"DEVELOPMENT AND VALIDATION OF STABILITY INDICATING ASSAY METHODS FOR BETULINIC ACID."

A THESIS SUBMITTED TO

NIRMA UNIVERSITY

IN PARTIAL FULFILLMENT FOR THE AWARD OF THE DEGREE OF

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

BY

ASHISH D. PANDYA (12MPH302), B. PHARM.

UNDER THE GUIDANCE OF

Dr. PRITI J. MEHTA – GUIDE
Professor, Department of Pharmaceutical Analysis

Ms. DIPAL M. GANDHI - CO-GUIDE
Assistant Professor, Department of Pharmacognosy

NIRMA UNIVERSITY
INSTITUTE OF PHARMACY

Department of Pharmaceutical Analysis
Institute of Pharmacy
Nirma University
Ahmedabad-382481
Gujarat, India.

May 2014

CERTIFICATE

This is to certify that the dissertation work entitled "Development and validation of stability indicating assay method for Betulinic Acid." submitted by Mr. Ashish D. Pandya with Regn. No. (12MPH302) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by the candidate at the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University under our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

Guide:

Dr. Priti J. Mehta
M. Pharm., Ph. D.
Professor & Head
Department of Pharmaceutical Analysis,
Institute of Pharmacy,
Nirma University

Co-Guide:

Ms. Dipal M. Gandhi
M. Pharm.,
Assistant Professor,
Department of Pharmacognosy,
Institute of Pharmacy,
Nirma University

Forwarded Through:

Prof. Manjunath Ghate
M. Pharm., Ph.D.
Director
Institute of Pharmacy,
Nirma University

Date: 21st May 2014

DECLARATION

I hereby declare that the dissertation entitled "Development and validation of stability indicating assay method for Betulinic Acid", is based on the original work carried out by me under the guidance of Dr. Priti J. Mehta, Professor, HOD, Department of Pharmaceutical Analysis and Ms. Dipal M. Gandhi, Assistant Professor, Department of Pharmacognosy, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

Mr. ASHISH D. PANDYA (12MPH302)
Department of Pharmaceutical Analysis
Institute of Pharmacy
Nirma University
Sarkhej - Gandhinagar Highway
Ahmedabad-382481
Gujarat, India

Date: 21st May 2014

ACKNOWLEDGEMENTS

Words are tools of expressions, but they fail miserably when it comes thanks giving. I might not be able to do adequate justice in this task of acknowledging my indebtedness to all those who have directly as well as indirectly made it possible to complete the project. Nevertheless, I shall endeavour to discharge this right to obligation in this thesis. This work has become successful by the blessings of GOD & my family.

A single flower cannot make a garland or a single star cannot make the beautiful shiny sky at night, same way research work can never be outcome of single individual's talent or efforts. During my this journey from objective to goal, I have experienced shower of blessings, guidance and inspiration from my teachers, parents, friends and all my well wishers.

"Have infinite patience and success is yours" *this is what* ***Dr. Priti J.Mehta****, Ph.D., HOD, Department of pharmaceutical Analysis, Institute of Pharmacy, Nirma University has enshowered on me. From the beginning of the work she has been a source of inspiration, guidance and encouragement. This work might have been possible only because of her guidance and constant moral support. She has become a ray of light that show me right path and lead me to achieve my goal. I am taking this opportunity to express my deepest gratitude to my esteemed guide* ***Dr.Priti J.Mehta*** *for her encouragement and guidance through the course of study. I am grateful for her timely attention, co-operation and for being a constant source of inspiration.*

With reverence and gratitude, I would like to thank ***Ms. Dipal Gandhi****, Department of Pharmacognosy, Institute of Pharmacy, Nirma University for providing constant guidance throughout this course in this college. I am also thankful to* ***Dr. Manjunath Ghate,*** *Ph.D. , Director, Institute of Pharmacy, Nirma University for being a source of inspiration and providing facilities. I would like to thank,* ***Mr.Nrupesh Patel,*** *Institute of Pharmacy, Nirma University for their continuous support, guidance and encouragement. I would like to thank,* ***Dr. Charmy S. Kothari*** *, Institute of Pharmacy, Nirma University for their continuous support, and valuable suggestions.*

I am extremely grateful to all of my seniors including ***Harshal, Krishna, Nirzari*** *for their continuous support and valuable guidance for my research work. Though friendship is far from any formalities this account will be incomplete without mention of my beloved friends* ***Jwal, pratik , Maharshi , Dhruv, Ankit, Guarav, Harshit, Palak ,Dhara*** *with whom I shared my dreams and they have encouraged me to complete it. I thank all of them for their care, share and help to me in this work. It was great pleasure for me to work with my colleagues.*

I owe special thanks to lab assistants of Analysis lab ***Satejbhai, Shreyashbhai,*** *and* ***Bipinbhai*** *for their help during my my M.Pharm. I always remember to Library staff for their caring nature and providing all sorts of help in library whenever required. I cannot find words to express my love and gratitude towards family who have given me lots of love, strength, motivation and It was their love, care and blessings that have carried me to the destination. Without their consistent prayers, affectionate blessings, selfless care and everlasting confidence in me, I would have never come to the stage of writing this acknowledgement. At the occasion of this presentation I pay my reverence to this institute, Institute of Pharmacy, Nirma University. I am undeniably proud to be associated with this college. Lastly I am thankful to the God for always being with me and for giving me good family, teachers, friends and well-wishers. Thanking each and every well wisher individually would make the task very difficult and so I keep up to them to accept my warm regards for them.*

Ashish D. Pandya

LIST OF ABBREVIATIONS

ABBREVIATION	FULL FORM
°C	Degree centigrade
±	Plus or Minus
<	Less than
Λ	Lambda
%	Percentage
Mg	Microgram
µL	Microliter
Abs.	Absorbance
API	Active Pharmaceutical Ingredient
AR	Analytical Reagent
BA	Betulinic acid
CAS No.	Chemical Abstract Service Number
Cm	Centimeter
Conc.	Concentration
FDA	Food and Drug Administration
CDER	Central Drug Evaluation and Research
D.indica	*Dillenia indica*
Fig.	Figure
FT-IR	Fourier Transform Infrared spectrometry
G	Gram
H	Hour
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
ICH	International Conference on Harmonization
IUPAC	International Union of Pure and Applied Chemistry
L	Liter
LOQ	Limit of Quantification
LOD	Limit of Detection

Max	Maximum
Mg	Milligram
No.	Number
Ng	Nanogram
Pg.No.	Page number
R_f	Retention Factor
RP	Reverse Phase
R.S.D.	Relative Standard Deviation
R_t	Retention Time
S.D.	Standard Deviation
Sec.	Second
Sr.No.	Serial number

LIST OF FIGURES

LIST OF TABLES

INDEX

ABSTRACT

A simple, selective, precise stability indicating high performance thin layer chromatography and high performance liquid chromatography of isolated Betulinic acid was developed and validated.

The stability indicating HPTLC method was developed and validated for quantification of Betulinic acid in different forced degradation conditions. Optimized mobile phase was used Chloroform: Toluene: Ethanol (4: 4: 1). The method is based on high performance thin layer chromatography using Precoated Silica Gel 60 F_{254} plates with 250 µm thickness. Detection was carried out at 525nm wavelength. The R_f value of isolated Betulinic acid was 0.65 ± 0.02. The linear regression analysis data for the calibration plots showed good linear relationship with r= 0.998 with respect to peak area in the concentration range 200-900 ng per spot. The method was validated for precision. The limit of detection and quantification were 26.93 ng per spot and 81.61 ng per spot, respectively.

The stability indicating HPLC method was developed and validated for quantification of Betulinic acid in different forced degradation conditions. Optimized mobile phase was used Acetonitrile: Water (93:7). The method is based on high performance liquid chromatography using Purosphere® STAR, Reversed Phase (C-18, 250mm × 4.6mm, 5µm) column. Detection was carried out at 207nm wavelength. The R_t value of isolated Betulinic acid was 7.55 ± 0.1. The linear regression analysis data for the calibration plots showed good linear relationship with r= 0.999 with respect to peak area in the concentration range 20-90 µg per ml. The method was validated for precision. The limit of detection and quantification were 0.3631 µg per ml and 1.101 µg per ml, respectively.

Isolated Betulinic acid was subjected to acid and base hydrolysis, oxidation, photo degradation, and thermal degradation. The drug undergoes degradation under oxidation, acidic and basic conditions. This indicates that the drug is susceptible to acid hydrolysis, base hydrolysis and oxidation. As the methods could effectively quantify the isolated Betulinic acid in different forced degradation conditions, it can be employed as stability indicating method.

Chapter-1
Introduction

1 INTRODUCTION

1.1 INTRODUCTION TO STABILITY OF PHYTOCONSTITUENTS

For process and preservation of Phytoconstituents needs sound knowledge and information of their chemical and physical properties. A good quality of the Phytoconstituents can be maintained, if they are processed and preserved properly.

Different Phytoconstituents are prone to degrade in different environmental conditions like radiation due to direct sun light, atmospheric oxygen, atmospheric moisture and different acidic and alkaline conditions. Number of Phytoconstituents absorbs moisture during processing and preservation and become susceptible to the microbial growth that impairs the quality of Phytoconstituents due to facilitation of enzymatic reactions (digitalis leaves, wild cherry bark.). Radiation due to direct sun light also causes destruction of active Phytoconstituents (ergot, cod liver oil, digitalis.). Atmospheric oxygen is also destructive to several Phytoconstituents and hence, they are filled completely in well closed containers or the air in container is replaced by an inert gas like nitrogen (shark liver oil, papain.).

To identify the ideal conditions for processing and preservation of Phytoconstituents, it is essential to know about stability characteristics of Phytoconstituents. Different methods like UV-Visible spectroscopy, High Performance Liquid Chromatography (HPLC), High Performance Thin Layer Chromatography (HPTLC) are available to study stability of Phytoconstituents in different degradation conditions and to quantify standard Phytoconstituents.

(A) **(B)**

(C) **(D)**

Figure 1.1: (A) *D. indica* tree; (B) *D. indica* flower; (C) *D. indica* fruit; (D)*D. indica* bark

1.2 INTRODUCTION TO *DILLENIA INDICA* (*D.indica*) BARK

1.2.1 Taxonomical classification of D. *indica* [12, 13]

The plant is classified as follows:

Kingdom : Plantae

Subkingdom : Tracheobionta

Superdivision : Spermatophyta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Dilleniidae

Order : Dilleniales

Family : Dilleniaceae

Genus : Dillenia

Species : *indica* Linnaeusor*speciosa*Thunberg

1.2.2 Vernacular names of *D. indica*

In addition to scientific names, *D. indica* and *D. pentagyna*also have multiple common (local) names shown in Table 1.1 [6-11].

Table 1.1: Vernacular names of *D. indica*

Language	***D. indica***
English	Elephant apple
Sanskrit	Bhavya, Ruvya
Hindi	Chalta, Girnar
Gujarati & Marathi	Karmbel, Mota Karmal, Mota Karambal
Bengali	Chalta, Hargesa
Nepali	Ramphal, Panchphal, Panchkule
Telugu	Peddakalinga
Tamil	Akku, Ugakkay, Uva, Uvav, Uvatteku

1.2.3 Pharmacognostical description of *D. indica*

The *D. indica*L. (1753) is an evergreen or semi-evergreen tree with a roundish top, tall up to about 25 m with a reddish brown smooth bark.

It has intense green coloured leaves, glossy on the upper side, alternate, usually grouped at the apex of the branches, of elliptic-oblong or obovate-oblong shape, 15-40 cm long and 7-14 cm broad, with prominent parallel lateral nervations and indented margins; the 2-6 cm long petioles are grooved and pubescent at the base.

The flowers are solitary, terminal, hanging on an about 8 cm long peduncle, great up to about 20 cm of diameter, with five roundish sepals, concave, thick and fleshy, 4-6 cm long, of a pale green colour.

The fruit is an aggregate fruit with a diameter of 5 to 15 cm which comes from the enlargement of more ovaries, 15-20, with persistent fleshy sepals, indehiscent (it does not open when ripe), with 5 or more, reniform, of brown colour, per each ovary. It easily reproduces by seed, which germinates in about one month at 20-25 °C, and by cutting; when by seed, the first fructification takes place after 8-10 years.

The leaves, the bark and the fruits are utilized in the traditional medicine especially as anti-inflammatory; the juice of the fruit is utilized for preparing cough syrups and, blended with water and sugar, for reducing the fever, the bark for poultices for the arthritis [14].

1.2.4 Occurrence and distribution of *D. indica*

D. indica is distributed to China (Yunnan), India, Indonesia, Malaysia, Sri Lanka, Thailand, Bhutan, Laos, Myanmar, Philippines and Vietnam, where it grows in the humid evergreen forests mainly along the banks of the rivers. In India, it is distributed in sub Himalayan tract, Assam, North Bengal, Bihar, Orissa, Madhya Pradesh and Gujarat[6,7]. The genus is honouredto the German botanist Johann Jacob Dillen (Latinized in Dillenius), (1687-1747); the Latin name of the species “indica” = of India, refers to one of its origin places.

1.2.5 Chemical constituents of D. *indica*

Stem bark of *D. indica* containsbetulin, β-sitosterol, 10% tannin, dillenetin, betunaldehyde, betulinic acid (BA), flavonoids like rhamnetin, dihydro-isorhamnetin, lupeol, myricetin, naringenin, quercetin derivatives and kaempferolglucoside[7,10].

Woods of *D. indica* contains BA, lupeol, β-sitosterol.

Leaves of *D.indica* found to contain flavonoids, triterpenoids, steroids, tannins, cycloartenone, n-hentriacontanol, sitosterol, betulin; chloroform extract contains BA[15]. Methanolic extract of leaves after fractionation with n-hexane and chloroform also yielded compounds like BA, β-sitosterol, stigmasterol as well as dillenetin[16].

Fruit of D. indica also contain BA, β-sitosterol and polysaccharide like anarabinogalactan[17,18].

1.2.6 Therapeutic importance of D. *indica*

These plants are found to have very good therapeutic values in various different diseases. Different parts are used traditionally and pharmacologically to cure ailments and diseases.

Traditionally, whole plant of *D. indica* usedin case of fever, as an aphrodisiac and also promotes virility; decoction of it can be used as an universal antidote[6,8-10].

Stem-bark of *D.indica* serve ascomponent of medicine for sores caused by mercury poisoning, carbuncle and as a prophylactic in the cholera season. Stem extract applied on and around the wound caused by spider bite helps to remove the poison.

Leaves of *D. indica* isused as an astringent. Different leaf preparations are used for treatments; like paste is applied on bone fracture, poultice is used in bleeding piles, decoction is used in skin disease and body pain; powder is given in treatment of breast cancer[19,20].

The fruits of *D.indica* are said to be relished by elephant and hence named as ‘Elephant Apple’. The green fruit (unripe) is acidic, sour, bitter, pungent, astringent, removes bile, phlegm and flatulence, cardiotonic, but the ripe fruit is sweet, sour, appetizing, tasty; removes ‘vata’ and ‘kapha’; dispels fatigue; stops abdominal pains, laxative, beneficial in colic associated with mucous[7,9,17].

1.3 INTRODUCTION TO BETULINIC ACID [21-29]

Drug Name: Betulinic Acid

Synonyms:

Betulic acid

Lup-20(29)-en-28-oic acid, 3beta -hydroxy

Mairin

Molecular Formula: $C_{30}H_{48}O_3$ [22]

Molecular Weight: 456.70 [22]

Chemical Structure:

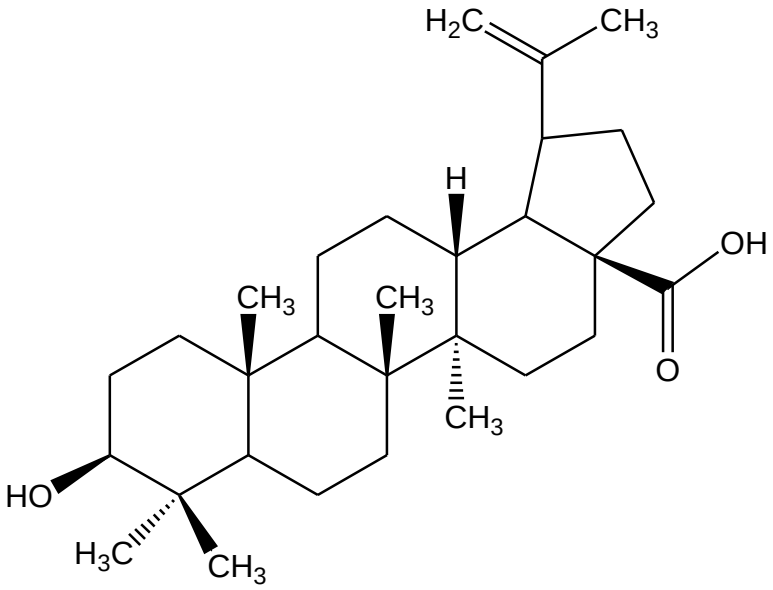


Figure 1.2: Chemical Structure of BA

IUPAC Name: (3β)-3-Hydroxylup-20(29)-en-28-oic acid [22]

CAS No.: 472-15-1 [22]

Category: [21]

Analgesic, Non-Narcotic, Anti-HIV agent, Anti-infective agent, Anti-inflammatory agent Anti-retroviral agent, Anti-inflammatory agent, Antimalarial, Antineoplastic agent, Antiparasitic agent, Antiprotozoal agent, Antirheumatic agent, Antiviral agent, Hormone substitute, and Hormone antagonist, Prostaglandin antagonist

Official Status:[22]

Official in Quality Standards of Indian Medicinal Plant; Vol. 7; ICMR 2008; p.g.no.: 78-85 and in The Merck Index, An encyclopaedia of chemicals, drugs and biological; Merck research laboratories; USA; 14th edition; 1192.

Physicochemical Properties[21-29]

- **Appearance:** White crystalline solids
- **Solubility:**
  Higher – Pyridine, Acetic acid
  Limited – Methanol, Ethanol, Chloroform, Ether
  Low – Water, Petroleum ether, Dimethyl formamide, Dimethyl sulfoxide, Benzene
- **Melting Point:** 290-293 °C
- **Partition Co-efficient(**LogP**):**5.34
- **Dissociation Constant(**pKa):4.75
- **Refractivity:** 132.63
- **Polarizability:** 54.8
- **Optical Rotation :**
  Etanol $[\alpha]_D$ **:** + 7°
  Pyridine $[\alpha]_D$**:** + 7.5°
  Chloroform $[\alpha]_D$**:** + 10.2°
  Methanol $[\alpha]_D$**:** + 10°

- **Storage Condition:** Store in cool, well-ventilated area. Keep away from direct sunlight. Keep container tightly sealed until ready for use. Recommended storage temperature is at +4°C.

Pharmacological Actions

- **Mechanism of Action:** BA selectively induces apoptosis in tumor cells by directly activating the mitochondrial pathway of apoptosis through p53 and CD95 independent mechanism.

- **Pharmacokinetics:** Peak serum concentrations of BA are observed at 0.146 and 0.228 hr for 250 and 500 mg/kg doses, respectively. At the 250 and 500 mg/kg doses, BA exhibit distribution volumes of 106 and 108 L/kg, respectively, and half-lives of 11.5 and 11.8 hr, respectively. The distribution of BA, administered at 500 mg/kg, is found to vary considerably among the various tissues over the course of 24 hr. High concentrations of BA are noted in fat tissues peaking after 24 hr at 2,260 ± 850 mg/g. Other high peak concentrations of BAare found in the bladder (3,523 ± 744 mg/g,8 hr), lymph node (4,218 ± 2,809 mg/g, 4 hr), mammary gland (1,184 ± 904 mg/g, 24 hr),ovary (3,055 ± 1,421 mg/g, 4 hr), spleen (1,287 ± 162 mg/g, 24 hr), and uterus (908 ± 165 mg/g, 24 hr).

- **Toxicological Information:** [29]
  Inhalation: There may be irritation of the throat with a feeling of tightness in thechest.
  Ingestion: There may beirritation of the throat.
  Skin: There may be mild irritation at the site of contact.
  Eyes: There may beirritation and redness.
  Delayed / Immediate Effects: No known symptoms.

- **Stability:** [29]

  Solids: Provided storage at 4 °C and the vial is kept tightly sealed, the product can be stored for up to 6 months from date of receipt.
  Solutions: Stock solutions, once prepared, are required to store in tightly sealed vials at -20°C and used within 1 month.

1.4 INTRODUCTION TO HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) [30, 31]

Planar chromatography (PC) is a multistage distribution process. It is a form of liquid chromatography in which the stationary phase is supported on a planar surface than column. Thin layer chromatography (TLC), also known as planar chromatography (PC), is one of the oldest methods in analytical chemistry still in use. In TLC, the different components of the sample are separated by their interaction with the stationary phase (bonded to the glass, aluminum, or plastic support) and the liquid mobile phase that moves along the stationary phase.

HPTLC has developed to the extent that separation and quantification can provide results that are comparable with other analytical methods such as High Performance Layer Chromatography (HPLC). HPTLC technique is most suited technique for content uniformity test and impurity profiling of the drugs as per compendial specification.

HPTLC uses the same type of silica gel 60 layers as TLC, with a thickness of 0.20- 0.25 mm. however the particle size is much smaller, typically ranging from 4-8 μm, with optimum 5-6 μm.

Table: 1.2 Differences between HPTLC and TLC

Parameters	HPTLC	TLC
Layer of Sorbent	100µm	250µm
Efficiency	High due to smaller particle size generated	Less
Separation	3 - 5 cm	10 - 15 cm
Analysis Time	Shorter migration distance and the analysis time is greatly reduced	Slower
Solid Support	Wide choice of stationary phases like silica gel for normal phase and C8 , C18 for reversed phase modes	Silica gel , Alumina & Kiesulguhr
Development Chamber	New type that require less amount of mobile phase	More amount
Sample spotting	Auto sampler	Manual Spotting
Scanning	UV-Visible and fluorescence scanner scans the entire chromatogram qualitatively and densitometer gives quantitative estimation	Not possible

Mechanism of HPTLC separation

- Adsorption
- Partition
- Ion-exchange

Features of HPTLC

- Simultaneous processing of sample and standard - better analytical precision and accuracy
- Simultaneously several samples of even divergent nature and composition can be studied
- Less need for internal standard
- Several analysts work simultaneously
- Lower analysis time and less cost per analysis
- Low maintenance cost
- Simple sample preparation- handle samples of divergent nature
- No prior treatment for solvents like filtration and degassing
- Low mobile phase consumption per sample
- No interference from previous analysis- fresh stationary and mobile phases for each analysis-no contamination
- Visual detection possible- open system
- Non UV absorbing compounds detected by post- chromatographic derivatization
- Possibility of multiple evaluation of the plate with different parameters because all fractions of the sample are stored on the plates.

Steps involved in HPTLC

1) **Selection of chromatographic layer**

- Precoated plates with different support materials and different sorbents are available.
- 80 % of analysis is done on silica gel GF.

2) **Sample and standard preparation**

3) **Chromatographic plate pre-washing**

- Plates exposed to high humidity or kept on hand for long time requires prewashing.
- Pre-washing is mainly done by dipping the plate in methanol.
- Activation of pre-coated plates is done by placing them in an oven at 110-120ºC for 30 min. prior to spotting.
- Aluminum sheets should be kept in between two glass plates and placing in oven at 110-
  120ºC for 15 minutes.
- Hot plates can also be used for HPTLC plates with aluminum as support material.

4) **Application of sample and standard**

- The solvent used to apply the sample to the TLC plate can have a decisive influence on the spot size.
- The least polar single solvent or mixture of solvents in which the analyte(s) are completely soluble or completely extracted from the sample matrix can be used.
- Usual concentration range is 0.1-1μg / μL - above this causes poor separation.

- Automatic applicators are available wherein N_2 gas sprays sample and standard from syringe on HPTLC plates as bands.
- Band wise application can be more accurately accomplished and provides better separation with high response to densitometer.

5) Chromatographic development

Selectivity of separation is greatly influenced by the choice of solvent or solvent mixture. Selection can be based on

- When the mobile phase is polar, polar compounds would be eluted first because of lower affinity with stationary phase while non-polar compounds retained because of higher affinity with the stationary phase.
- One's own experience and literature
- Trial and error

Components of mobile phase should be mixed thoroughly and before introduction to the chamber. Multi component mobile phase once used not recommended for further use. After development, plate is removed from the chamber and mobile phase is removed from the plate. Drying can be done either at room temperature or at alleviated temperatures if solvents like water or acids are used.

6) Detection and quantification of spot Detection

- Spots of fluorescent compounds can be seen at 254 nm (short wave length) or at 366 nm (long wave length).
- Spots of non-fluorescent compounds can be seen when fluorescent stationary phase is used like as silica gel 60 F_{254}.
- Non UV absorbing compounds can be visualized by dipping the plates in 0.1 % iodine solution.
- When individual component does not respond to UV-derivatization required for detection.

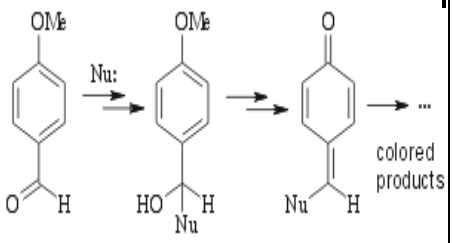
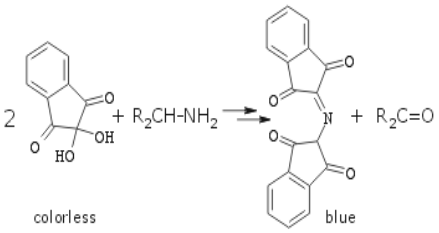
Table: 1.3 HPTLC spot detection general techniques

Detection techniques	
Non- Destructive	**Destructive**
Visible detection	Charring reaction Thermo-chemical reaction
Reversible reaction	
Ultra-Violet detection	
Non-reversible reactions	

Table: 1.4 Different HPTLC plate staining techniques[32,33]

TLC Staining procedure	Stain chemistry / physics	Comments
254nm UV light	UV light excites a fluorescent additive in TLC plate. Compounds screen some of the UV, making fluorescence weaker. Sometimes, visible fluorescence is exited by UV making a spot brighter, and so is colored differently than the background	A compound must have a chromophore.
Permanganate stain	Oxidative stain. Strong heating required.	The most universal stain. Harsh oxidizer. oxidizable compound such as alcohol, ether, ester, alkene, alkyne,

	$KMnO_4$(pink) + [H] ⟶ MnO_2(brown) + KOH + H_2O $KMnO_4$(pink) + R-SH ⟶ Mn^{2+}(colorless) + $R\text{-}SO_3K$ + H_2O	alkyl aromatic, ketone, carboxylic acid, amine, amide brown-yellow spots (MnO_2). Strongly reductive compounds such as thiols, phosphines and even dienes will show up as white spots (Mn^{+2}) BEFORE heating. Alkanes and pyridine won't show-up at all.
Iodine chamber	Different absorption of brown colored iodine on clean silica as opposed to silica loaded with analytes. Spots may be darker or lighter than background. Thiols will oxidize to disulfides: $2R\text{-}SH + I_2 = R\text{-}S\text{-}S\text{-}R + 2HI$	Hit and miss stain. Works in about 50% of all cases. Works with alkanes! Thiols and phosphines will immediately show up as white spots.
PMA stain (Phosphomolybdic acid stain)	Oxidative stain. Strong heating required. Upon exposure to reducing organics Mo^{5+} and Mo^{4+} compounds are formed (molybdenum blue, so called Keggin structure) which are green or blue colored	Expensive. Fairly universal stain. Some amines, amides and oxidation-resistant aromatics won't show up. Stain solution is somewhat light-sensitive.
CAM stain (Hanessian's Stain; Cerium Ammonium Molybdate stain)	Oxidative stain. Strong heating required. Procedure same as above	Very universal stain. More sensitive than PMA stain (above).

Anisaldehyde Stain	Some heating required. Chemistry of this stain is unknown. Probably something along the following lines: 	Works for allylic alcohols (green spots), phenols (violet spots), aldehydes, ketones, carbohydrates, esters like alkylphtalates (blue/red spots) and some. Alkenes, alkynes and aromatic compounds will NOT show up.
Ninhydrin Stain	Gentle warming required. Blue-pink color is due to the formation of Ruhemann's purple: 	Specific stain for amino acids (see group TLC below) and primary amines. Secondary amines stains light yellow which is difficult to see. Tertiary amines do not stain.

Quantification

- **Densitometry**

Densitometry is a means of measuring the concentration of the chromatographic zones on the developed HPTLC layer without damaging the separated substance. There are three possible scanning modes, single beam, single wavelength, double beam using a beam splitter and dual wavelength, double beam combined into a single beam.

The single beam format is most popular, as the beam of electromagnetic radiation hits the chromatographic layer, some passes into and through the layer whilst the remainder is reflected back from the surface.

Reflectance occurs due to the opaqueness of the layer. This reflected radiation is measured by the photomultiplier unit or photoelectric cell in the instrument. The spectrodensitometric scanner scan separate tracks and wavelength produces vast amount data. These data includes peak heights and areas, and position of zones (start, middle and end) for every resolved component on every chromatographic track on the HPTLC plate.

A baseline adjustment is applied so that all peaks can be accurately integrated ready for possible quantification. Calibration of single and multiple levels with linear or non-linear regressions are possible when target values are to be verified such as stability testing and dissolution profile single level calibration is suitable.

Video imaging and densitometry

The developed chromatogram is illuminated from above with visible, 254nm (UV) or 360 nm (UV) light, depending on the radiation required to visualize the analytes

Illumination from below the plate can often improve the brightness of the image. With the plate suitably lit, an image acquisition device, usually CCD (charged coupled device) camera with zoom attachment is positioned vertically above. The CCD camera transmits a digital signal to a computer and video printer.

7) Scanning

8) Documentation of chromatographic plate

Development chambers

There are a variety of different types of chambers, each designed with particular features to control to the greater or lesser extent the parameters of chromatogram development reproducibility.

As solvent vapour saturation, sorbent vapour adsorbed, solvent vapour "demixing" and solvent front and edge effects on the chromatographic layer can have a bearing on separation achieved, it is important to eliminate unwanted effects and to utilize those features that will improve resolution.

1.5 INTRODUCTION TO HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is a chromatographic technique that is useful for separating ions and molecules that are dissolved in a solvent.

a) Mechanism

Retention by interaction of non polar hydrocarbon chain of stationary phases with non polar parts of the sample molecules.

HPLC instrument consists of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by injecting a plug of the sample mixture onto the column.

b) Mobile phase:

- Methanol **(**CH_3OH**)**
- Acetonitrile (ACN)
- Water (H_2O)
- Buffer
- Tetrahydrofuran (THF)

It is very essential to degas the solvents which are used to get stable baseline, enhance the sensitivity of the detector and for stable pump operation.

c) Types of the Pumps:

1) Syringe pumps
2) Reciprocating pump
    - Single piston reciprocating pump
    - Dual piston reciprocating pump
    - Reciprocating diaphragm pump
3) Pneumatic pump
    - Direct pressure pump
    - Amplifier pump

Table: 1.5 Advantages and Disadvantages of HPLC Pump

Types of pump	Advantage	Disadvantage
Syringe (Position displacement)	1) Pulse free delivery at high pressure. 2) FR independent	1) Limited solvent capacity. 2) Change in solvent is inconvenient.
Reciprocating pump	1) FR independent of viscosity of MP. 2) Suitable for continuous operation.	1) Detection of noise due to pulsating out flow.

Pneumatic pump	1) Rugged 2) Inexpensive 3) easy to operate 4) Pulse free	1) FR dependent of viscosity of MP. 2) Change in solvent is inconvenient. 3) Gradient

d) HPLC Columns:

It is the heart of HPLC. A stable and high performance column is essential requisite for rugged, reproducible method. The various stationary phases used in HPLC columns are mentioned in table 1.6.

Table: 1.6 Various stationary phases used in HPLC columns

Group	Type	Particle diameter (µm)
Amino	Normal	5, 10
Nitrile	Norma	5, 10
Amine and Nitrile	Normal	40
Octyl	Reverse phase	3, 4, 5, 10
Octadecyl	Reverse phase	4, 5, 10, 40

e) Sample Injection System:

Injection systems, includes manual injector, standard auto sampler, high-performance auto sampler, high-performance auto sampler SL plus, micro well-plate auto sampler, preparative auto sampler and dual loop auto sampler as well as the thermostat. The figure 1.2 shows load and inject position of the the injection system.

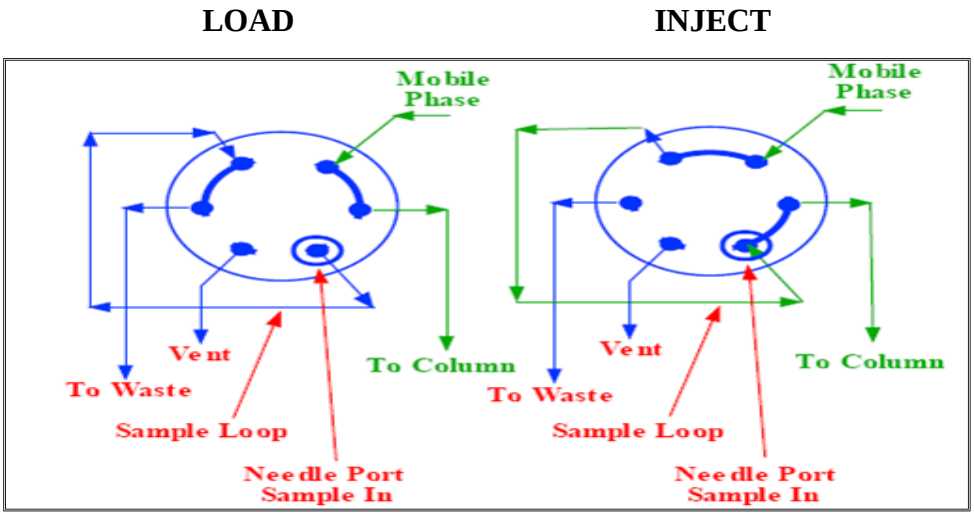


Figure: 1.3 Sample Injection Systems

f) Detectors:

It is considered as the eye of LC because it measures the separated components.

Two types of detectors:

1) Bulk property detectors: They are based on some bulk properties of eluent, such as RI and are not suitable for gradient elution. They are usually less sensitive than solute property detectors.
2) Solute property detectors: Performed by measuring some types of physical or chemical property that is specific to solute only and so can be used with gradient elution.

1.6 INTRODUCTION TO STABILITY INDICATING ASSAY METHODS (SIAMS)

a) Stability Indicating Assay Methods (SIAMs)

A stability-indicating method is defined as an analytical method that accurately quantitates the active ingredients without interference from degradation products, process impurities, excipients, or other potential impurities **or** A method that accurately quantitates significant degradants may also be considered stability-indicating.

A proactive approach to developing a stability indicating HPLC method should involve forced degradation at the early stages of development with the key degradation samples used in the method development process. Forced degradation should be the first step in method development. If forced degradation studies are performed early, method development and identification of primary degradation products and unknown impurities can be run in parallel.

Stability-indicating methods according to United States-Food and Drug Administration (USFDA) stability guideline of 1987 were defined as the "Quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured." This definition in the draft guideline of 1998 reads as: "Validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference."

- **Purpose:**
  1) To provide evidences on how quality of drug substance or product varies with time under the influence of variety of environmental conditions such as temperature, pH, humidity and light.

2) To establish:
   - The retesting period of drug substance or drug product.
   - The shelf life of drug substance or drug product.
   - The storage condition of drug substance or drug product.

b) Types of stability indicating assay method (SIAM):[34]

- **Specific Stability Indicating Assay Method:**
  It can be defined as 'A method that is able to measure unequivocally the drug(s) in the presence of all degradation products, in the presence of excipients and additives, expected to be present in the formulation.'

- **Selective Stability Indicating Assay Method:**
  Whereas it can be defined as 'A method that is able to measure unequivocally the drug(s) and all degradation products in the presence of excipients and additives, expected to be present in the formulation'.

1.6.1 Various regulatory guidelines available to carry out SIAM [35-40]

Table:1.7 Various ICH guidelines for stability testing and impurity profiling

Guidelines reference	Title
Q1A (R2)	Stability testing of new DS and DP
Q1B	Photo stability of new DS and DP
Q1E	Evaluation of stability data
Q2 (R1)	Validation of analytical procedures

Q3A (R2)	Impurities in new DS
Q3B (R2)	Impurities in new DP
Q5C	Stability testing of biotech/biological products
Q6A Specification:	New DP/DS (to determine content of DS/DP)

Table: 1.8 Selection of FDA and EMEA guidelines and pharmacopeia chapter referencing SIAM

Guidelines reference	Title
USP <1086>	Impurities in official articles
USP <11>	USP reference standard
USP <1150>	Pharmaceutical stability
USP <1191>	Stability consideration in dispensing practice
USP <797>	Pharmaceutical compounding of sterile products, storage and beyond use dating
EMEA March 2001	Note for guidance on in use stability testing of human medicinal products
EMEA Dec 2004	Guidance on chemistry of new API
FDA guidance for industry	Analytical procedures and method validation

1.6.2 Development of Validated Stability Indicating Assay Methods (SIAMs) [34]

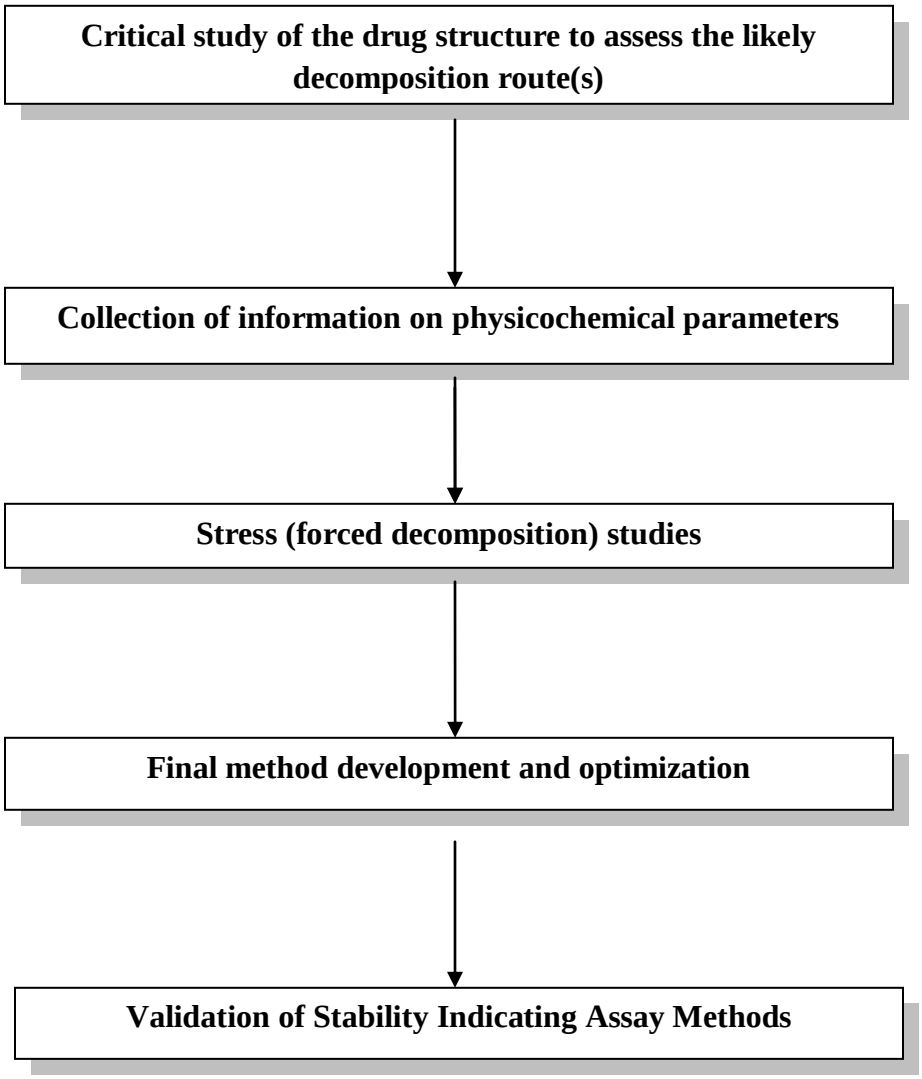


Figure: 1.4 Overview of validated SIAMs

1.7 METHOD VALIDATION PARAMETERS

The different parameters which are to be considered in analytical method validation as per USP (1225), CDER (Central Drug Evaluation & Research) and ICH guidelines can be summarized as follows.

Table: 1.9 Method validation parameters

USP (1225)	ICH	CDER
Accuracy	Accuracy	Accuracy
Precision	Precision	Precision
Repeatability	Repeatability	Repeatability
-	Intermediate precision	Intermediate precision
Reproducibility	Reproducibility	Reproducibility
Specificity	Specificity	Specificity
Limit of detection	Limit of detection	Limit of detection
Limit of quantification	Limit of quantification	Limit of quantification
Linearity	Linearity	Linearity
Range	Range	Range
Ruggedness	-	-
Robustness	Robustness	Robustness
-	-	Stability of solution

The different parameters of analytical method development are discussed below as per ICH guideline [36]

1) **Specificity**

   Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

   Acceptance criteria: No interference peaks from blank or placebo at the R.T. of the drug and the peak purity should pass.

2) Linearity

The linearity of an analytical procedure is its ability (within given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample.

Acceptance criteria: Correlation coefficient should not be less than 0.999.

3) Range

The range of analytical procedure is the interval between the upper and lower concentration of analyte in the sample which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

4) Accuracy

The accuracy of analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

Acceptance criteria: % Recovery should be in the range of 98-102%.

5) Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between the series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Acceptance criteria: % Relative standard deviation should be less than 2.

6) Detection Limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated under stated experimental conditions.

Acceptance criteria: S/N ratio > 3; not specified in other cases

7) Quantitation Limit

The quantitation limit of an individual analytical procedure is defined as the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

Acceptance criteria: S/N ratio > 10:1; not specified in other cases

8) Robustness

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

Acceptance criteria: The method must be robust enough to withstand slight changes and allow routine analysis of sample.

Table: 1.10 Characteristics and acceptance criteria for validation

Characteristics	Acceptance criteria
Accuracy/trueness	Recovery 98-102% (individual)
Precision	RSD < 2%
Repeatability	RSD < 2%
Intermediate precision	RSD < 2%
Specificity/selectivity	No interference
Detection limit	S/N > 3
Quantitation limit	S/N > 10
Linearity Correlation coefficient	$r^2 > 0.999$
Range	80 –120 %
Solution Stability	> 24 h or >12 h

Chapter-2
Literature Review

2.1 LITERATURE REVIEW ON BETULINIC ACID

Several literatures have been reported to determine biological properties like anti-HIV and anti-leukemic activity of *Dillenia indica* plant extract which contains Betulinic acid. HPLC and HPTLC methods have been reported for quantification of Betulinic acid in fruit extract and bark extract.

Table: 2.1 Literature review on Betulinic acid

Chemotherapeutic and Anti-HIV Potential of Betulinic acid				
Sr. No.	**Topic of Article**	**Author**	**Journal**	**Reference No.**
1.	Betulinic Acid and Its Derivatives: A Review on their Biological Properties	P. Yogeeswari, D. Sriram	Current Medicinal Chemistry 12 (2005) 657-666.	41
2.	Chemistry, Biological Activity, And Chemotherapeutic Potential Of Betulinic Acid for the Prevention And Treatment of Cancer And HIV Infection	R. H. Cichewicz, S. A. Kouzi	Wiley Periodicals 24(2004) 90–114.	42
3.	Activation of Mitochondria and Release of Mitochondrial Apoptogenic Factors by Betulinic Acid	S.Fulda, C.Scaffidi, S.A.Susini, P.H.Krammer, G. Kroemer, M.E.Peter	The Journal of Biological Chemistry 273 (1996) 33942–33948	43

4.	Betulinic Acid Derivatives as Human Immunodeficiency Virus Type 2 (HIV-2) Inhibitors	Z. Dang, W. Lai, K.Qian, P. Ho, K. Lee, Chin-Ho Chen, and L. Huang	Journal of Medicinal Chemistry 52(2009) 7887–7891.	44
5.	Betulinic acid derivatives as HIV-1 antivirals	C. Aiken, ChinHoChen	Trends in Molecular Medicine 11 (2005) 57-60.	45
Quantification of Betulinic acid by HPLC method				
6.	Betulin-Derived Compounds as Inhibitors of Alphavirus Replication	L. Pohjala, S. Alakurtti, T. Ahola, And P. Tammela	Journal of Natural Product 72(2009) 1917–1926.	46
7.	Anti-leukemic activity of Dillenia indica L. fruit extract and quantification of Betulinic acid by HPLC	D. Kumar, S. Mallick, J. R. Vedasiromoni, B.C.Pal	Phytomedicine 17 (2010)431–435.	47
9.	Anti-AIDS agents 73: Structure–activity relationship study and asymmetric synthesis of 3-O-monomethylsuccinyl-betulinic acid derivatives	Keduo Qian, K. N.Goto, Donglei Yu, L. M.Natschke, T. J. Nitz, N. Kilgore, G.P. Allaway, K.H. Lee	Bioorganic & Medicinal Chemistry Letters 17 (2007) 6553–6557.	48

10.	Esters of betulin and betulinic acid with amino acids have improved water solubility and are selectively cytotoxic toward cancer cells	M. D. Zalesinska , J. Kulbacka , J. Saczko, T. Wysocka , M. Zabel, P. Surowiak , M.Drag	Bioorganic & Medicinal Chemistry Letters 19 (2009) 4814–4817.	49
11.	Simultaneous determination of betulin and betulinic Acid in white birch bark using	G.Zhao, W.Yan, D.Cao	Journal of Pharmaceutical and Biomedical Analysis 43(2007)959–962.	50

Quantification of Betulinic acid by validated HPTLC Method				
12.	Validated High-Performance Thin-Layer Chromatographic Method for the Quantification of Betulinic Acid from Two Indian Plants of the Species Dillenia and Ziziphus	D.M. Gandhi, P. J. Mehta	Journal of Planar Chromatography 26 (2013) 331–335.	51
13.	The role of degradant profiling in active pharmaceutical ingredients And drug products	K.M.Alsante, A.Ando, R.Brown, J.Ensing, T.D.Hatajik, WeiKong , Y.Tsuda	Advanced Drug Delivery Reviews 59(2007)29–37.	52

Stability indicating assay method for different Phytoconstituents				
Sr. No	**Topic of Article**	**Author**	**Journal**	
1.	A validated stability-indicating **HPLC** method for simultaneous determination of **Silymarin** and **Curcumin** in various dosage forms	M. A. Korany, R. S. Haggag , A.A. Ragab , O. A. Elmallah	Arabian Journal of Chemistry 12(2013) 17-32.	53
2.	Anti-diabetic activity and stability study of the formulated leaf extract of **Zizyphus spina-christi (L.) Wild** with the influence of seasonal variation	C. G. Michela, D.I. Nesseemb, M. F. Ismailc	Journal of Ethnopharmacology 133 (2011) 53–62.	54
3.	**HPTLC** method for guggulsterone-II. Stress degradation studies on **guggulsterone**	H.Agrawal,N.Kaul, A.R.Paradkar, K.R.Mahadik	Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 23–31.	55
4.	A validated stability-indicating **HPLC** with photodiode array detector (PDA) method for the stress tests of ***Monascus purpureus fermented*** **rice, red yeast rice**	Yong-Guo Li , *Hong Liu , Zheng-Tao Wang*	Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 82–90.	56

Chapter-3
Aim and Objectives of Present work

Betulinic acid is an Antineoplastic and anti-HIV phytoconstituent obtained from the plant *Dillenia indica*. Betulinic acid is Official in Quality Standards of Indian Medicinal Plant; Vol. 7; ICMR 2008; p.g.no.: 78-85 and in The Merck Index, An encyclopaedia of chemicals, drugs and biological; Merck research laboratories; USA; 14th edition; 1192.

Literature review shows that HPLC and HPTLC methods are reported for estimation and quantification of Betulinic acid alone or in combination with other phytoconstituents like betulin, but there is not any stability indicating assay method has been reported yet. Thus, it was endeavoured to develop a complete degradation profile for Betulinic acid using HPTLC and HPLC methods.

OBJECTIVES OF PRESENT WORK

- To collect the bark sample.
- Extraction of Betulinic acid from bark of *Dillenia indica*.
- Identification and confirmation of isolated Betulinic acid.
- To develop stability indicating HPTLC and HPLC methods for quantification of isolated Betulinic acid obtained from *Dillenia indica* bark.
- Validate the stability indicating HPTLC and HPLC methods as per ICH guidelines.

Chapter-4
Identification of Betulinic acid

4. IDENTIFICATION OF BETULINIC ACID

Identification of Betulinic acid was carried out by Melting point, Thin Layer Chromatography and FT-IR spectroscopy.

Instrumentation

- Melting Point Apparatus : T603160, (EIE Instruments, Pvt. Ltd.)
- FT-IR Spectrophotometer: JASCO FT/IR-6100, (Inc. Japan)

4.1 IDENTIFICATION BY MELTING POINT

Melting point of Betulinic acid has been determined using melting point apparatus. The melting point of the pure drug was taken by capillary method.

Table: 4.1 Melting point determination data for Betulinic acid

Drug	Reported Melting Point[22]	Observed Melting Point
Betulinic acid	290-293 °C	291-293 °C

4.2 IDENTIFICATION BY THIN LAYER CHROMATOGRAPHY [51]

50 µg/ml solution of Betulinic acid (Both Isolated and Standard) was prepared in Acetonitrile and spotted on TLC plate. Chloroform: Toluene: Ethanol (4:4:1) was used as mobile phase. Anisaldehyde: H_2SO_4 was used as Derivatizing agent.

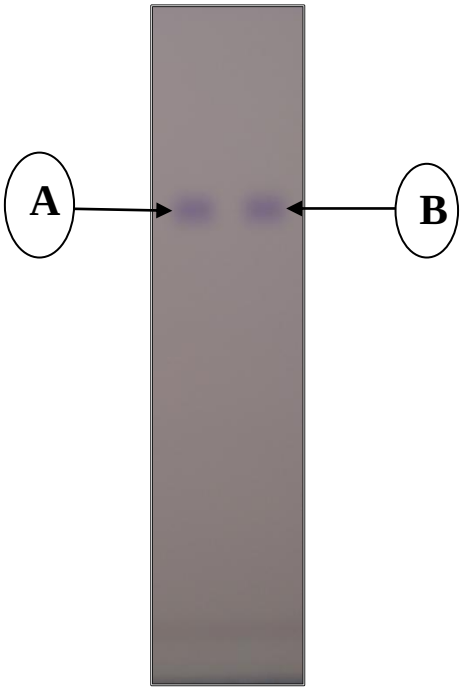


Figure: 4.1 TLC plate for Comparison of Standard Betulinic acid and isolated Betulinic acid.

[A- Standard Betulinic acid obtained from Sigma Aldrich. (R_f - 0.65)]

[B- Isolated Betulinic acid from *Dillenia indica* bark (R_f – 0.65)]

4.3 IDENTIFICATION BY INFRARED SPECTROSCOPY

IR Spectra of Standard Betulinic acid [From Sigma Aldrich] and Isolated Betulinic acid [From *Dillenia indica* bark] was taken using FT-IR spectrophotometer. IR Spectra obtained was verified with reported IR spectra available in literature.

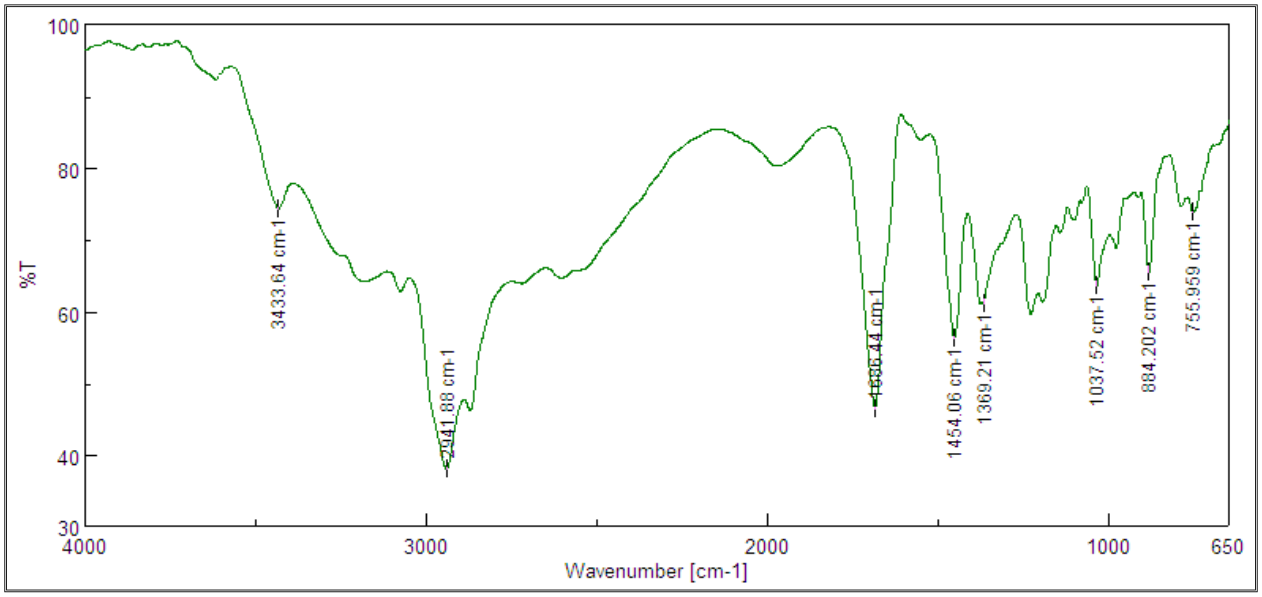


Figure: 4.2 FT-IR Spectra of Betulinic acid [Standard received from Sigma Aldrich]

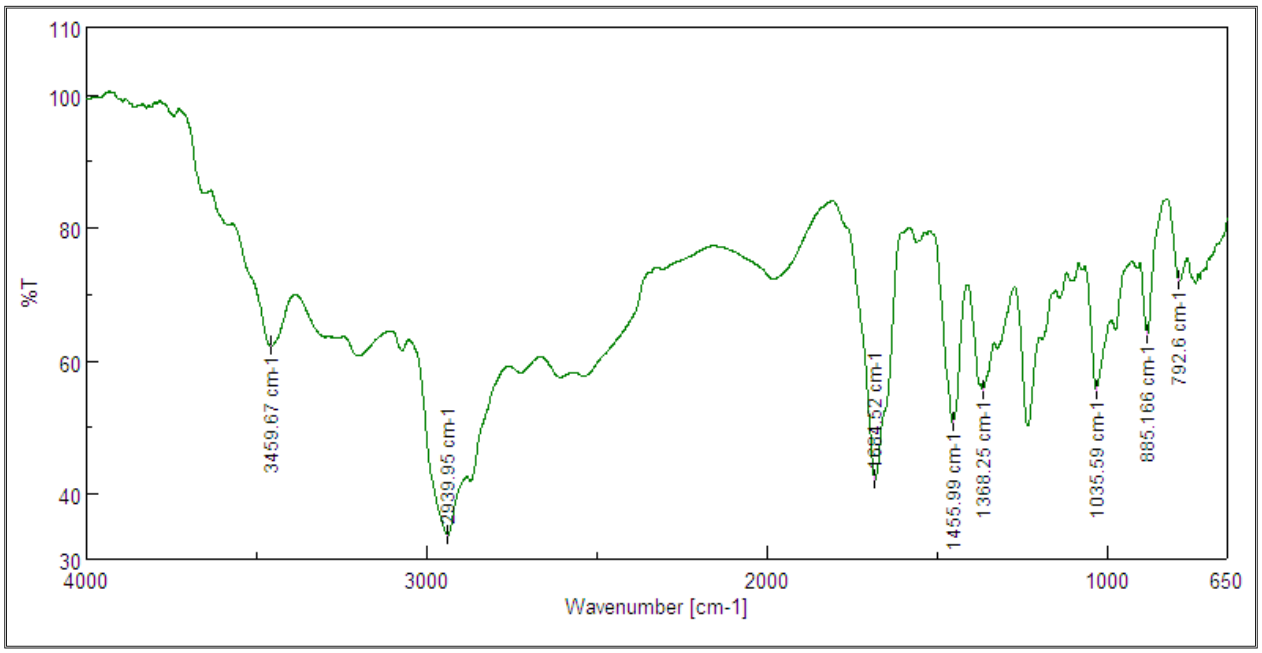


Figure: 4.3 FT-IR Spectra of Betulinic acid [Isolated from *Dillenia indica* bark]

Table: 4.2 Reported and Observed FT-IR Peaks of Betulinic acid

Functional Group	Reported FT-IR Peaks (cm^{-1}) [57]	Observed FT-IR Peaks (cm^{-1})
-OH Stretching	3438	3459
C-H Unsaturated Stretching	2943	2939
C=C Stretching	1693	1684
CH_3 Bending	1451	1455
COO- Stretching (Strong peak)	1377	1368

Mixed C-C Bending, C-H Bending, C-O Bending	1032	1035
C-C Bending	884	885

Chapter-5 Experimental Work

5.1 INSTRUMENTATION

5.1.1. High Performance Thin Layer Chromatography [HPTLC]

- Pre-coated TLC silica gel aluminum plate 60 F_{254} (10 × 10 cm, 0.2 mm Layer thickness) E Merck Ltd (India).
- Camag 100 µl Applicator syringe (Hamilton, Bonaduz, Schweiz).
- Camag Linomat-V applicator with N_2 pressure.
- Camag twin trough chamber (20 x 10 cm and 10 × 10) with stainless steel Lid.
- Camag TLC scanner 3.
- UV cabinet with dual wavelength (254 and 366 nm).
- Software- winCATs.

5.1.2. High Performance Liquid Chromatography [HPLC]

- Pump : Jasco PU-2080 PLUS
- Photo Diode Array Detector(PDA) : MD – 2015 PLUS - Multi wavelength detector
- Ultraviolet – Visible Detector (UV) : UV- 2075 PLUS – UV/Visible Detector
- Column used - Purosphere® STAR , Reversed Phase (C-18, 250mm × 4.6mm, 5 µm)

5.2 MATERIALS AND REAGENTS

5.2.1. Materials

- **Bark of *Dillenia indica*** was collected in July, 2011 from Vaghai Botanical garden of Dang district, (GPS: E 71° 19' N 22° 26') Gujarat, India. Bark sample was authentically identified with the help of Department of Botany, Gujarat University, Gujarat, India.

5.2.2. Reagents and Chemicals

- **For Extraction :** Methanol and Benzene used of AR Grade (Analytical Reagent) (Merck Ltd. India)
- **For HPTLC Method:** Toluene, Chloroform, Ethanol, Anisaldehyde, Concentrated Acetic acid, Concentrated Sulphuric acid, used of AR Grade (S.D. Fine chemicals Ltd., Mumbai, India.)
- **For HPLC Method:** Acetonitrile HPLC Grade (Merck Ltd. India), Triple Distilled Water was used.
- **For Solution preparation:** Acetonitrile, Concentrated Sulphuric acid, NaOH pellets, 30% H_2O_2 solution used of AR Grade (S.D. Fine chemicals Ltd., Mumbai, India.)

5.3 ISOLATION OF BETULINIC ACID FROM *DILLENIA INDICA* BARK

5.3.1 Preparation of bark powder using bark of *Dillenia indica*:

Bark of *Dillenia indica* was collected in December, 2012 from Vaghai Botanical garden of Dang district, (GPS: E 71° 19' N 22° 26') Gujarat, India. Bark sample was sent for authentication. Authentication was done by Dr. Jasrai (Botanist) Department of Botany, Gujarat University, Gujarat, India. The collected bark sample was dried in sunlight for 2 weeks. Dried sample was ground with the help of pulverizer and the powder was passed from 60 # sieve. The colour of the dried sample was cocoa brown. Total amount of bark powder collected was 400gm. The bark powder was stored in cool, dry and dark place in an air-tight container.

5.3.2 Extraction and isolation procedure using bark of *Dillenia indica*:

Pulverized bark powder was taken in 2000ml round bottom flask. Methanol (AR Grade) was added to bark powder and refluxed for around 6 hours. The Methanolic extract with bark powder was kept aside overnight. Next day Methanolic extract was collected from round bottom flask and fresh methanol was added to bark powder. This was repeated for six to seven days to extract out Betulinic acid from bark powder as much as possible. Extraction was performed until the methanolic extract gave negative results for **Liberman Buchardt** and Salkowski **Tests.** (Tests for phytosterols and triterpenoids)**.** Methanolic extract was collected in 2000ml round bottom flask with condenser attached to it. Methanolic extract was evaporated on heating metal until we got concentrated Methanolic extract of bark powder.

Concentrated Methanolic extract was collected in separating funnel. To the Methanolic extract Benzene was added. Methanolic extract was shacked well and kept aside for 30 minutes to separate benzene fraction from separating funnel. Transfer benzene fraction to the porcelain dish. Porcelain dish containing benzene fraction was kept on water bath at 75°C to evaporate benzene. On further evaporation concentrated benzene fraction gave isolated Betulinic acid (1.70 gm) which was filtered out.

The content of Betulinic acid was found to be the highest, **0.43%**, in *Dillenia indica* bark. As we got 400gm of *Dillenia indica* bark, amount of Betulinic acid present in bark is 1.72gm. % Yield was calculated for isolated Betulinic acid.

$$\textbf{\% Yield} = \frac{\text{Amount obtained after isolation}}{\text{Amount present in bark}} * 100$$

$$= 1.70/1.72*100$$

$$= \mathbf{98.837\ \%}$$

5.4 OPTIMIZED HPTLC METHOD [51]

- **Stationary Phase** :- Pre-coated TLC silica gel aluminium plate 60 F_{254}
- **Mobile Phase** :- Toluene : Chloroform : Ethanol [4: 4: 1 v/v/v]
- **Scanning Wavelength** :- 525nm
- **Derivatizing Agent** :- Anisaldehyde : H_2SO_4
- **Chamber Saturation Time: -** 20min.
- **Band Width** :- 5mm
- **Application Rate** :- 150nL/s
- **Distance Run** :- 80mm
- **Slit Dimension** :- 4mm x 0.25mm
- **Scanning Speed** :- 20mm/s
- **Running Time** :- 17 min

5.5 OPTIMIZED HPLC METHOD [Reported]

- **Stationary Phase**: Purosphere® STAR , Reversed Phase (C-18, 250mm × 4.6mm, 5 µm)
- **Mobile Phase:** Acetonitrile : Water (93 : 07 v/v)
- **Flow rate:** 1ml / minute
- **Injection volume:** 20 µl
- **Wavelength:** 207 nm
- **Run time:** 10 minutes

5.6 PREPARATION OF SOLUTIONS FOR HPLC AND HPTLC ANALYSIS

5.6.1. Preparation of standard stock solution of isolated Betulinic acid

Isolated BA (50mg) was accurately weighed and transferred to50ml volumetric flask. It was dissolved in 25ml of Acetonitrile. Flask was sonicated for 10minutes and solution was diluted up to the mark with Acetonitrile to obtained stock solution of concentration 1000 µg/ml.

From this solution, working standard solution was prepared by taking 5ml standard stock solution and dilute it with Acetonitrile up to 100ml to obtain concentration of 50 µg/ml.

5.6.2. Preparation of Mobile Phase for HPLC method

Acetonitrile (HPLC Grade) was taken in previously cleaned and dried HPLC mobile phase reservoir. Similarly Triple distilled water was also taken to Mobile phase reservoir. Both the reservoirs were sonicated for 15minutes before use.

5.6.3. Preparation of mobile phase for HPTLC method

Toluene (8ml), Chloroform (8ml) and Ethanol (2ml) were taken in measuring cylinder and mixed well. Final mobile phase was sonicated for 10 minutes before use.

5.6.4. Preparation of derivatizing agent for HPTLC method

10ml of concentrated acetic acid was taken to which 0.5ml of Anisaldehyde solution was added. 30ml of methanol was added to previous mixture. Drop vise 5ml of concentrated sulphuric acid was added with continuous stirring. Solution was diluted with methanol up to 100ml. Light yellow coloured solution was obtained which was used to derivatize HPTLC plate for quantification of Betulinic acid. Fresh derivatizing agent must be prepared after every 5 days.

5.7 METHOD VALIDATION FOR HPTLC AND HPLC

5.7.1 Preparation of calibration curve of Betulinic acid

For HPTLC method standard stock solution of 1000 µg/ml from isolated Betulinic acid was prepared as per section 5.6.1. From that 5ml of stock solution was taken to 25ml volumetric flask and volume was made up to 25ml with Acetonitrile to produce final concentration of 200 µg/ml. From the solution (200 µg/ml) different volumes (1ml, 1.5ml, 2ml, 2.5ml, 3ml, 3.5ml, 4ml, 4.5ml) was pipette out in 10ml volumetric flask and made up the volume up to 10ml to produce final concentration of 20 µg/ml, 30 µg/ml, 40 µg/ml, 50 µg/ml, 60 µg/ml, 70 µg/ml, 80 µg/ml and 90 µg/ml respectively. 10 µl of each solution was spotted on the TLC plate to obtained final concentration 200-900 ng/spot. The plate was developed in optimizes conditions as per section 5.4. Peak area was plotted against corresponding concentrations to obtain the calibration graph.

For HPLC calibration curve was plotted by injecting 20-90 µg/ml solutions and peak area was plotted against corresponding concentrations to obtain the calibration graph.

5.7.2 Precision

5.7.2.1. Repeatability of standard solution scan for HPTLC

Repeatability of sample scan was determined by scanning 500 ng/Spot, 6 times and peak area was recorded. Repeatability of sample scan was measured in terms of %RSD.

5.7.2.2. Repeatability of standard solution application for HPLC and HPTLC

Repeatability of sample application was determined by spotting the standard solution (500 ng/spot) 6 times on the same plate and peak area was recorded. Similarly for HPLC 50 µg/ml solution of isolated BA was applied 6 times. Repeatability of sample application was measured in terms of %RSD.

5.7.3. Specificity

HPTLC: Interference from degradation products is formed by stress condition, was checked by Specificity study. In HPTLC method purity of the isolated Betulinic acid was checked by scanning in the range of 400-700 nm with the help of spectra scanner model of the WinCATs software. The peak purity of isolated Betulinic acid was determined by comparing the spectrum at three different regions of the spot i.e. peak start (S), peak apex (M), and peak end (E) positions which confirm that the peak represents pure single component for Betulinic acid.

HPLC: In HPLC method purity of isolated Betulinic acid was checked by detecting the peak using PDA detector. The peak purity of isolated Betulinic acid was determined by examine the data like retention time ($R_{t)}$, Purity tail, Purity front, Number of plates, Tailing Factor which confirm that the peak represents pure single component for Betulinic acid.

5.7.4 Limit of Detection and Limit of Quantification for HPTLC and HPLC

For determination of LOD/LOQ, all linearity range solution was prepared in triplicate as described in section 5.7.1. HPTLC and HPLC Chromatograms of all these solutions are recorded. 3 linearity curves of peak area v/s concentration (µg/ml or ng/spot) were plotted. From these curves, mean slop value and SD of intercept was calculated. From all these values, LOD/LOQ were calculated using following equation.

LOD= 3.3 σ / S

LOQ = 10 σ / S

σ = Standard deviation of intercept

S = Slope of the linearity curve

5.8 SAMPLE PREPARATION FOR FORCED DEGRADATION STUDY

5.8.1. Acid hydrolysis for isolated Betulinic acid

Acid hydrolysis study was carried out by using 0.1N of HCl in reflux condition at 80°C conditions for different time to get sufficient degradation. Aliquots were withdrawn periodically, neutralized with NaOH and subjected to analysis after dilution with Acetonitrile to obtain concentration of 50µg/ml and solution was analyzed for HPTLC and HPLC methods.

5.8.2. Base hydrolysis for isolated Betulinic acid

Base hydrolysis study was carried out by using 0.1N of NaOH in reflux condition at 80°C conditions for different time to get sufficient degradation. Aliquots were withdrawn periodically, neutralized with HCl and subjected to analysis after dilution with Acetonitrile to obtain concentration of 50µg/ml and solution was analyzed for HPTLC and HPLC methods.

5.8.3. Oxidative degradation for isolated Betulinic acid

The oxidative stress study was carried out in 3% H_2O_2 and for different time interval at room temperature (ambient) till sufficient degradation was observed. Aliquots were withdrawn periodically and subjected to analysis after dilution with Acetonitrile to obtain concentration of 50µg/ml and solution was analyzed for HPTLC and HPLC methods.

5.8.4. Thermal degradation for isolated Betulinic acid

Drug powder was subjected to dry heat at 80ºC for different time to get sufficient degradation. Samples were withdrawn periodically and subjected to analysis after dilution with Acetonitrile to obtain concentration of 50µg/ml and solution was analyzed for HPTLC and HPLC methods.

5.8.5. Photolytic degradation for isolated Betulinic acid

Photolytic studies were done in solid state by spreading a thin layer of drug in a Petri plates and it was exposed directly to sunlight for different time to get sufficient degradation. Samples were withdrawn periodically and subjected to analysis after dilution with Acetonitrile to obtain concentration of 50µg/ml and solution was analyzed for HPTLC and HPLC methods.

5.8.6. Optimized forced degradation condition

Degradation Type	Stressor	Time Period	Temperature
Acid Hydrolysis	0.1N HCl	4h.	Reflux, 80 °C
Base Hydrolysis	0.1N NaOH	4h.	Reflux, 80 °C
Oxidative Degradation	3% H_2O_2	4h.	Room Temperature
Thermal Degradation	Dry Heat	24h.	80 °C
Photolytic Degradation	Direct sunlight	8h.	-

Chapter-7
Conclusion and Future Scope

For process and preservation of Phytoconstituents needs sound knowledge of their chemical and physical properties. Stability indicating assay method provides information of stability of Phytoconstituents in different environmental conditions like acid and base hydrolysis, oxidative degradation, photo degradation, thermal degradation.

Dillenia indica plant is distributed in sub Himalayan tract, Assam, North Bengal, Bihar, Orissa, Madhya Pradesh and Gujarat. Betulinic acid is the most important active Phytoconstituent present in *Dillenia indica* plant. Maximum amount (0.43%) of Betulinic acid is present in *Dillenia indica* bark from which extraction was carried out. Betulinic acid selectively induces apoptosis in tumor cells by directly activating the mitochondrial pathway of apoptosis through p53 and CD95 independent mechanism.

Forced degradation study was carried out according to ICH guidelines Q1A (R2) and Q1B. The objective of the study was to find out the degradation behavior of isolated Betulinic acid and to quantify the isolated Betulinic acid in different environmental conditions described in ICH guidelines Q1A (R2) and Q1B. A stability indicating HPTLC and HPLC methods were developed and validated to quantify isolated Betulinic acid in different stress conditions like acid and base hydrolysis, oxidative degradation, photo degradation, thermal degradation.

The stability indicating HPTLC method was developed and validated for quantification of Betulinic acid in different forced degradation conditions. Optimized mobile phase was used Chloroform: Toluene: Ethanol (4: 4: 1). The method is based on high performance thin layer chromatography using Precoated Silica Gel 60 F_{254} plates with 250 µm thickness. Detection was carried out at 525nm wavelength. The R_f value of isolated Betulinic acid was 0.65 ± 0.02.

Forced degradation HPTLC method for isolated Betulinic acid was performed in different stress conditions like acid and base hydrolysis, oxidative degradation, photo degradation, thermal degradation. Forced degradation HPTLC method quantifies isolated Betulinic acid in all described stress conditions. Results shows that highest degradation was observed (30.04%) in oxidative condition (3% H_2O_2, Room Temperature, 4h). Acid hydrolysis was carried out by using 0.1 N HCl by refluxing at 80°C for 4h which shows 15.32% degradation.

Base hydrolysis was carried out by using 0.1 N NaOH by refluxing at 80°C for 4h which shows 13.04% degradation. There is no significant degradation was observed in photo degradation condition (Direct sunlight, 8h) and thermal degradation condition (Dry Heat, 24h, 80°C).

The stability indicating HPLC method was developed and validated for quantification of Betulinic acid in different forced degradation conditions. Optimized mobile phase was used Acetonitrile: Water (93:7). The method is based on high performance liquid chromatography using Purosphere® STAR, Reversed Phase (C-18, 250mm × 4.6mm, 5µm) column. Detection was carried out at 207nm wavelength. The R_t value of isolated Betulinic acid was 7.55 ± 0.1.

Forced degradation HPLC method for isolated Betulinic acid was performed in different stress conditions like acid and base hydrolysis, oxidative degradation, photo degradation, thermal degradation. Forced degradation HPTLC method quantifies isolated Betulinic acid in all described stress conditions. Results shows that highest degradation was observed (31.25%) in oxidative condition (3% H_2O_2, Room Temperature, 4h). Acid hydrolysis was carried out by using 0.1 N HCl by refluxing at 80°C for 4h which shows 21.40% degradation. Base hydrolysis was carried out by using 0.1 N NaOH by refluxing at 80°C for 4h which shows 17.12% degradation. There is no significant degradation was observed in photo degradation condition (Direct sunlight, 8h) and thermal degradation condition (Dry Heat, 24h, 80°C).

FUTURE SCOPE:

As Betulinic acid does not contains any chromophore and conjugates double bond in its structure, Forced degradation method can be developed further by using different derivatization techniques.

Chapter-8
Bibliography

1. Jawla, S.; Gupta, A. K.; Singla, R.; Gupta, V. General awareness and relative popularity of allopathic, ayurvedic and homeopathic systems.*Journal of Chemical and Pharmaceutical Research* **2009,***1* (1), 105-112.
2. Dickison, W. C. A note on the wood anatomy of *Dillenia* (Dilleniaceae). *International Association of Wood Anatomists Bulletin* **1979,** 2-3.
3. Dubey, P. C.; Sikarwar, R. L. S.; Khanna, K. K.; Tiwari, A. P. Ethnobotany of *Dillenia pentagyna* Roxb. in vindhya region of Madhya Pradesh, India.*Natural Product Radiance* **2009,***8* (5), 546-548.
4. Pradhan, B. K.; Badola H. K. Ethnomedicinal plant use by Lepcha tribe of Dzongu valley, bordering Khangchendzonga Biosphere Reserve, in North Sikkim, India. *Journal of Ethnobiology and Ethnomedicine* **2008,***4* (22), 1-18.
5. Sharma, U. k.; Pegu, S. Ethnobotany of religious and supernatural beliefs of the mising tribes of Assam with special reference to the 'Dobur Uie'. *Journal of Ethnobiology and Ethnomedicine***2011,***7* (16), 1-13.
6. Khanum, A.; Khan, I.; Ali, A. *Ethnomedicine and Human Welfare*Ukaaz Publication, **2007**, Vol. 4.
7. Khare, C. P. *Indian Medicinal Plants;* Springer, **2007**, p 214.
8. Panda, H. *Handbook on Medicinal Herbs with uses;***2009**, p 279.
9. Nadkarni, K. M.; Nadkarni, A. K. *Dillenia indicaLinn.* and *Dillenia pentagyna*.*The Indian Materia Medica;* Popular Prakashan PVT. LTD.: Mumbai, Maharashtra, India. **1954**, pp 448-449.
10. Shah, G. L. *Dillenia indica* and *Dillenia pentagyna*. In *Flora of Gujarat.***1978,** p 49 & 214.
11. Rastogi, R. P.; Mehrotra, B. N.; Sinha, S.; Seth, R. *Compendium of Indian Medicinal Plants;*, **2001**; p 53 & 54.
12. Metcalfe, C. R.; Chalk, C. *Anatomy of the Dicotyledons;*Clarendon Press: Oxford, **1983**.
13. Kartesz, J. T. United states department of agriculture : Natural resources conservation services. http://plants.usda.gov/java/profile?symbol=DIIN6 (accessed March 10, **2013**).

14. Puccio, P. http://www.photomazza.com/?Dillenia-indica (accessed August 6, **2013**).
15. Mukherjee, K. S.; Badruddoza, S. Chemical constituents of *Dillenia indica Linn.* and *Vitex negundo Linn*. *Journal of Indian Chemical Society***1981,***58,* 97-98.
16. Md, M. A.; Tareq, S. M.; Apu, A. S.; Basak, D.; Islam, M. S. Isolation and identification of compounds from the leaf extract of *Dillenia indica* Linn. *Bangladesh Pharmaceutical Journal***2010,***13* (1), 49-53.
17. ENVIS CENTER ON FLORAL DIVERSITY. http://www.bsienvis.nic.in/forms/SearchResult.aspx?search=Dillenia%20indica&SearchType=all (accessed March 31, **2013**).
18. Riyas, A. K. Medicinal Plants. http://ayurvedaherbalplants.blogspot.in/2010/05/dillenia-indica.html (accessed March 31, **2013**).
19. Janick, J.; Paull, R. E. The encyclopedia of fruit and nuts. 1st Ed.; CABI: London, **2008**.
20. Anisuzzaman, M.; Rahman, A. H. M. M.; Harun, R. M.; Naderuzzaman, A. T. M.; Islam, A. K. M. R. An ethnobotanical study of Madhupur tangail. *Journal of Applied Sciences Research* **2007,***3* (7), 519-530.
21. Cichewicz, R. H.; Kouzi, S. A. Chemistry, biological activity and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. *Medicinal Research Review* **2004,***24,* 90-114.
22. *The Merck Index, An Encyclopedia of Chemicals , Drugs and Biologicals;* USA, **2006**, *14*, p 1192.
23. Nick, A.; Wright, A. D.; Rali, T.; Sticher, O. Antibacterial triterpenoids from *Dillenia papuana* and their structure–activity relationships. *Phytochemistry***1995,***40,* 1691-1695.
24. Bringmann, G.; Saeb, W.; Assi, L. A.; Francois, G.; Narayanan, A. S. S.; Peters, K.; Peters, E. M. Betulinic acid: Isolation from *Triphyophyllum peltatum* and *Ancistrocladus heyneanus*, antimalarial acitivity and crystal structure of the benzyl ester. *Planta Medica***1997,***63,* 255-257.
25. Peng, C.; Bodenhausen, G.; Qiu, S.; Fong, H. H. S.; Farnsworth, N. R.; Yuan, S. Computer-assisted structure elucidation: Application of CISOC-SES to the resonance assignment and structure generation of betulinic acid. *Magnetic Resonance in Chemistry***1998,***36,* 267-278.

26. Cinta, P. S.; Leopold, N.; Kiefer, W. Vibrational spectroscopy of the HIV inhibitor betulinic acid and its birch bark natural source. *Talanta* **2002,** 625-631.

27. Kumar, D.; Mallick, S.; Vedasiromoni, R. J.; Pal, C. B. Anti-leukemic activity of *Dillenia indica L.* fruit extract and quantification of betulinic acid by HPLC. *Phytomedicine* **2010,** 431-435.

28. Uppalapati, L.; Rao, J. T. Antimicrobial efficiency of fixed oil and unsaponifiable matter of *Dillenia indica Linn*. *Indian Drugs Pharm Ind*, **1980,** 35-38.

29. Tocris Bioscience. http://www.tocris.com/literature/3906_sds.pdf?1364875600 (accessed August 2, **2013**).

30. Wall, P.E. Thin Layer Chromatography- A Modern practical approach, Royal chemical society, 7th Edition, 126-175.

31. Sethi, P.D. HPTLC- Quantitative Analysis of Pharmaceutical Formulations, 1st Edition, CBS publisher.

32. http://www.reachdevices.com/Techniques/3409 (accessed November 21, **2013**)

33. T*hin-Layer Chromatography Reagents and Detection Methods, Vol. 1a:* H. H. Jork, W. Funk, W. Fischer, and H.Wimmer, VHC, Weinheim, Germany, **1990**.

34. Singh, S. S.; Bakshi, M. Development of Validated Stability Indicating Assay Methods: Critical Review. *J. Pharm. Biom. Anal*. **2002**, 28, 1011 – 1040.

35. Food and Drug Administration. Guidance for Industry: Analytical Procedures and Methods Validation (Draft guidance). **2000**, 501–504.
36. ICH guidelines for Validation of Analytical Procedures: Text and Methodology Q2 (R1), International Conference on Harmonization, IFPMA, Geneva, **2005**.
37. FDA, Guidance for Industry Q1A (R2) Stability Testing of New Drug Substances and Products. **2003**.
38. FDA, Guidance for Industry ICH Q1B: Stability Testing: Photo stability Testing of New Drug Substances and Products.
39. ICH. Stability Testing of New Drug Substances and Products Q1A (R2), International Conference on Harmonization, IFPMA, Geneva, **2003.**
40. ICH. Photo stability testing of New Drug Substances and Products Q1B, International Conference on Harmonization, IFPMA, Geneva, **1996.**
41. Yogeeswari, P.; Sriram, D. Betulinic Acid and Its Derivatives: A Review on their Biological Properties, Current Medicinal Chemistry, **2005**, 657-666.
42. Cichewicz, R. H.; Kouzi, S. A. Chemistry, Biological Activity, And Chemotherapeutic Potential Of Betulinic Acid for the Prevention And Treatment of Cancer And HIV Infection, Wiley Periodicals, **2004**, 90–114.
43. Fulda, S.; Scaffidi, C.; Susini, S.A.; Krammer, P.H.; Kroemer, G. ; Peter, M.E.; Activation of Mitochondria and Release of Mitochondrial Apoptogenic Factors by Betulinic Acid, The Journal of Biological Chemistry,**1996**, 33942–33948.
44. Dang, Z.; Lai, W.; Qian, K.; Lee, K.; Huang, L. Betulinic Acid Derivatives as Human Immunodeficiency Virus Type 2 (HIV-2) Inhibitors, Journal of Medicinal Chemistry,**2009**, 7887–7891.
45. Aiken, C. Betulinic acid derivatives as HIV-1antivirals, Trends in Molecular Medicine, **2005**, 57-60.
46. Pohjala, L.; Alakurtti, S.; Ahola, T.; Tammela, P. Betulin-Derived Compounds as Inhibitors of Alpha virus Replication, Journal of Natural Product, **2009**,1917–1926.
47. Kumar, D.; Mallick, S.; Vedasiromoni, J. R.; Pal, B.C. Anti-leukemic activity of Dillenia indica L. fruit extract and quantification of Betulinic acid by HPLC, Phytomedicine,**2010**,431–435.

48. Nitz, T. J.; Kilgore, N.; Allaway, G.P.; Lee, K.H. Anti-AIDS agents 73: Structure–activity relationship study and asymmetric synthesis of 3-O-monomethylsuccinyl Betulinic acid derivatives, Bioorganic & Medicinal Chemistry Letters, **2007**, 6553–6557.
49. Kulbacka, J.; Saczko, J.; Wysocka, T.; Zabel, M. Esters of betulin and Betulinic acid with amino acids have improved water solubility and are selectively cytotoxic toward cancer cells, Bioorganic & Medicinal Chemistry Letters,**2009**,4814–4817.
50. Zhao, G.; Yan, W.; Cao, D. Simultaneous determination of Betulin and Betulinic Acid in white birch bark using, Journal of Pharmaceutical and Biomedical Analysis, **2007**, 959–962.
51. Gandhi, D.M.; Mehta, P. J. Validated High-Performance Thin-Layer Chromatographic Method for the Quantification of Betulinic Acid from Two Indian Plants of the Species Dillenia and Ziziphus, Journal of Planar Chromatography, **2013**, 331–335.
52. Alsante, K.M.; Ando, A.; Brown, R. The role of degradant profiling in active pharmaceutical ingredients And drug products, Advanced Drug Delivery Reviews, **2007**, 29–37.
53. Korany, M. A.; Haggag, R. S.; Ragab, A.A. ; Elmallah, O. A. A validated stability-indicating HPLC method for simultaneous determination of Silymarin and Curcumin in various dosage forms, Arabian Journal of Chemistry, **2013**,17-32.
54. Michela, C. G.; Nesseemb, D.I.; Ismailc, M. F. Anti-diabetic activity and stability study of the formulated leaf extract of Zizyphus spina-christi (L.) Wild with the influence of seasonal variation, Journal of Ethnopharmacology, **2011**, 53–62.
55. Agrawal, H.; Kaul, N.; Paradkar, A.R.; Mahadik, K.R. HPTLC method for guggulsterone II. Stress degradation studies on guggulsterone, Journal of Pharmaceutical and Biomedical Analysis, **2004**, 23–31.
56. Yong-Guo Li , *Hong Liu , Zheng-Tao Wang*, A validated stability-indicating HPLC with photodiode array detector (PDA) method for the stress tests of *Monascus purpureus fermented* rice, red yeast rice, Journal of Pharmaceutical and Biomedical Analysis, **2005**,82–90.
57. Cınta, S.; Leopold, N.; Kiefer, W. Vibrational spectroscopy of Betulinic acid HIV inhibitor and of its birch bark natural source, Talanta, **2002**, 625–631.