationship.

System suitability[58, 59]

Tests are based on the concept that the equipment, electronics, analytical operation and samples constitute an integral system that can be evaluated as such.

Chromatographic parameters

TABLE NO: 27

	PARAMETERS	VALUES
1	Wavelength	254nm
2	Flow rate	1.2ml/mt
3	Column	C_{18}
4	Injection volume	20 µl

Preparation of Calibration curve

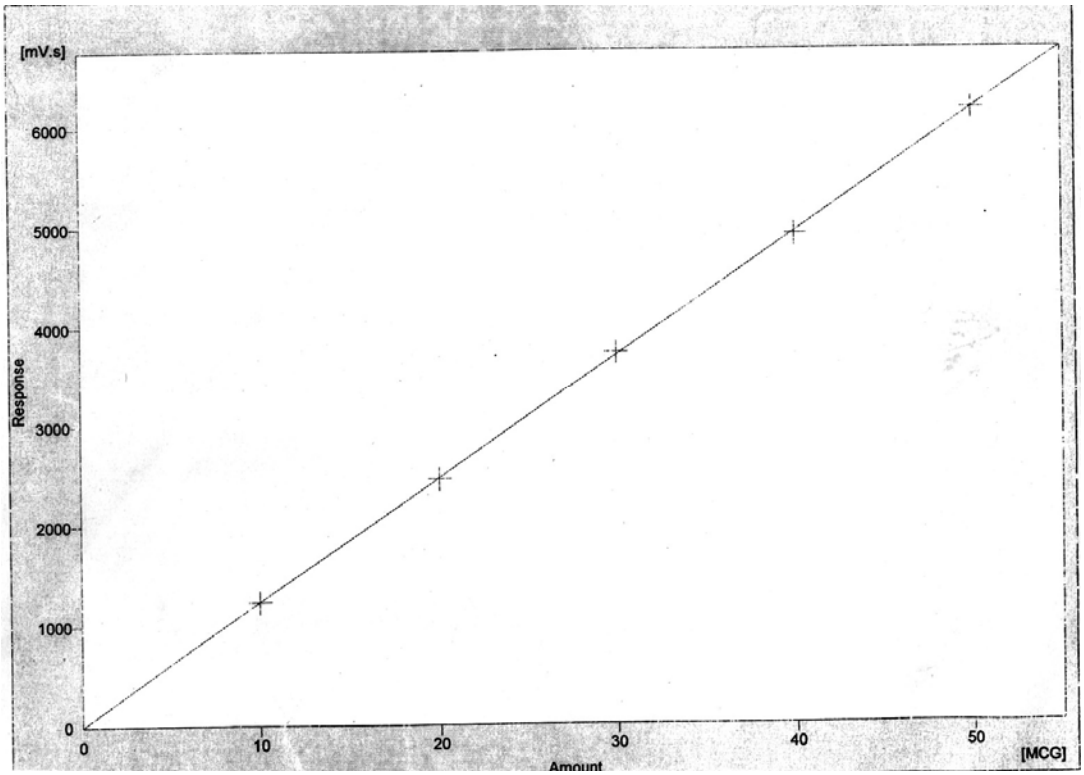
For the preparation of calibration curve, aliquots of 1ml, 2ml, 3ml, 4ml, 5ml were pippeted out and the volume was made up to 100ml with methanol to produce concentrations in the range of 10-50µg/ml. Each solution was injected and a chromatogram was recorded. The peaks were recorded. Calibration curve was constructed by plotting concentration vs. peak area and was recorded in table no: 28 and and the chromatogram was shown in chromatogram no: 1

TABLE NO: 28

Concentration	Peak area
10	1231.309
20	2456.657
30	3730.062
40	4906.374
50	6163.697

Calibration curve

CHROMATOGRAM NO: 1



Assay

10 tablets were accurately weighed and ground to fine powder. Powder equivalent to 50mg of nitazoxanide was accurately weighed, dissolved and made up with methanol. From this 5ml was pippeted and transferred to 100ml standard flask and the volume was made up with mobile phase. 20µl of the solution was injected and the chromatogram was recorded. In the similar manner chromatogram of pure drug as same concentration was also recorded in table no: 29 and was shown in chromatogram 2, 3, 4, 5, 6 and 7.

Calculation

$$\frac{\text{Test absorbance}}{\text{Std absorbance}} \times \frac{\text{Wt of std drug(mg)}}{50} \times \frac{5}{100} \times \frac{50}{\text{wt of tablet powder (mg)}} \times \text{Avg wt oftablet (mg)}$$

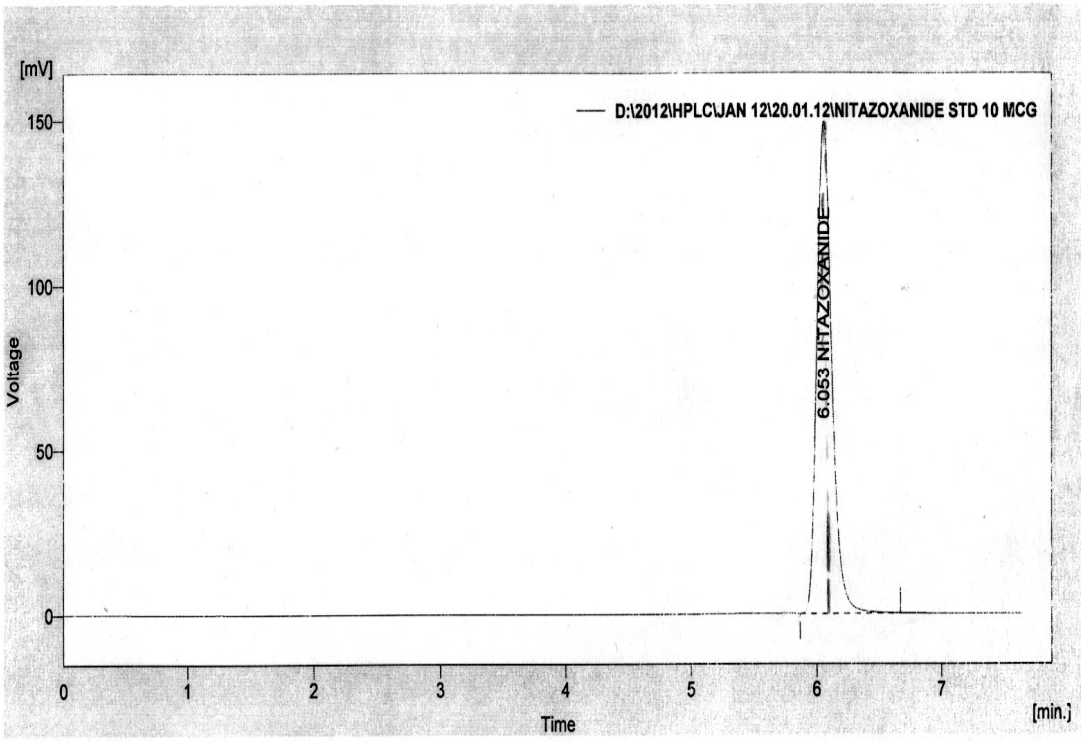
DATA FOR ASSAY OF TABLET

(Label claim: 500mg)

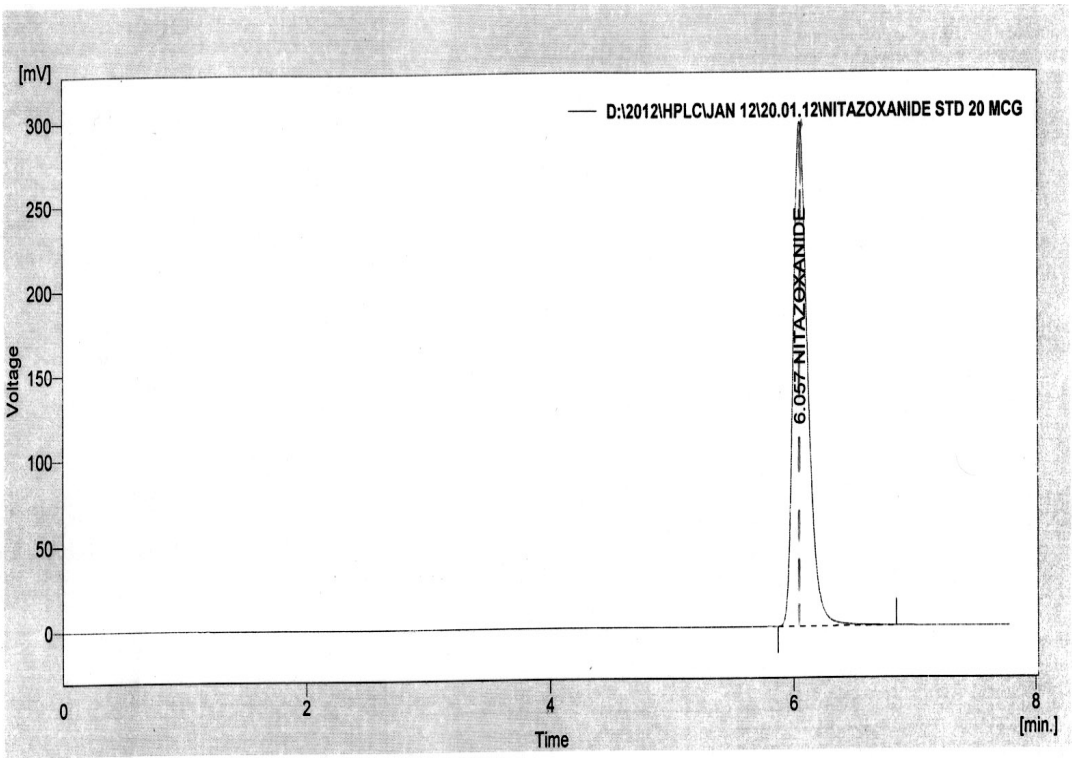
TABLE NO: 29

Sl no:	Brand name	Avgwt of tablet (mg)	Wt of std drug (mg)	Avg peak area for test	Wt of tab powder (mg)	Avg peak area for std	Avg content (mg)
1	NIZO NIDE - 500mg	1285.1	50.4	3220.388	68.2	6160.406	496.58

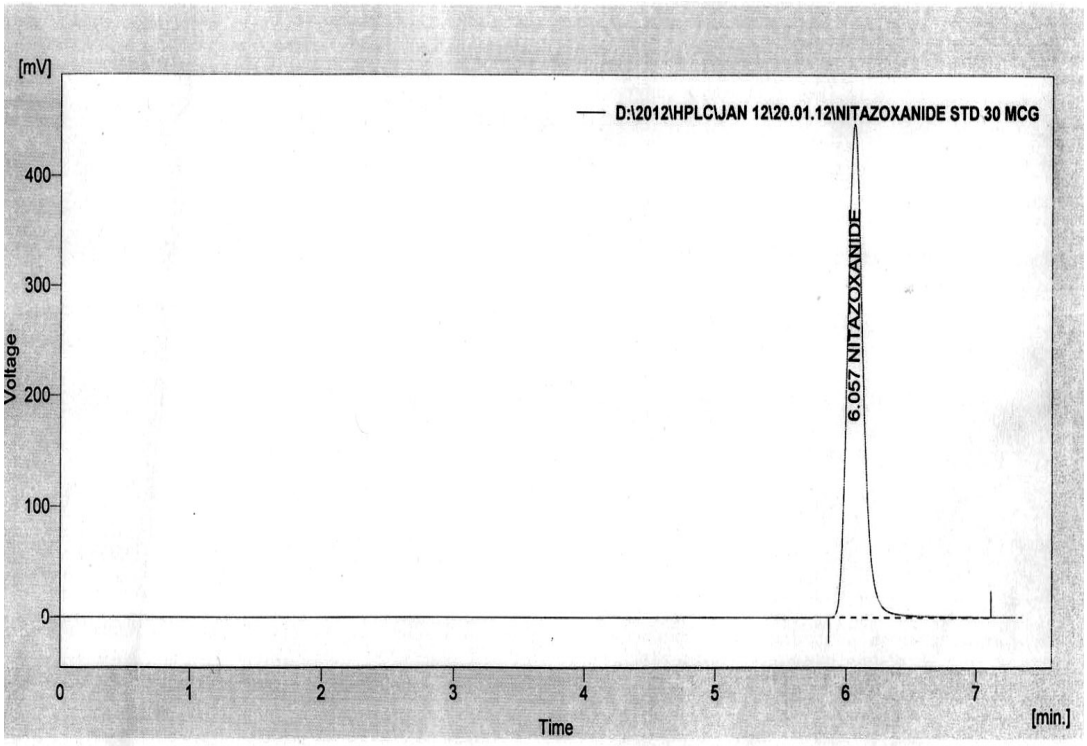
CHROMATOGRAM: 2



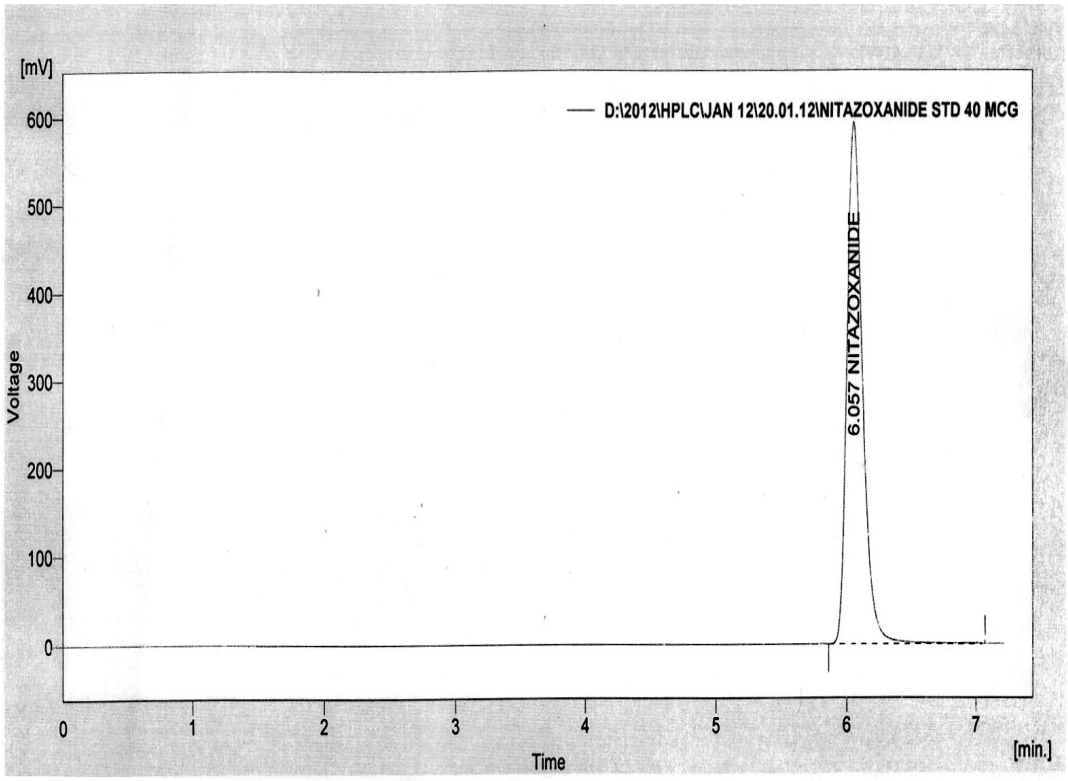
CHROMATOGRAM: 3



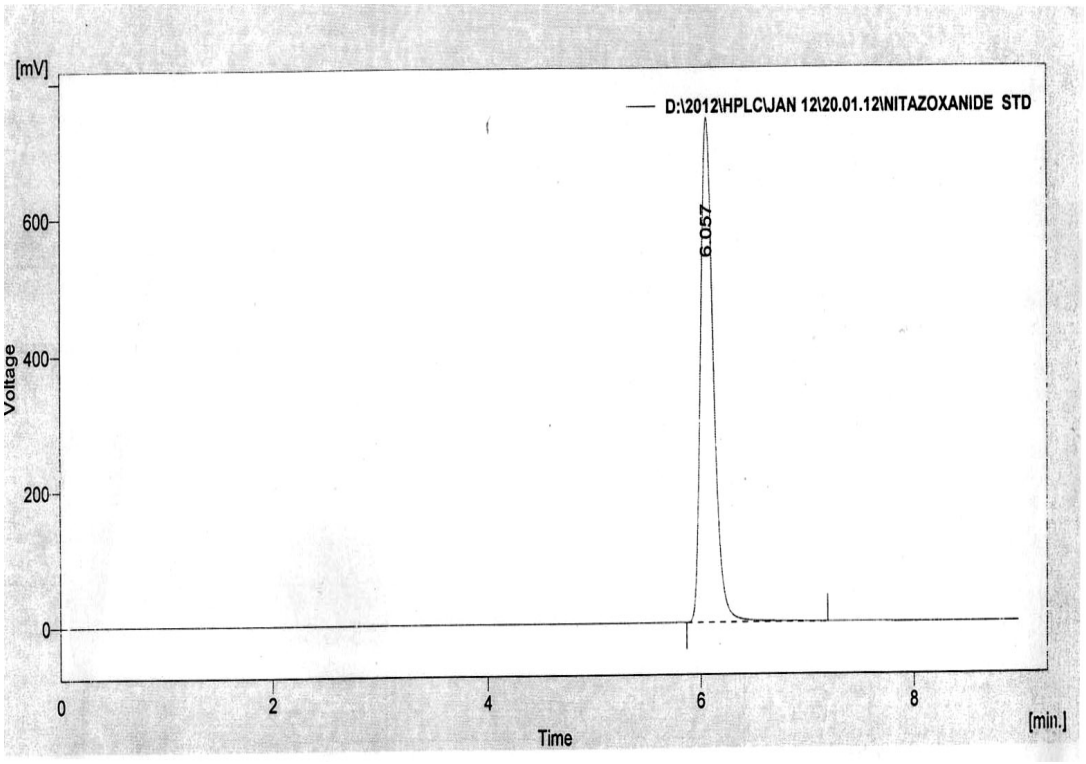
CHROMATOGRAM: 4



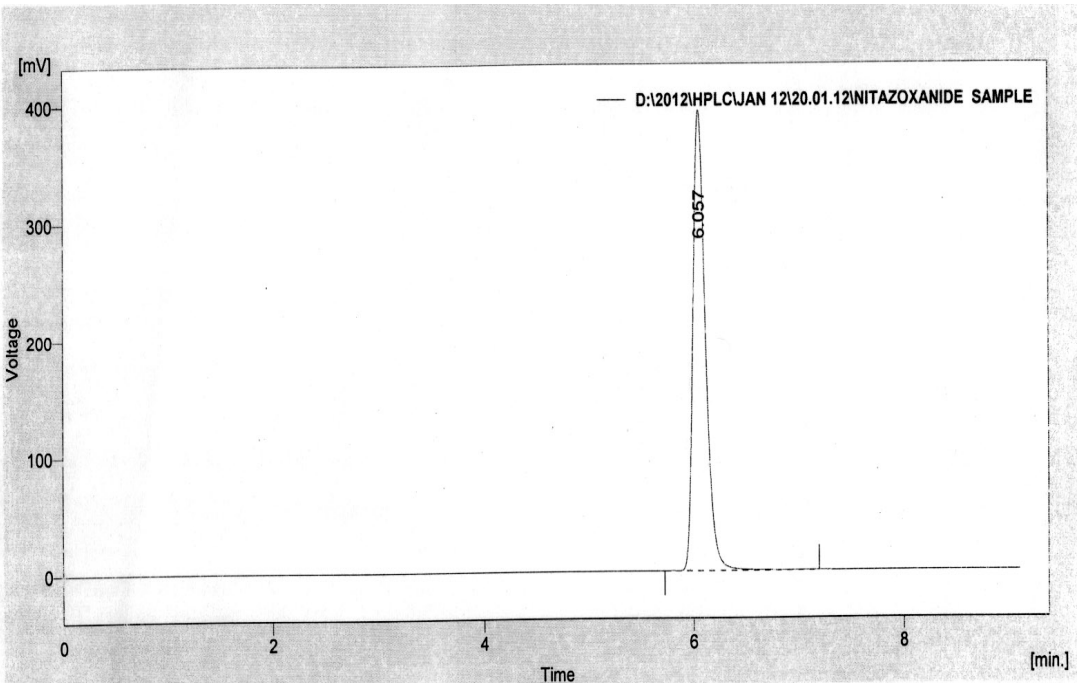
CHROMATOGRAM: 5



CHROMATOGRAM: 6



CHROMATOGRAM: 7



System suitability parameters

TABLE NO: 30

Sl no:	Parameters	Observed values	Acceptance criteria
1	No: of theoretical plates (N)	1917.22	Not less than 2000
2	Tailing factor (T)	1	≤ 2.0
3	Linearity	--	--
4	Correlation coefficient	0.9999507	0.9999
5	Limit of Detection	0.0747	--
6	Limit of Quantitation	0.0246	--
7	% RSD	0.185	--