HPLC Method Devlopment and Method Validation of Drug Rutin And Silymarin and Nanoformulation For Asthma Treatment

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I. INTRODUCTION

Bronchial asthma is a chronic inflammatory condition of the airways characterized by airway hyper responsiveness and fluctuating airflow restriction in response to irritating stimuli, which is frequently reversible spontaneously or with therapy.[1]In bronchial asthma, airway blockage is reversible, however in COPD (Chronic Obstructive Pulmonary Disorder), the obstruction is either not reversible or only partially reversible with bronchodilators. Common symptoms include wheezing, coughing, tightness in the chest, and shortness of breath.[1]Asthma is a complex disorder that is influenced by both genetic and environmental factors. Despite the fact that the etiology of airway inflammation is unknown, tremendous progress has been achieved in our fundamental understanding of asthma pathology.[2]Since the late 1980s, the number of persons suffering from asthma has risen, as has the fatality rate.

CAUSES OF ASTHMA

Allergies:

Allergies Animal proteins (mostly from cats and dogs), dust mites, cockroaches, and fungi are common indoor allergy sources.[3]The desire for energy-efficient housing may have increased exposure to these causes of asthma.[3] Allergic reactions caused by antibodies in the blood frequently result in the airway inflammation associated with asthma.

Genetics:

If your family has a history of asthma or allergic diseases, you have a higher risk of developing the disease.[4]

Allergies:

Having allergies increases the likelihood that you will experience asthma attacks.[5]

Environment :

Asthma can develop from exposure to airborne allergens and irritants. These substances include allergies, pollutants, vapors, and second- or third-hand smoke. Infants and early toddlers are especially vulnerable since their immune systems are still growing.[5,6]

Respiratory infections:

The developing lungs of young children can be harmed by some respiratory diseases, including respiratory syncytial virus (RSV).[6]

PATHOPHYSIOLOGY

Normally, an allergic reaction to an allergen causes an asthma attack, which is then mediated by immunoglobulin E. When allergens such as pollen or animal dander are present, IgE is generated [7]. Sensitization occurs following initial exposure, which results in the formation of IgE antibodies specific to allergens that stick to the surface of mast cells. When the allergen binds to the allergen-specific IgE antibodies on the surface of mast cells after repeated contact, inflammatory mediators such as leukotrienes, histamine, and prostaglandins are produced. These inflammatory mediators cause bronchospasm, which initiates an asthma episode. In an untreated attack, mast cells, T-helper cells, and eosinophils enter the airways[4]. In addition to heightened airway tone and hyper responsiveness, goblet cells' excessive mucus production

Diagnostic test for Asthma:

- **Spirometry**: Spirometry is a noninvasive test that involves inhaling deeply and pushing out air via a hose that is attached to a device known as a spirometer.
- **Eczema** (bumpy rashes caused by allergies)
- **Chest X-ray**

Nano-formulation

Nano-formulations typically range in size from 10 to 100 nm, with the drug dissolved, entrapped, encapsulated, or connected to the drug carrier. When developing nano-drugs, several important properties must be considered. The formulation must allow the medicine to reach the site of action from the point of delivery while also protecting it from the negative effects of environmental conditions such as pH, enzyme attack, and probable biochemical breakdown. Furthermore, the formulation should release the payload in its active state in and around the target region, allowing for smaller doses to produce a strong pharmacological impact [16]

Relevance and Motivation

Asthma is a chronic respiratory illness affecting over 260 million individuals globally. According to the World Health Organization (WHO), it is one of the most frequent Non- communicable diseases, posing a considerable public health burden. Developing successful formulations is crucial to improving the quality of life for millions of patients who use inhalers, tablets, and other antiasthmatic medications. Inhaled treatment is the most common technique to treat asthma. However, achieving consistent and accurate drug administration to the lungs remains problematic, particularly in chronic care. Advanced formulations can enhance drug absorption and targeting, leading to fewer side effects and better therapeutic outcomes.[17]The project provides an opportunity to employ High Performance Liquid Chromatography (HPLC) ideas, with a focus on ensuring formulations are strong. Nanoparticles, liposomal delivery methods, and dry powder inhalers (DPIs) can improve therapeutic efficacy while lowering systemic drug exposure. Current formulations may fail to address important issues such as patient compliance (due to the complexity of inhaler use), environmental concerns (CFC-free formulations),and drug resistance.[18]

Innovative formulations that target severe asthma or address steroid resistance have the potential to close significant treatment gaps. Regulatory incentives for novel drugs, notably orphan drug classification for severe asthma variants, help to spur the development of new antiasthmatic formulations. The growing market for respiratory drugs, fueled by higher diagnostic rates, has great promise. Improved formulations can gain significant market share and regulatory approval, allowing for a faster launch. An anti-asthmatic formulation project is incredibly significant.[19]

II. LITERATURE REVIEW

Aaditya Ganeshpurkar (2017): He studied the pharmacological potential of rutin and recognized flavonoids as a distinct class of medicinal chemicals due to their various therapeutic qualities. He discovered that rutin has been examined for a range of pharmacological properties, including anti-asthmatic action and other connected effects. Rutin's anti-asthmatic activity was investigated in conscious guinea pigs with ovalbumin sensitization. Airway resistance was measured throughout both the early and late phases of the response. Rutin dramatically reduced airway resistance, immediate-phase response, histamine, phospholipase A2, and eosinophil peroxidase. Neutrophils and eosinophils were not conscripted into the lung as much. Rutin, together with vitamins C and K, has been suggested for whooping cough.[13]

Peter F. Surai (2024): He discovered that inflammation is the immune system's response to damaging stimuli like infections and damaged cells. When inflammation resolution is delayed or hampered as a result of misregulation, the process progresses from acute to chronic inflammation, resulting in the development of a variety of chronic diseases. After conducting multiple in vitro and in vivo tests, he found that sylimarin and its primary ingredient, silibin, have remarkable anti-inflammatory properties.SM/SB inhibits TLR4/NF-κB-mediated signaling pathways and reduces the expression of pro-inflammatory mediators (TNF-α, IL-1β, IL-6, IL-12, IL-23, CCL4, CXCL10), which is the key reason for its anti-inflammatory effect.[12]

P.M.Deshmukhe, M.S Charade (2021) : They found that HPLC is the most extensively utilized separation technology for detecting, separating, and quantifying pharmaceuticals. Several chromatographic factors were studied to optimize the process, including sample pretreatment, mobile phase selection, column selection, and detector selection. The HPLC method can analyze the majority of pharmaceuticals in multicomponent dosage forms because to its advantages such as speed, specificity, accuracy, precision, and ease of automation. They also discovered that developing and validating HPLC methods is crucial in new drug discovery, development, and manufacture, as well as a wide range of other human and animal investigations. Analytical procedures must be validated during drug development and production to ensure that they are appropriate for their intended use. [8,9,10]

David Ong Cherk Yong et.al, (2019): has worked on Preparation, characterization and in-vitro efficacy of quercetin loaded liquid crystalline nanoparticles for the treatment of asthma and from that they found effectively encapsulate quercetin into liquid crystalline nanoparticles, demonstrating

their sustained release characteristics. Additionally, pro-inflammatory mediators such IL-1β, IL-6, and IL-8 were effectively reduced by quercetin LCN and sm-LCN; hence, the anti-inflammatory action of quercetin was further increased by its encapsulation in LCN. This indicates that quercetin LCN may be used as a novel medication delivery system for the management of asthma. This method will address the problems related to quercetin, including its short half-life, limited solubility, and poor bioavailability. The present study's findings support its translation into other related pulmonary inflammatory disorders, such as lung cancer and chronic obstructive pulmonary disease (COPD), which will assist give pulmonary clinics a new focus[14]

Deepak kaushik,SaritaGarg(2023) : they found that the mulberry is rich of flavonoids and active constituents like rutin and querchetin. The plant is recognized in traditional Chinese medicine for its antiphlogistic, diuretic, expectorant, and antidiabetic characteristics. The mulberry leaf is high in flavonoids, which have a variety of biological functions, including antioxidant potential. Quercetin, isoquercetin, rutin, isoquercitrin, quercitrin, luteolin, chlorogenic acid, and other flavonoids can be found in their leaves.To identify this elements they use hplc technique. The RP-HPLC method was designed and validated to identify these components. HPLC (Shimadzu Technologies LC series) with UV-visible detector at 259nm and C18 column (250mm 4.6mm, 5µm particle size) were employed to achieve chromatographic separation (Phenomenex Luna).[15]

AIM& OBJECTIVE

Aim:HPLC Method Development and Validation of Rutin-Sylimarin and Nano-formulation for Asthma Treatment.

Objective:

1.HPLC Method Development:

Develop a precise, accurate, and robust High-Performance Liquid Chromatography (HPLC) method for the simultaneous quantification of rutin and silymarin in their pure form and in combination.Optimize chromatographic parameters such as mobile phase composition, flow rate, detection wavelength, and column type to ensure reliable separation and quantification.Ensure the method is suitable for routine quality control analysis.

2.HPLC Method Validation:

Validate the developed HPLC method according to ICH (International Council for Harmonisation) guidelines, ensuring it meets parameters such as accuracy, precision, specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), and robustness.Demonstrate that the method is reliable for different matrices, including formulations and biological samples.

3.Nanoformulation Analysis:

Characterize the nanoformulations of rutin and silymarin developed for asthma treatment.Evaluate the entrapment efficiency, drug release profile, and stability of the nanoformulations using the validated HPLC method.

4.Application to Asthma Treatment:

Investigate the therapeutic potential of rutin and silymarinnanoformulations in managing asthma by analyzing key pharmacokinetic parameters and formulation efficacy.Ensure that the method enables monitoring of drug levels in biological samples, facilitating pharmacokinetic and pharmacodynamic studies.

5.Scientific Contribution:

Provide a validated analytical method that supports further research on rutin and silymarin as potential therapeutic agents for asthma.Contribute to the development of advanced drug delivery systems for enhanced therapeutic outcomes in asthma manageme

PLAN OF WORK

1. **Selection of Drugs**

Online Journals, chemical and analytical abstracts were studied to find out drugs for which reported methods or the reported methods were observing, many methods were got costly and time consuming. Market survey was carried to check the availability of these drugs.

The criteria for selection of drugs are explained in individual drug profile.

2. **Drug: Rutin-Silymarin**

3. **Selection of analytical techniques**:
   - HPLC method

4. **Plan of Study:**

   a) **Literature Review**

- Study existing methods for Rutin-Silymarinestimation in bulk and pharmaceutical formulations.
- Review stability-indicating methods and HPLC method development principles.
- Identify research gaps to design a novel and reliable method.

b) **Procurement of Materials**
- Obtain Rutin-Silymarin bulk drug and pharmaceutical formulations.
- Procure required chemicals, reagents, solvents (HPLC grade), and reference standards.
- Arrange necessary laboratory consumables (columns, filters, etc.).

c) **Optimization of Chromatographic Conditions**
- Select appropriate stationary phase (column) based on drug characteristics.
- Optimize the mobile phase composition (e.g., organic solvents, pH, buffers).
- Determine ideal flow rate, detection wavelength, injection volume, and run time.
- Conduct preliminary trials to achieve sharp, symmetrical peaks with good resolution.

d) **Forced Degradation Studies**

Expose Rutin-Silymarin to stress conditions as per ICH guidelines:
a. Acidic and basic hydrolysis.
b. Oxidative stress.
c. Thermal degradation.
d. Photolytic degradation.
- Collect and analyze degraded samples to identify and separate degradation products.

e) **Method Validation**

Validate the developed method according to ICH Q2(R1) guidelines for:
a. **Specificity:** Evaluate the method's ability to separate Rutin-Silymarin from impurities and degradation products.
b. **Linearity:** Determine the concentration range showing a linear response.
c. **Accuracy:** Perform recovery studies at multiple levels.
d. **Precision:** Assess repeatability and reproducibility.
e. **Sensitivity:** Calculate LOD and LOQ.
f. **Robustness:** Analyze results under small variations in experimental conditions.
g. **System Suitability:** Verify resolution, peak shape, and other system parameters.

DRUG PROFILE

RUTIN (Vitamin P or Rutoside)

General Information:

- **Chemical Formula**: C27H30O16
- **Molecular Weight**: 610.52 g/mol
- **Structure**: A glycoside combining quercetin (flavonoid) and rutinose (disaccharide).
- **Source**: Found in fruits (e.g., citrus), vegetables (e.g., asparagus), and medicinal plants such as buckwheat.

Pharmacological Properties:

1. **Category**:
   - Flavonoid; Antioxidant
   - Vasoprotective agent
   - Anti-inflammatory agent
2. **Mechanism of Action**:
   - Scavenges reactive oxygen species (ROS) to reduce oxidative stress.
   - Inhibits enzymes like cyclooxygenase and lipoxygenase involved in inflammation.
   - Strengthens capillaries, reducing capillary permeability and fragility.
3. **Therapeutic Uses**:
   - Treats capillary fragility and varicose veins.
   - Potential anti-asthmatic effects via anti-inflammatory and antioxidant mechanisms.
   - Neuroprotective and cardioprotective applications.
4. **Pharmacokinetics**:
   - **Absorption**: Limited oral bioavailability due to poor water solubility.
   - **Metabolism**: Primarily metabolized in the gut by microbiota into active flavonoid metabolites.
   - **Excretion**: Excreted via urine and bile.
5. **Toxicity**:
   - Generally considered safe; high doses may cause mild gastrointestinal discomfort.

Challenges in Formulation:

Poor solubility and low bioavailability necessitate advanced formulations like nanoformulations for enhanced therapeutic efficacy

SILYMARIN (Milk Thistle Extract)

General Information:

- **Chemical Formula**: Mixture of flavonolignans, primarily silybin, silydianin, and silychristin.
- **Molecular Weight**: ~482.44 g/mol (silybin A and B).
- **Source**: Extracted from seeds of *Silybummarianum* (Milk Thistle).

Pharmacological Properties:

1. **Category**:
   - Flavonolignan; Hepatoprotective agent
   - Antioxidant and anti-inflammatory agent
2. **Mechanism of Action**:
   - Scavenges ROS and enhances the cellular antioxidant defense by increasing glutathione levels.
   - Stabilizes cell membranes and prevents toxin penetration in hepatocytes.
   - Modulates inflammatory cytokines (e.g., TNF-α, IL-1β) and inhibits fibrosis.
3. **Therapeutic Uses**:
   - **Hepatoprotective**: Used in liver disorders (e.g., cirrhosis, hepatitis, drug-induced hepatotoxicity).
   - **Antioxidant/Anti-inflammatory**: Potential role in managing oxidative stress-related conditions such as asthma.
   - **Antifibrotic**: Reduces lung and liver fibrosis.
4. **Pharmacokinetics**:
   - **Absorption**: Poor oral bioavailability due to low water solubility.
   - **Metabolism**: Extensively metabolized in the liver via conjugation (sulfation/glucuronidation).
   - **Excretion**: Primarily excreted through bile and feces.
5. **Toxicity**:
   - Safe at therapeutic doses; high doses may cause mild gastrointestinal issues or allergic reactions.

Challenges in Formulation*:*

Limited oral bioavailability necessitates strategies like liposomes, nanoparticles, or complexation with phospholipids (e.g., phytosomes).

III. METHODOLOGY

Stage	Methodology	Description
1.Method Development	**Selection of HPLC Instrumentation and Conditions**	- Use of a suitable HPLC system with UV/VIS detector, typically at 254 nm or 280 nm for flavonoid detection.
	Selection of Chromatographic Column	- Column type (C18 reversed-phase column preferred). Commonly used column: 250 mm x 4.6 mm, 5 µm particle size.
	Mobile Phase Optimization	- Selection of mobile phase (water:acetonitrile:phosphoric acid or methanol, water, and acid mixture).
	Flow Rate and Temperature	- Optimized flow rate (typically 1.0 mL/min) and column temperature (25°C to 30°C) for resolution and analysis time.
	Wavelength Selection	- UV detection at 254 nm or 280 nm for rutin and silymarin quantification.
	Injection Volume	- Injection volume (typically 10-20 µL).
2.Method Optimization	**Mobile Phase Composition**	- Optimizing buffer and organic solvent ratio to achieve baseline separation and minimize interference.
	Gradient vs Isocratic Elution	- Decide between gradient (changing composition over time) or isocratic elution depending on the complexity of sample.
	Sample Preparation	- Extracting rutin and silymarin from plant or pharmaceutical formulations. Use of appropriate solvents (methanol, ethanol, etc.).
3. Method Validation	**Specificity**	- Ensure that the method can distinguish rutin and silymarin from other components in the

		sample matrix.
	Linearity	- Create calibration curves for both rutin and silymarin over a suitable concentration range (e.g., 0.5–100 µg/mL).
	Precision	- **Repeatability (intra-day precision):** Multiple injections of the same sample.
		- **Intermediate precision (inter-day precision):** Samples run on different days by different analysts.
	Accuracy	- Perform recovery studies by spiking known amounts of rutin and silymarin into the sample and comparing measured vs spiked values.
	Limit of Detection (LOD) and Limit of Quantification (LOQ)	- Establish LOD and LOQ based on signal-to-noise ratio (S/N > 3 for LOD, S/N > 10 for LOQ).
	Robustness	- Assess method robustness by varying parameters (e.g., flow rate, temperature, mobile phase composition) within ±10%.
	System Suitability Testing	- Evaluate peak symmetry, resolution, theoretical plates, and column efficiency to ensure the system is suitable for analysis.
4. Stability Studies	**Sample Stability**	- Evaluate the stability of both rutin and silymarin in the sample matrix under storage conditions (room temperature, 4°C, freeze).
	Solution Stability	- Test for any degradation of standards and sample solutions over time under various conditions (light, heat, pH).
5. Data Analysis	**Calibration Curve Analysis**	- Calculate correlation coefficient (r^2) for linearity and quantify the analytes in samples.
	Quantification	- Using the calibration curve, quantify rutin and silymarin in the sample.
6.Application in Real Samples	**Sample Analysis**	- Apply the developed and validated HPLC method to analyze rutin and silymarin in real samples (e.g., plant extracts, tablets).

FACILITIES AVAILABLE, INSTRUMENTS REQUIRED

Instruments Required:

UV Spectrophotometer, HPLC System, HPLC Column, pH meter, Electronic Balance,Sonicator, etc.

Instruments /equipment required:

Electronic Balance (Shimadzu AUW 220D), Magnetic Stirrer (Remi Equipment Pvt. Ltd.), Centrifuge (Remi Equipment Pvt. Ltd.), UV-Visible Spectrophotometer (Agilent Technology Carry 60 UV-vis UV-1800 Shimadzu, Japan), Micropipettes (Swastik Instrument Private Ltd. Mumbai), Mechanical Shaker (Remi Equipment Pvt. Ltd), Rotary Evaporator (Indosati Scientific lab equipment), Ultrasonicator (Quality Equipment and Instrument), etc.

Chemicals required:

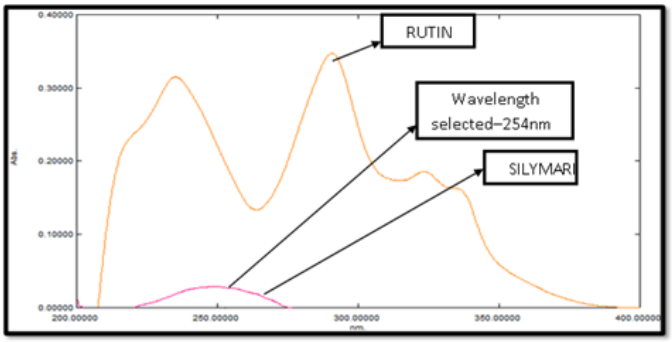
Mobile phase solvent:(Acetonitrile, Methanol, Phosphate buffers)

SELECTIONOFELUTION MODE

Reverse phase chromatography was chosen because of the recommended use for ionic and moderate to nonpolar compound. Reverse phase chromatography is not only simple,convenient but also better interms of efficiency, stability and reproducibility.C18 column was selected because

it is least polar compound to C4 and C8 column. C18 column allows elution of polar compound. In addition to this, UV detector is used, which allows easy detection of the compounds in UV transparent organic solvents. A 250mm × 4.6mm column packed with 5µm particles was preferred as a starting point for method development. Isocratic mode was chosen due to simplicityin application and robustness with respect to longer column stability. This configuration provides a large value for the number of theoretical plates giving better separation.

SELECTION OF WAVE LENGTH



Boththedrugsshowedgoodabsorbanceat254nmsoitwasselectedf orestimation of RUTIN and SILYMARIN.

SELECTION OF MOBILE PHASE

The mobile phase should be sufficiently transparent at the detection of wavelength. Methanol,acetonitrile and water(pH4 adjusted with or thophosphoricacid) are the solvent used for mobile phase provided optimum polarity for proper separation and resolution for Rutinfloxacin and Silymarinetasone Furoate.

The ratio selected is Methanol: Acetonitrile and water(pH4 adjusted with orthophosphoric acid) 55:30:15 v/v/v.

HPLC Trials for selection of mobile phase

TableNo.8.4HPLCTrialsforselectionofmobilephase:

Noof Trials	Mobile Phase	Observation
Trial1	Methanol: water(70:30v/v)	Onlyonepeakwasobserved
Trial2	Acetonitrile:water(50:50v/v)	Twopeakwasobserved Butnotsatisfactory
Trial3	Acetonitrile:water(65:35v/v)	Twopeakwasobserved Butnotsatisfactory
Trial4	Acetonitrile: Water(90:10 v/v)	Mergepeakswithtailing
Trial5	Methanol:Acetonitrile: Water (40:50:10 v/v)	Twopeakwasobserved Butno goodresolution
Trial6	Methanol:Acetonitrile: Water (50:40:10 v/v)	Twopeakwasobserved Butno goodresolution
Trial7	Methanol:Acetonitrile:Water (45:35:20 v/v)	TwoPeakwasObserved but tailing was 2.391 and 2.413 &RTwas 4.126and 6.916
Trial8	Methanol:Acetonitrile:Water(pH4 adjusted with OPA (45:35:20 v/v)	TwoPeakwasObserved but tailing was 2.369 and 2.437 &RT was4.178and 7.054
Trial9	Methanol:Acetonitrile:Water (55:30:15 v/v)	TwoPeakwasObserved but tailing was 2.364 and 2.491 &RTwas 3.846and 5.375
Trial10	Methanol:Acetonitrile:Water(pH4 adjusted with OPA (55:30:15 v/v)	Two Peak was Observed Tailingwas1.644and1.610 RTwas3.870and 5.377

Trial1

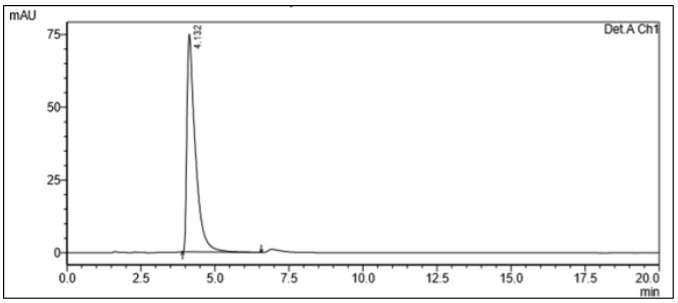


Fig.8.2Chromatogram of RUTIN and SILYMARIN in Methanol : Water (70:30 v/v)

Trial2

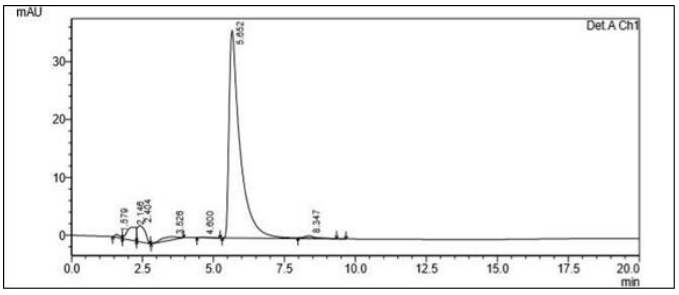


Fig 8.3 Chromatogram of RUTIN and SILYMARIN in Acetonitrile : Water(50:50 v/v)

Trial3

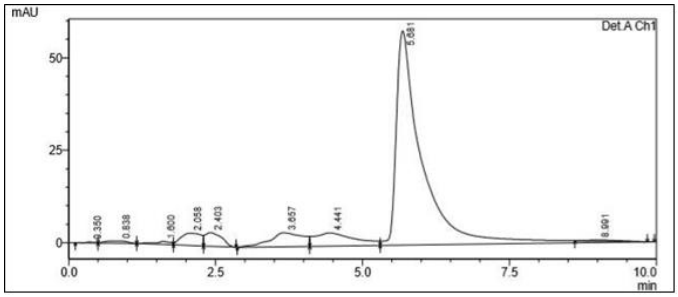


Fig8.4Chromatogram of RUTINand SILYMARIN inAcetonitrile :Water(65:35 v/v)

Trial4

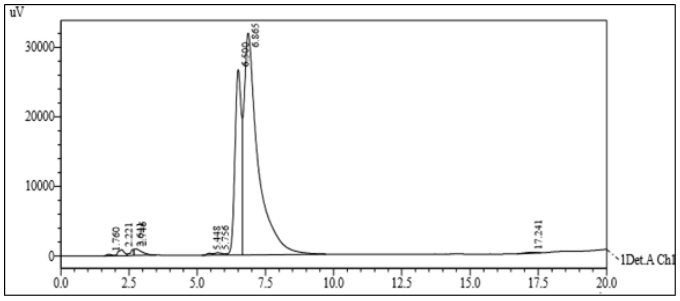


Fig8.5 Chromatogramof RUTIN and SILYMARIN in Acetonitrile :Water(90:10 v/v)

Trial5

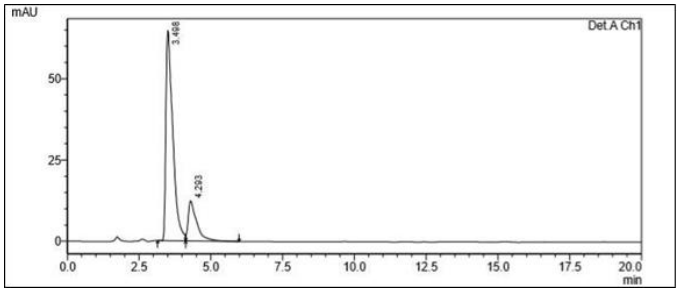


Fig8.6 Chromatogram of RUTIN and SILYMARIN in Methanol: Acetonitrile:Water (40:50:10 v/v)

Trial6

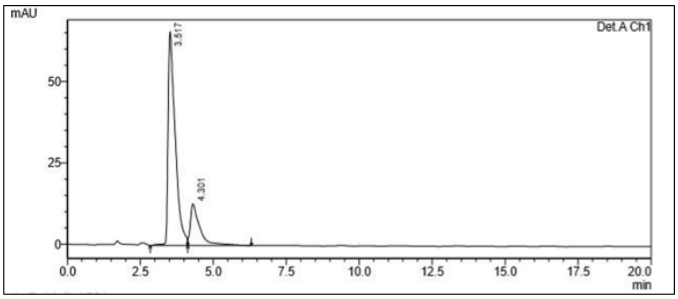


Fig8.7 Chromatogram of RUTIN and SILYMARIN in Methanol: Acetonitrile: Water (50:40:10 v/v)

Trial7

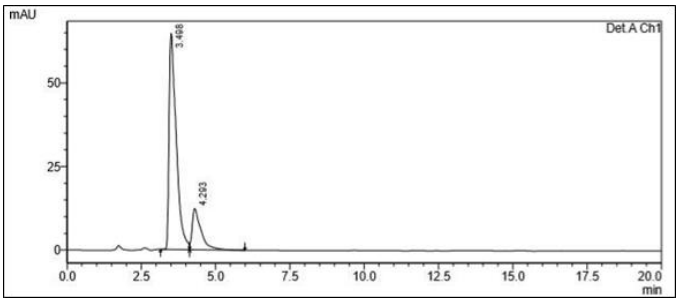


Fig 8.8 Chromatogram of RUTIN and SILYMARIN in Methanol: Acetonitrile: Water (45:35:20 v/v)

Trial8

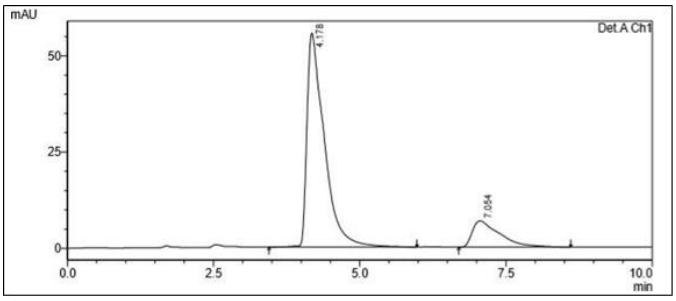


Fig 8.9 Chromatogram of RUTIN and SILYMARIN in Methanol: Acetonitrile: Water (adjust to pH 4 with OPA) (45:35:20 v/v)

Trial9

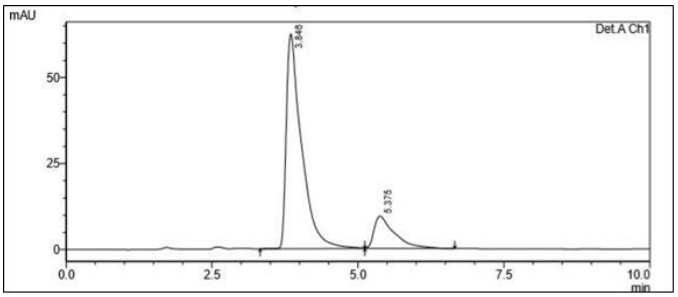


Fig 8.10 Chromatogram of RUTIN and SILYMARIN in Methanol: Acetonitrile: Water (55:30:15 v/v)

Trial10

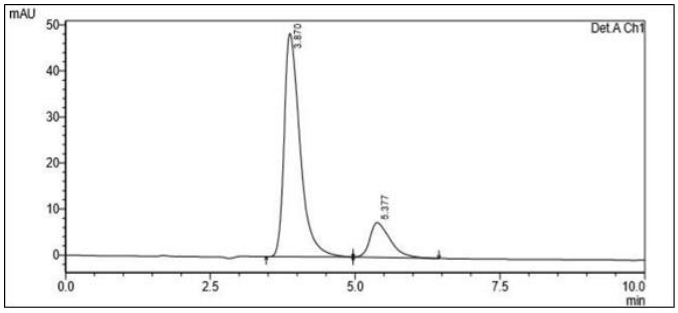


Fig 8.11 Chromatogram of RUTIN and SILYMARIN in Methanol: Acetonitrile: Water (adjust to pH 4 with OPA) (55:30:15 v/v)

Confirmation-1(RUTINFLOXACIN)

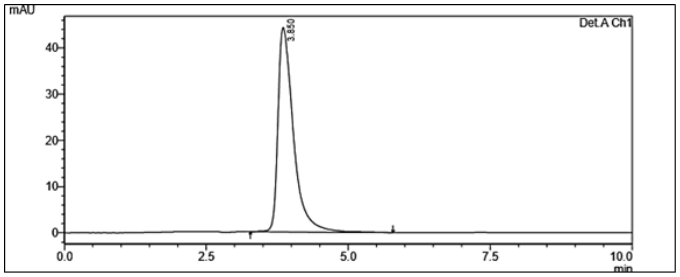


Fig 8.12 Chromatogram of Confirmation of RUTIN FLOXACIN in Methanol: Acetonitrile: Water (pH 4 adjusted with OPA (55:30:1

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