DEVELOPMENT OF ANALYTICAL METHOD FOR ESTIMATION OF BIOMARKERS IN HEPASAVE TONIC: A POLYHERBAL FORMULATION

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IN

PHARMACEUTICAL ANALYSIS

BY

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CERTIFICATE

This is to certify that the dissertation work entitled "Development of Analytical method for Estimation of biomarkers in HEPASAVE Tonic: A Polyherbal Formulation" submitted by Ms. KRITI H. SHARMA with Regn. No. 13MPH307 in partial fulfilment 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.

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I hereby declare that the dissertation entitled "Development of Analytical method for Estimation of biomarkers in HEPASAVE Tonic: A Polyherbal Formulation" is based on the original work carried out by me under the guidance of Dr. Dipal Gandhi, Assistant professor, 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

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DEDICATED TO THE ALMIGHTY GOD

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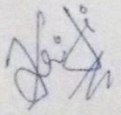

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Abbreviations

Chemicals

AG	Andrographolide
GA	Gallic acid
KT	Kutkin
VS	Vasicine
OPA	o-phosphric acid

Symbols

°C	Degree centigrade
λ_{max}	Wavelength maxima
µl	Microliter
ml	Mililiter
mg	Miligram
µg	Microgram
ng	Nanogram
cm	Centimeter
mm	Milimeter
µm	Micrometer
R_f	Retention factor
R_t	Retention time
r^2	Correlation coefficient
<	Less than
>	More than
%	Percentage
min	Minute

sec	Second
hr	Hour
Temp	Tempearture
v/v/v/v	Volume/volume/volume/volume
w/v	Weight by volume
Sr, No.	Serial number
Conc.	Concentration

Others

TLC	Thin layer chromatography
HPTLC	High performance thin layer chromatography
HPLC	High performance liquid chromatography
UV	Ultra Violet
MP	Melting point
SP	Stationary phase
NP	Normal phase
RP	Reverse phase
USP	United States pharmacopoeia
IP	Indian pharmacopoeia
BP	British pharmacopoeia
ICH	International conference on Harmonization
IUPAC	International Union of Pure and Applied Chemistry
SD	Standard Deviation
RSD	Relative Standard Deviation
LOD	Limit of detection
LOQ	Limit of quantitation

DEVELOPMENT OF ANALYTICAL METHOD FOR ESTIMATION OF BIOMARKERS IN HEPASAVE TONIC: A POLYHERBAL FORMULATION

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Abstract

The Polyherbal formulation, HEPASAVE SYRUP, is an Ayurvedic Proprietary Medicine, used particularly as a Powerful Hepato-protective, an Antioxidant, and a Bitter tonic. The formulation composed of *Amalaki fruit i.e. Phyllanthus emblica Ab., Haritaki fruit i.e., Terminalia chebula Ab., Bibhitaka fruit i.e., Terminalia bellerica AB., Vasa leaf i.e., Adhatoda vasica Ab., Bhunimba herb i.e., Andrographis paniculata, Katuka root i.e., Picrorrhiza kurroa, Nimba stem bark i.e., Azadirachta indica Ab, Amruta stem i.e., Tinospora cordifolia, Sarpankha herbTephrosia purpurea Ab.,* and also a Flavored syrup base. The quality control of formulation is required to be set in terms of developing HPTLC and HPLC method to ascertain the quality of formulation. Few Biomarkers have been selected for the study which includes AG, GA, KT and VS. A couple of HPTLC and HPLC methods have been reported for the simultaneous estimation of Andrograpolide and Gallic acid in combination with other biomarkers, and a couple of such methods have been reported for the simultaneous estimation of Andrographolide and Kutkin, together with other biomarkers. New, simple and rapid analytical methods like, HPTLC and RP- HPLC developed for the simultaneous estimation of Andrographolide (AG), Gallic acid (GA), Kutkin (KT) and Vasicine (VS) in HEPASAVE can be applied to the routine quality control studies. A new, simple, accurate, sensitive, precise, reproducible and robust High-performance thin layer chromatography (HPTLC) method was developed using Mobile phase Toluene: Ethyl acetate: Formic acid: Methanol (3: 3: 0.8: 0.4, v/v/v/v) and sample Ethyl acetate extract and was further utilized for the HPTLC study. The band dimensions

were kept 6 mm in length, 10 mm from the bottom edge, 10 mm from the side edge; the application rate was 0.1µL/s, chamber saturation time utilized was 35 min at room temperature (25 ± 2 °C and 40% RH), the run length and time were kept at 55 mm for 25 min, Densitometric scanning was carried out using Deuterium/Tungsten lamp, in the absorbance mode at 254 nm for Quantitative evaluation (CAMAG winCATS software). The scanning speed of 20 mm s^{-1} was employed and a slit dimension of 5 mm x 0.1 mm was employed. The results obtained from scanning showed different spots of AG, GA and KT obtained at R_f values of 0.72 min, 0.61 min and 0.17 min, respectively. Vasicine peak was not obtained by the proposed HPTLC method. The data for calibration plots showed good linear relationship for Andrographolide with $r^2 = 0.995$, Gallic acid with $r^2 = 0.9932$ and Kutkin with $r^2 = 0.9917$ in the concentration range of 200ng to 800ng, 80ng to 320ng and 2000ng to 6000ng, respectively with respect to peak area. The present method was validated by linearity, accuracy, precision according to ICH guidelines. The limits of detection and quantitation were determined. The limit of detection for AG, GA and KT were found to be 2.3111, 0.8950 and 0.0095, respectively and the limit of Quantitation was found to be 7.0034, 2.7120 and 0.0287, respectively. Statistical analysis of the data showed that the method is reproducible and selective for the quantitation of Andrographolide, Gallic acid and Kutkin simultaneously. The amount of AG, GA and KT in the formulation were quantified and were found to be 1.27 %w/v, 1.15 %w/v and 0.014 %w/v, respectively. The amount of Andrographolide, Gallic acid and Kutkin in the Ethyl acetate extract were found to be 7.6 %w/v, 6.92 %w/v and 0.22 %w/v, respectively.

Following the HPTLC method, the simultaneous estimation of Andrographolide, Gallic acid and Kutkin in Polyherbal formulation by RP- HPLC was carried out in a C18 PUROSPHERE STAR Hyber column with 250 × 4.5 mm i.d., 5 µm particle size. The column temperature and sample temperature was kept at 25ºC. The diluent used was Water : ACN (75:25), pH 3.45 maintained by 0.1% solution of o- phosphoric acid. The mobile phase used for the separation of the biomarkers was Water : ACN (75:25), pH 3.45 maintained by 0.1% solution of o- phosphric acid, flown at 1.0 ml/min. The injection volume employed was 20 µL, run time was kept 35 min and the detection wavelength was 254 nm. The blank used for the sample solutions was methanol. Prepared standard Andrographolide, Gallic acid and Kutkin solutions as well as the final Ethyl acetate extract were injected to HPLC column as per earlier mentioned chromatographic conditions. The chromatographic peaks of AG, GA, KT in formulation extract were compared with retention time of standard

AG, GA and KT. Peak areas were recorded of prepared standard solutions. From the peak area of standard AG, GA and KT obtained from the RP- HPLC conditions, the amounts of these Biomarkers in extract as well as in the formulation were computed simultaneously. The amount of Andrographolide, Gallic acid and Kutkin in the Formulation were found to be 0.56 %w/v, 0.554 %w/v and 0.44 %w/v, respectively. The amount of Andrographolide, Gallic acid and Kutkin in the Ethyl acetate extract were found to be 3.354 %w/v, 3.28 %w/v and 1.8 %w/v, respectively.

Hence, we can conclude that an ICH- validated HPTLC method and an RP- HPLC method was developed for the first time for the quantitation of AG, GA and KT simultaneously.

CHAPTER- 1

INTRODUCTION

1. INTRODUCTION

1.1. LIVER DISORDERS

Liver is the largest of the internal organs in Human body, performing more than 5,000 functions like, cleansing the toxins from the blood, converting food into nutrients, controlling the hormone levels etc. There are various liver diseases that can be caused by virus, damage from the drugs or chemicals, obesity, diabetes or an attack from one's own immune system. Such liver diseases cause the organ to become inflamed, which can progress to scarring or cirrhosis. It is very critical that patients with cirrhosis, seek help because people with cirrhosis are at an increased risk of liver cancer or liver failure. Liver cancer and liver failure can be treated by a Multidisciplinary approach including radiation, medication or surgery, which also includes Liver transplant. Common Liver diseases can lead to Cirrhosis, which can lead to Liver Cancer or Liver Failure. Viral hepatitis is an inflammation of the liver caused by one of three virus forms, named as A, B or C. Hepatitis A is generally caused by consuming contaminated food or water. Hepatitis B is transmitted through the bodily fluids which triggers an immune reaction, and causes a lower level inflammation and liver damage. Hepatitis C spreads through contact with infected blood. Fatty liver disease is an excessive accumulation of fat in Liver (more than 5 to 10 percent of the Total liver weight). This condition is most common in overweights, diabetics or people with certain metabolic syndrome. Autoimmune hepatitis is a condition in which the body attacks the liver and inflames or scars the liver. Just like it is in the case of any cancer, an early diagnosis is critical. But when diagnosed at the right time, it can be treated with the help of radiation, medication or surgery (including Liver transplant). [1]

1.2. HERBAL MEDICINES USED IN LIVER DOSORDERS

Herbal medicines are in great demand in the developed world for primary health care because of their efficacy, safety and lesser side effects. A detailed investigation and documentation of plants used in health traditions and pharmacological evaluation of these plants and their taxonomical relatives can lead to the development of invaluable plant based drugs for many dreaded diseases. A large number of plants and purified natural substances have been screened for liver disorders. The main plants used in such formulations are *Eclipta*

alba (bhringraj), Andrographis paniculata (kalmegh), Boerrhaavia diffusa (punarnava), Picrorhija kurroa (kutaki), Cichorium intybus (kasani), Phylanthus niruri (Bhumyamlaki), Tephrosia purpurea (Sharpunkha), Zingiber officinale (Shunthi) etc. and it is found that bhringraj is more likely to be used in the hepatoprotective formulations. Most of the preparations used for the treatment of Liver disorders are formulated as liquid dosage forms. The major companies involved in manufacture of these herbal formulations, are Himalaya Drug Company, Charak Pharmaceuticals Pvt.Ltd., Dabur India Ltd., etc. The reported side effects of these herbal formulations were found to be negligible. Nevertheless, much additional work is needed to open up new biomedical application of these plants. [2]

Some of the main Medicinal plants in the Liver- protective formulations are shown in the Table 1.1.

Table 1.1. Medicinal plants commonly used in the Liver- protective formulations [3]

Common name	Botanical name	Family	Part used	Distribution
Kasamard	*Cassia occidentalis*	Caesalpinaceae	Whole plant	Throughout India, distributed in the waste lands.
Kasani	*Cichorium intybus*	Asteraceae	Whole plant	Cultivated throughout India
Katuki	*Picrorhiza scrophulariflora*	Scrophulariaceae	Dried rhizomes	In the Himalayas from Kashmir to Sikkim
Amrita	*Tinospora cordifolia*	Menispermaceae	Stem	Throughout India in forest
Arjuna	*Termenalia arjuna*	Combretaceae	Bark	Throughout India
Aswangdha	*Withania somnifera*	Solanaceae	Root,leaves	Throughout drier parts of India
Amalaki	*Emblica officinalis*	Euphorbiaceae	Roots, bark, leaves, fruits	Throughout India in deciduous forests and on hill slopes up to 200m

Parpoti	*Zanonia indica*	Cucurbitaceae	Leaves, fruit	Throughout India in forests
Punarnava	*Boerhavia diffusa*	Nyctaginaceae	Whole plant	Throughout in India as weeds Throughout in India as weed
Hanspadi	*Desmodium triflorum*	Fabaceae	Whole plant	Throughout in India as weed
Haritaki	*Terminalia chebula*	Combretaceae	Fruit	Throughout India in deciduous forest
Haridra	*Curcuma longa*	Zingiberaceae	Rhizomes	Throughout India
Kalmegh	*Andrographis paniculata*	Acanthacae	Whole plant	Throughout India in plains and also in forests
Kurchi	*Halorhenna pubescens*	Apocynaceae	Bark, leaves, seed	Throughout India in deciduous forests up to 900m
Chirata	*Swertia chirayita*	Gentianaceae	Whole plant	In the temperate Himalayas, especially in Nepal
Vasaka	*Adhatada vasika*	Acanthaceae	Leaves, root, flower, stem, bark	Throughout India upto an altitude of 1300m

Most popular hepatoprotective herbal formulations contain the following plants which have been proven to have Hepatoprotective effects are:

1. Andrographis paniculata (Kalmegh)

Andrographolide, the active constituent reported to be present in *Andrographis paniculata*, showed a significant dose dependent protective activity against paracetamol-induced toxicity on ex vivo preparation of isolated rat hepatocytes. It has been reported that it significantly increases the percent viability of the hepatocytes as tested by tryptan blue exclusion and oxygen uptake tests. It completely antagonized the toxic effects of

paracetamol on certain enzymes (GOT, GPT and alkaline phosphates) in serum as well as in isolated hepatic cells. Andrographolide was found to be more potent than silymarin, a standard hepatoprotective agent. [3]

2. Boerhavia diffusa (Punarnava)

It has been reported that an alcoholic extract of whole plant *Boerhavia diffusa,* given orally exhibits hepatoprotective activity against experimentally induced carbon tetrachloride hepatotoxicity in rats and mice. Also that the extract produces an increase in normal bile flow in rats suggesting a strong choleretic activity. The extract does not show any signs of toxicity up to an oral dose of 2g/kg in mice. [3]

3. Eclipta alba (Bhringraj)

The hepatoprotective effect of the ethanol/water (1:1) extract of *Eclipta alba* has been studied at subcellular levels in rats against (CCl_4) -induced hepatotoxicity. It has been reported that the loss of hepatic lysomal acid phosphatase and alkaline phosphatase by (CCl_4) was significantly restored by Ea. Hepatoprotective activity of Ea is by regulating the levels of hepatic microsomal drug metabolizing enzymes. [3]

4. Picrorhija kurroa (Katuki)

Picrorrhiza Kurroa is one of the herbs recommend to support the liver not only in everyday situations, but in cases where severe viral infections exists: a study by Vaidya et al found protection against viral hepatitis, and other studies reported have demonstrated its helpfulness in protecting against alcohol. [3]

5. Cichorium intybus (Kasani)

It has been reported to be a popular ayurvedic remedy for the treatment of liver diseases. It is commonly known as kasani and it is a part of polyherbal formulations used in the treatment of liver diseases. It was reported that an optimum properties have been seen with

a dose of 75 mg/kg given 30 min after CCl_4 intoxication. In preclinical studies, its alcoholic extract was reported to be effective against chlorpromazine induced intoxication. [3]

6. Tephrosia purpurea (Sharpunkha)

It has been reported that an alkaline preparation of *Tephrosia purpurea* is used in the treatment of liver and spleen diseases, which has been shown to be protective against CCl_4 and D-galactosamine poisoning. [3]

7. Zingiber officinale (Shunthi)

It has been reported that *Zingiber officinale can be* used as an antiemetic. Its adsorbent aromatic and carminative properties on entero-intestinal tract have been reported to cause adsorption of toxins and acids enhanced gastric motility. [3]

8. Phylanthus amaris (Bhumyamlaki)

Main application of Bhumyamlaki has been reported to be in viral infection of the liver, specifically Hepatitis B. Thyagaran et al. reported that 22 to 37 cases of hepatitis B were cured after using the herb for a month.

Herbal medicines are now in great demand in the developing world for primary health care not because they are inexpensive but also for better cultural acceptability, better compatibility, with the human body and minimal side effects. Global acceptance of Ayurveda is gearing up there has been a steep rise in the demand for medicinal plants from India. Presently the United States is the largest market for Indian botanical products accounting for about 50% of the total exports. However recent findings indicate that all herbal medicines may not be safe. Globally, there have been concerted efforts to monitor quality and regulate the growing business of herbal drugs and traditional medicine. Health authorities and governments of various nations like United States congress, US Food and Drug Administration (FDA) and World Health Organization (WHO) are keen regarding traditional medicine and have been active in creating strategies, guidelines and standards of botanical medicines. WHO, USFDA, European Scientific Cooperative on Phytomedicine (ESCOP), have published standard sets of guidelines to address the concerns. The

publication of Indian Herbal Pharmacopoeia and Ayurvedic Pharmacopoeia are a positive step towards the standardization of herbal drugs. Regulatory norms have to be stringent for nutraceutical preparations and herbal drugs so that the variation between similar contents in different formulations may cease and a scientific approach with definite constituents may be possible for herbals as well. [2]

1.3. INTRODUCTION TO HPTLC

1.3.1. Principles of HPTLC [4]

- Planner chromatography is a multistage distribution process. It is a form of liquid chromatography in which the stationary phase is supported on a planer surface rather than a 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, aluminium, or plastic support) and the liquid mobile phase that moves along the stationary phase.
- High performance thin layer chromatography has developed to the extent that separation and quantification can provide results that are comparable with other analytical methods such as 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.

The main differences between HPTLC and TLC methods are sorted out in the Table 1.2.

Table 1.2. Difference between HPTLC and TLC [4]

Parameters	HPTLC	TLC

Layer of Sorbent	100µm	250µm
Particle size	5-6 µm	10-12 µm
Pore diameter	60 Å	60-100 Å
Sensitivity limit	In Upper pg	In ng
Efficiency	High due to smaller particle size generated	Less
Separations	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 requires less amount of mobile phase	More amount
Sample spotting	Auto sampler	Manual spotting
Scanning	Use of UV/ Visible/ Fluorescence scanner scans the entire chromatogram qualitatively and quantitatively and the scanner is an advanced type of densitometer	Not possible

Mechanisms of HPTLC separation

- Adsorption
- Partition
- Ion-exchange

1.3.2. Features of HPTLC [4]

- 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

1.3.2. Steps involved in HPTLC [4]

The general steps involved in HPTLC have been shown in a flow chart in Figure 1.1.

1. Selection of chromatographic layer (stationary phase)

2. Sample and standard preparation

3. Chromatographic Plate pre-washing

4. Chromatographic Plate pre-conditioning

5. Application of sample and standard

6. Chromatographic development

7. Detection of spots

8. Scanning

9. Documentation of chromatographic plate

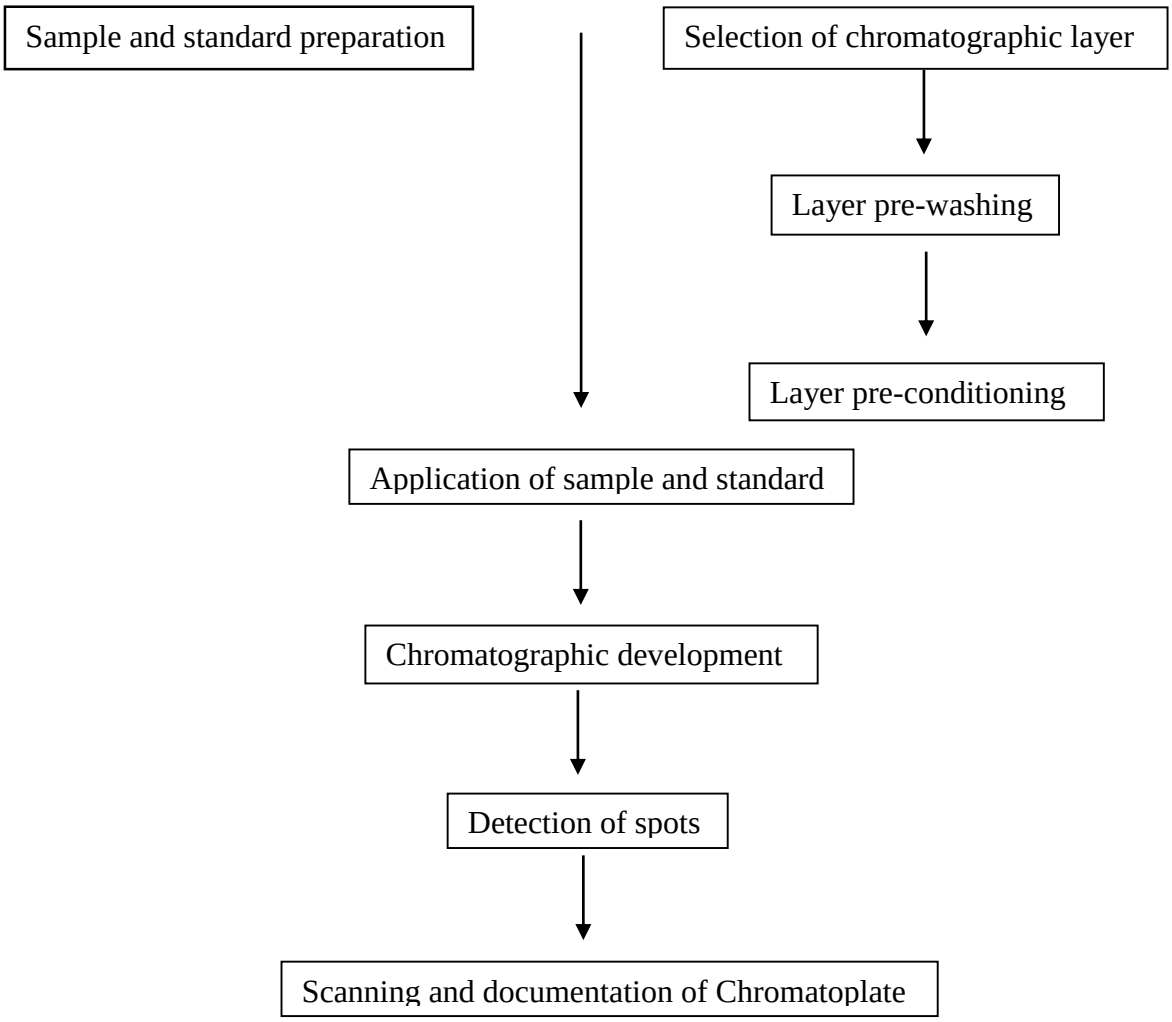


Figure 1.1. Steps involved in HPTLC

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. Pre-washing and activation of pre-coated plates

- 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

3. 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

4. Selection of mobile phase

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

- Trial and error
- Practical experience
- Literature
- 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
- 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

5. Selection of 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.

The types of HPTLC chambers are:

- Nu-chamber
- Ns-chamber
- Twin-through chamber
- Su-chamber
- Ss- chamber
- Horizontal chamber
- U-chamber
- Automatic development chamber (ADC)
- Vario chamber
- Forced flow development chamber (OPLC)

Amongst these Twin-Trough chamber is most commonly used and it requires only 10-15 ml of mobile phase.

6. Pre-conditioning (Chamber saturation)

Unsaturated chamber takes longer time for run and may lead to high R_f values. Saturation of chamber is done by lining with filter paper for ~30 minutes prior to development which allows uniform distribution of solvent vapors in the chamber, so less solvent is required for the sample to travel

7. Chromatographic development and drying

- 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

8. Detection and visualization

The types of detection in HPTLC are shown in the Figure 1.2.

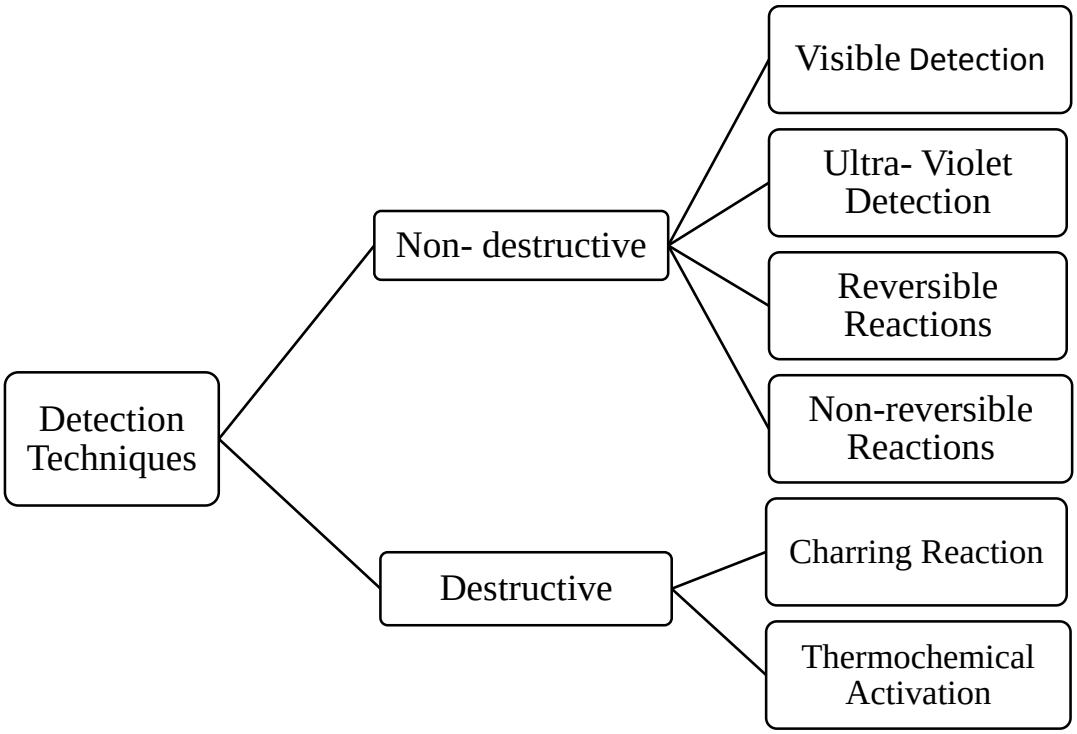


Figure 1.2. Detection methods in HPTLC

- Detection under UV light gives benefit of no- destruction.
- 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 GF
- Non UV absorbing compounds can be visualized by dipping the plates in 0.1% iodine solution
- When individual component does not respond to UV – derivatisation is required for detection

9. Quantification [5]

1) 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 spectro- densitometric 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. Statistics such as RSD or CV are reported automatically.

2) Video imaging and Densitometry

The developed chromatogram is illuminated from above with visible, 254 nm (UV) or 366 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.

1.4. INTRODUCTION TO HPLC

1.4.1. Principle:

- High Performance Liquid Chromatography, or HPLC, is the most common analytical separation tool and is used in many aspects of drug manufacture and research. HPLC is used for:

1. Qualitative and quantitative analysis of unknown mixtures – determining what is there, and how much.
2. Separation of mixtures for later analysis –preparative HPLC.

- Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity.
- In chromatography a small volume of a mixture of chemicals is passed through a column using a solvent and different molecules exit the column at different times – this is called separation.
- The separation of a compound involves its physical interaction with a stationary phase and a mobile phase. In chromatography a tube is filled with stationary phase (typically surface-modified silica particles or silica "gel") and a mobile phase (solvent) is passed through the system. In HPLC the stationary phase is extremely small.
- Even though the stationary phase is small, a small, high-surface-area stationary phase maximizes the interaction between the substance to be separated and the stationary phase, which results in better separation. [6]

1.4.2. HPLC Instrumentation and working

- The primary parts of an HPLC are a solvent PUMP, an injector, the column, and a detector/recorder.
- Each of these components are connected in a series to each other by steel tubing. The pump controls the flow of solvent through the system.
- Upon leaving the pump, solvent enters the injector, then passes through the column, and finally through the optical unit of a detector.

- The injector allows a convenient and controlled introduction of sample directly onto the column, while the detector indicates when a particular analyte (also called a band) elutes (or leaves the column).
- If desired, the separated components can be individually collected for later analysis. The two important things to know about each analyte appearing as a peak in the chromatogram are 1 the retention time and 2 the peak area.

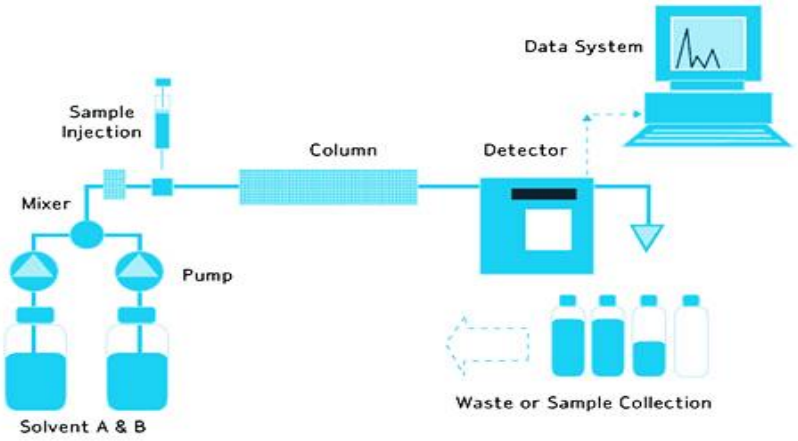


Figure 1.3. Schematic diagram of HPLC

- The stationary phase in RP- HPLC comprises high surface area particles of silica that have a 'greasy' coating (just one molecule thick!) on the surface. A chromatographic separation works in the following way:

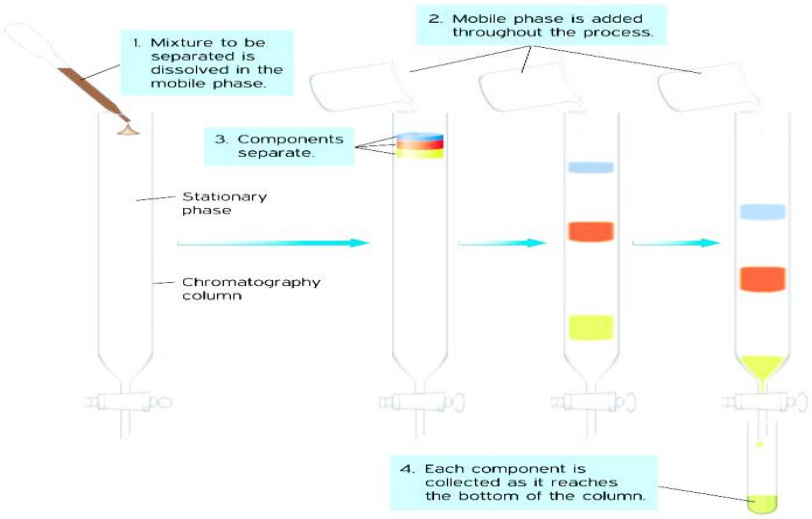


Figure 1.4. Diagram showing the chromatographic separation inside the RP- HPLC column

- The most important pieces of information needed for each peak are the retention time (informs about what the analyte is) and the peak area (informs about the amount of analyte).
- The retention time should be the same for the standard and the sample to separate the analyte from the other components of the sample. [6]

1. Preparation of the sample and standard solutions with specific concentrations and other require solutions, like Mobile phase, for the analysis.

2. Column Equilibration and Blank Run

3. Connect the guard and the column to the solvent delivery system according to the HPLC system requirements and equilibrate under the following initial conditions.

a. Solvent

b. Flow rate (in mL/min) (see Note 6)

c. Detection wavelength (see Note 7)

d. Temperature (see Note 8)

4. Once a stable baseline is obtained, it is generally advisable to perform two blank runs to ensure proper equilibration of the column.

5. Sample Injection and Analysis

- Once a stable baseline is obtained, and the blank runs are completed, inject sample (either manually or via an automatic injector) and use a gradient or isocratic elution for the sample (see Note 9). The large majority of components should normally elute within the gradient time.

1.4.3. Factors affecting RP- HPLC Analysis of an Analyte [6]

- Each individual method is relatively straightforward to perform. The scope lies in the wide range of operating parameters that can be changed in order to manipulate the resolution of sample and sample mixtures in RP-HPLC.

- These parameters include the chemistry of column, the column packing geometry, the column dimensions, the ionic additives (that is, the buffers), the organic solvent, the mobile phase flow rate, the gradient time and gradient shape, and the operating temperature, which are described as follows:

1. Column chemistry:

- The most commonly employed experimental procedure for the RP-HPLC analysis generally involves the use of a C18-based column.
- The chromatographic packing materials that are generally used are based on microparticulate porous silica which allows the use of high linear flow velocities resulting in favourable mass transfer properties and rapid analysis times.
- The most commonly used column is C18, whereas n-butyl (C4) and n-octyl (C8) also find important application based on the type of analyte being analysed.

2. Column dimensions:

- The desired level of efficiency and sample loading size determines the dimension of the column to be used. For small analyte molecules, increased resolution will be obtained with increases in column length. For example, for applications such as tryptic mapping, column lengths between 15–25 cm and id of 4.6 mm are generally employed.
- However, for larger analyte molecules, low mass recovery and loss of biological activity may result with these columns as a result of irreversible binding and/or denaturation (in case of proteins). In these cases, shorter columns of between 2 and 20 cm in length can be used.
- For preparative applications in the 1–500 mg scale, such as the purification of synthetic peptides,so-called semipreparative columns of dimensions 30 cm × 1 cm id and preparative columns of 30 cm × 2 cm id can be used.
- The selection of the internal diameter of the column is based on the sample capacity and detection sensitivity.
- Whereas most analytical applications are carried out with columns of internal diameter of 4.6 mm i.d., for samples derived from previously unknown analytes where there is a limited supply of material, the task is to maximize the detection

sensitivity. In these cases, the use of narrow bore columns of 1 or 2 mm i.d. can be used that allow the elution and recovery of samples in much smaller volumes of solvent.

- Capillary chromatography is also finding increasing application where capillary columns of internal diameter between 0.2–0.4 mm and column length of 15 cm result in the analysis of femto-mole of sample.

3. Column packing geometry:

- The geometry of the particle in terms of the particle diameter and pore size,is also an important feature of the packing material.
- Improved resolution can be achieved by decreasing the particle diameter and the most commonly used range of particle diameters for analytical scale RP-HPLC is 3–5 µm. There are also examples of the use of nonporous particles of smaller diameter.
- For preparative scale separations, 10–20 µm particles are utilized.

  The pore size of RP-HPLC sorbents is also an important factor that must be considered.

1. **Ionic additive:**
   - RP-HPLC is generally carried out with a mobile phase with Phosphate or Acetate buffers. However, for high sensitivity applications, the amount of Acetate buffer can be adjusted downward because o- phosphoric acid, perchloric acid, formic acid, hydrochloric acid, acetic acid, and hepta-flouro-butyric acid have also been used.
2. **Organic solvent:**
   - One of the most powerful characteristics of RP-HPLC is the ability to manipulate solute retention and resolution through changes in the composition of the mobile phase. In RP-HPLC, the analyte retention is a result of the interactions with the column packing.
   - The three most commonly employed organic solvents in RP-HPLC are acetonitrile, methanol, and 2-propanol, which all exhibit high optical transparency in the detection wavelengths used for analysis.
   - Acetonitrile provides the lowest viscosity solvent mixtures and 2-propanol is the strongest eluent

3. Mobile phase flow- rate:

The typical experiment with an analytical scale column would utilize flow rates ranging between 0.5–2.0 mL/min. With microbore columns (1–2 mm id) flow rates of 50–250 µL/min are used,whereas for capillary columns of 0.2–0.4 mm id,flow rates of 1–4 µL/min are applied. At the preparative end of the scale with columns of 10–20 mm id, flow rates ranging between 5–20 mL/min are required.

4. Detection of analytes can be carried out at its specified wavelength maxima value using the U.V detectors. The use of photodiode array detectors can enhance the detection capabilities by the on-line accumulation of complete solute spectra. The spectra can then be used to identify the peaks specifically on the basis of spectral characteristics and for the assessment of peak purity.

5. Operating temperature:

The operating temperature can also be used to manipulate resolution. Although the separation of specific analyte is normally carried out at specific temperature, solute retention in RP-HPLC is influenced by temperature through changes in solvent viscosity. However, if the efficient recovery of both mass and biological activity is of paramount importance,the use of elevated temperatures is not an option.

6. Gradient time and gradient temperature:

The choice of gradient conditions will depend on the nature of the molecules of interest. Generally the use of longer gradient times provides improved separation. However, these conditions also increase the residence time of the solute at the column surface, which may then result in an increase in the degree of degradation of the analyte as well as the column surface.

1.4.4. Advantages of RP- HPLC over other techniques [6]

RP-HPLC is a very powerful technique because of a number of factors that include:

1. The excellent resolution that can be achieved under a wide range of chromatographic conditions for very closely related molecules as well as structurally quite distinct molecules;

2. The experimental ease with which chromatographic selectivity can be manipulated through changes in mobile phase characteristics;

3. The generally high recoveries and, hence, high productivity; and

4. The excellent reproducibility of repetitive separations carried out over a long period of time, which is caused partly by the stability of the sorbent materials under a wide range of mobile phase conditions.

1.5. INTRODUCTION TO METHOD VALIDATION [7]

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

Validation is an act of proving that any procedure, process, equipment, material, activity or system performs as expected under given set of conditions and also give the required accuracy, precision, sensitivity, ruggedness etc. When extended to an analytical procedure, depending upon the application, it means that a method works reproducibly, when carried out by some different persons, in same or different laboratories, using different reagents, different equipments, etc.

For analytical method validation of pharmaceuticals, guidelines from the International Conference on Harmonization (ICH), United States Food and Drug Administration (US FDA), American Association of Official Analytical Chemists (AOAC) United States Pharmacopoeia (USP), and International Union of Pure and Applied Chemists (IUPAC) provide a framework for performing such validations in a more efficient and productive manner.

1.5.1. Data Elements Required for Validation

Both the USP and ICH recognize that is it not always necessary to evaluate each and every analytical performance parameter. The type of method and its intended use dictates which parameters needs to be investigated, as illustrated below:

Table 1.3. ICH Validation Guideline [8]

Type of analytical procedure characteristics	Identification	Testing for impurities, quantitate limit		Assay, dissolution (measurement only)
1. Accuracy	-	+	-	+
2. Precision				
2.1. Repeatability	-	+	-	+
2.2. Interm Precision	-	+(1)	-	+(1)
2.3. Reproducibility	-	-(2)	-	-(2)
3. Specificity	+	+	+	+(4)
4. Detection limit	-	-	+	-
5. Quantitation limit	-	+	-	-
6. Linearity	-	+	-	+
7. Range	-	+	-	+

- Signifies that this characteristic is not normally evaluated.

+ Signifies that this characteristic is normally evaluated.

(1) Intermediate precision is not needed in some case, when reproducibility is checked.

(2) May be needed in some cases.

(3) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).

(4) May not be needed in some cases.

The different parameters which are to be considered in analytical method validation of an as per USP and ICH guidelines can be summarized as:

1. Specificity
2. Linearity
3. Range
4. Accuracy
5. Precision
6. Detection Limit
7. Quntitation Limit
8. Robustness
9. System Suitability Testing

1.5.1.1. Linearity

The linearity of an analytical method is its ability to elicit test results that are directly or by a well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range.

1.5.1.2. Range

The range of analytical method is the interval between upper and lower level of analyte including levels that have been demonstrated to be determining with precision and accuracy using the method. The range is normally expressed in the same units as the test results (e.g., percentage, parts per million) obtained by the analytical method. Results were expressed in terms of Correlation co-efficient.

1.5.1.3. Accuracy

Accuracy of an analysis is determined by systemic error involved. It is defined as closeness of agreement between the actual (true) value and analytical value and obtained by applying test method for a number of times.

Accuracy may often be expressed as % Recovery by the assay of known, added amount of analyte. It is measure of the exactness of the analytical method.

1.5.1.4. Precision

The precision is measure of either the degree of reproducibility or repeatability of analytical method. As per ICH guideline, precision of a method is "express the closeness of agreement (degree of scatter) between a series of successive measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions".

It provides an indication of random error. The precision of an analytical method is usually expressed as the standard deviation, Relative standard deviation or coefficient of variance of a series of measurements.

The standard deviation is calculated from the following formula

$$SD = \sqrt{\frac{\sum (X_i - X)^2}{N - 1}}$$

Where,

Xi = Individual measurement in the set

X = Arithmetic mean of the set

N = Number of replicates taken in the set

$$RSD = SD / X$$

%RSD or coefficient of variance (CV) is expressed as

$$\%RSD = CV = (SD \times 100) / X$$

a. Repeatability (Precision on replication):

It is a precision under the same conditions (Same analyst, same apparatus, short interval of time and identical reagents) using same sample.

b. Intraday and Interday Precision:

Variation of results within same day is called Intraday precision and variation of results amongst days is called Interday precision.

1.5.1.5. Limit of Detection (LOD)

It is the lowest amount of analyte in sample that can be detected but not necessarily quantities under the stated experimental conditions. ICH describes three methods for LOD determination:

(I) Based on visual evaluation: It is determined by the analysis of samples with known concentrations of analyte and establishing the minimum level at which the analyte can be reliably detected.

(II) Based on signal to noise ratio: Determination of the signal to noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal to noise ratio of 2:1 or 3:1 is generally considered acceptable for estimating the detection limit.

(III) Based on standard deviation of the response and the slope: The detection limits may be expressed as:

$$DL = 3.3 \sigma / S$$

Where, σ = the standard deviation of the response

S = the slope of calibration curve

1.5.1.6. Limit of Quantification (LOQ)

It is the lowest concentration of analyte in the sample that can be determined with the acceptable precision and accuracy condition. . ICH defines three methods for LOQ determination:

(I) Based on visual evaluation: It is determined by the analysis of samples with known concentrations of analyte and establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

(II) Based on signal to noise: Determination of the signal to noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably quantified. A signal to noise ratio of 10:1 is generally considered acceptable for estimating the quantities limit.

(III) Based on standard deviation of the response and the slope:

The quantitation limits may be expressed as:

$$DL = 3.3 \sigma / S$$

Where, σ = the standard deviation of the response

S = the slope of calibration curve

1.5.1.7. Limit of Quantification (LOQ)

It is the lowest concentration of analyte in the sample that can be determined with the acceptable precision and accuracy condition. . ICH defines three methods for LOQ determination:

(I) Based on visual evaluation: It is determined by the analysis of samples with known concentrations of analyte and establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

(II) Based on signal to noise: Determination of the signal to noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably quantified. A signal to noise ratio of 10:1 is generally considered acceptable for estimating the quantities limit.

(III) Based on standard deviation of the response and the slope: The quantitation limits may be expressed as:

$$DL = 10 \sigma / S$$

Where, σ = the standard deviation of the response

S = the slope of calibration curve

1.5.1.8. Specificity and Selectivity

Specificity of an analytical method is the ability to measure accurately and specifically the analyte in presence of components that may be expected to be present in the sample matrix.

Specificity is expressed as degree of bias of test results obtained by analysis of sample containing added impurities, degradation product, related chemical compounds or placebo ingredients when compared with test results from samples without added samples. Bias may be expressed as difference in assay results between two group samples. Thus specificity is a measure of the degree of interference in the analysis of complex sample mixture.

Selectivity is the ability of analytical method to differentiate various substances in the sample.

CHAPTER- 2

INTRODUCTION TO FORMULATION

2. INTRODUCTION TO FORMULATION- HEPASAVE SYRUP

The Polyherbal formulation, HEPASAVE SYRUP, is an Ayurvedic Proprietary Medicine, used particularly as a Powerful Hepato-protective, an Antioxidant, and a Bitter tonic. The formulation can be administered with a dosage regimen of 10ml three times a day for the Adults and 5 ml three times a day for the Children as prescribed by the concerned physician. The polyherbal formulation remains in the best form when stored below 30˚C and when protect from exposure to Direct Sunlight. The herbal constituents of the formulation include *Amalaki fruit i.e. Phyllanthus emblica Ab., Haritaki fruit i.e., Terminalia chebula Ab., Bibhitaka fruit i.e., Terminalia bellerica AB., Vasa leaf i.e., Adhatoda vasica Ab., Bhunimba herb i.e., Andrographis paniculata, Katuka root i.e., Picrorrhiza kurroa, Nimba stem bark i.e., Azadirachta indica Ab, Amruta stem i.e., Tinospora cordifolia, Sarpankha herb i.e., Tephrosia purpurea Ab.,* and also a Flavored syrup base. The constituents under study for this project were Gallic acid (*Amalaki fruit i.e., Phyllanthus emblica Ab*.), Gallic acid (*Haritaki fruit i.e., Terminalia chebula Ab.*), Gallic acid (*Bibhitaka fruit i.e., Terminalia bellerica Ab*.), Vasicine (*Vasa leaf i.e., Adhatoda vasica Ab*.), Andrographolide (*Bhunimba herb i.e., Andrographis paniculata*), Kutkin (*Katuka root i.e., Picrorrhiza kurroa*). An introduction to the plants included in the formulation as herbal components is given as follows:

2.1. MEDICINAL PLANTS USED IN THE PREPARATION OF HEPASAVE SYRUP

A Pharmacognostical description of each medicinal plant used in the formulation includes:

2.1.1. Amalaki fruit i.e., Phyllanthus emblica Ab.

Biological source: The drug consists of fruits obtained from the plant known as *Phyllanthus emblica* (Family: Euphorbiaceae/ Phyllanthaceae), which grows in tropical and subtropical parts of China, India, Indonesia, and Malay Peninsula. [10]

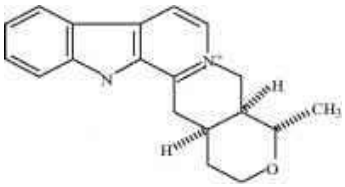
Synonym: *Emblica officinalis, Emblic myrobalan*, Emblic, Emblic myrobalan, Myrobalan, Indian gooseberry, Malacca tree, or Amla (from Sanskrit word Amalaki). [10]

Vernacular name: Indian Goose berry (English), Amla (Hindi), Nelli (Malayalam), Usiri Kaya (Telugu) [10]

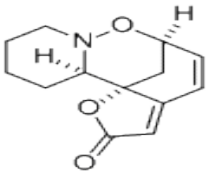
Morphology: It is a small to medium sized tree grown up to 8 to 18 meters in height, and having asymmetrical shape with spreading branches. Greenish yellow colored flowers and fruits are present. The fruits are fleshy with sour, astringent taste, spherical in shape with six vertical bands. [11]

Figure 2.1.: Morphology of (a) *Phyllanthus emblica tree,* (b) *Phyllanthus emblica fruit*

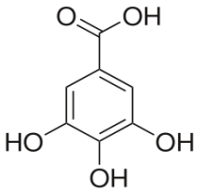
Chemical constituents: [11]



(a)



(b)



(c)

Figure 2.2. The chemical structure of (a) Phyllantine, (b)Phyllantidine and (c)Gallic acid

Traditional uses: Treatment of inflammatory problems anywhere in the body, of lung infections, of asthma, as a brain tonic, of urinary tract infections, to prevent hair loss and nourish the hair, in conjunctivitis, as a skin care ingredient, protection against heat or light, to strengthen digestion, absorption and assimilation of food, for assimilation of iron for healthy blood, to calm mild to moderate hyper-acidity, stimulate the liver, eliminate toxins from the body, maintain cholesterol, to sharpen the intellect and mental functioning, to improve blood circulation, to strengthen and nourish the lungs, to relieve from constipation, to keep menstruation regular and healthy, for enhancement of fertility and conception, to support natural diuretic action, supports immunity of the skin against bacterial infection and helps enhance glow and luster, strengthens the hair follicles against hair thinning with age, for flushing out chemicals and additives from the liver, in regeneration of cells, to improve vision, to strengthen the muscles, to reduce diseases and slowing of aging process. [9]

Pharmacological activities: Antagonistic activity against genotoxic chemicals, Anticlastogenicity in vitro, Antimicrobial activity in vitro, Antioxidant activity in vitro, Anti-inflammatory activity in vivo and in vivo, Hepatoprotective activity, Prevention of hepatocarcinogenesis in vitro and in vivo, Hypolipidaemic activity in vivo and in vitro, Enhancer of natural killer cell (NK) activity in vitro, Inhibition of human immunodeficiency virus- 1 (HIV-1) reverse transcriptase in vitro, Prevention of experimental acute pancreatitis in vivo, Protection against radiation-induced chromosome damage in vitro. [12]

2.1.2. Haritaki fruit i.e, Terminalia chebula Ab.

Biological source: The drug consists of fruits obtained from plant known as *Terminalia chebula Ab* (Family: Combritaceae), native to Southern Asia from India and Nepal, China, Sri Lanka, Malaysia and Vietnam. [14]

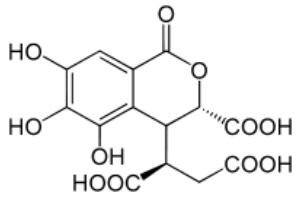
Synonym: Yellow Myrobalan, Chebulic Myrobalan. [14]

Vernacular name: Harra, Harad (Hindi), Hirad (Marathi), Katukka (Malayalam), Kayastha, Jivapriya (Sanskrit). [14]

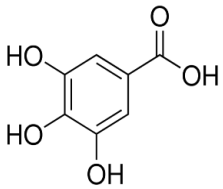
Morphology: *T. chebula* is a medium-sized deciduous tree with a height of up to 30 m, wide spreading branches and a broad roundish crown. The fruit are glabrous, ellipsoids ovoid drupes, yellow to orange brown in colour, encloses a single angle stone. [14]

Figure 2.3. Morphology of (a) *Terminalia chebula tree,* (b) *Terminalia chebula fruit*

Chemical constituents: [14]



(a)



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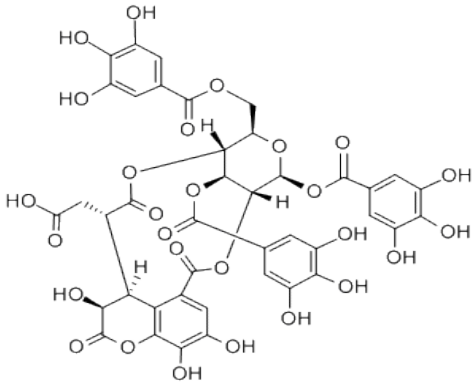
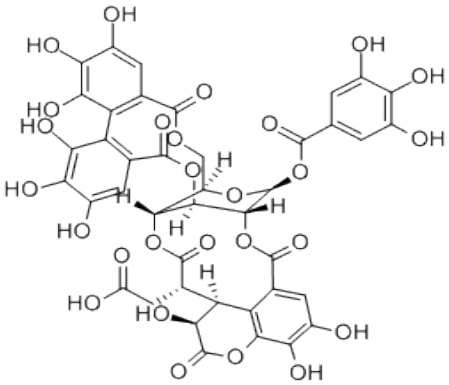


(c)



(d)

Figure 2.4. Chemical structure of (a) Chebulic acid, (b) Gallic acid, (c) Chebulinic acid, and (d) Chebulagic acid

Traditional uses: To reduce swelling, Hasten the healing process, Clean the wounds and ulcers, Astringent for loose gums, gastrointestinal ailments, tumours, enlargement of liver-spleen, in anaemia, in asthma, in hepatitis and obesity, improves memory, in urinary stones, improves appetite and helps in digestion. [15]

Pharmacological activities: Antibacterial activity, Antifungal activity, Antiamoebic and immunomodulatory activities, Antiplasmodial activity, Molluscicidal activity, Anthelmintic activity, Antiviral activity, Antimutagenic and anticarcinogenic activities, Antioxidant activity, Antidiabetic and retinoprotective activities, Antianaphylactic and adaptogenic activities, Antinociceptive activity, Antiulcerogenic activity, Anti-arthritic activity, Wound healing activity, Cytoprotective and antiaging activities, Radioprotective activity, Cardioprotective activity, Hepatoprotective activity, Chemomopreventive activity, Hypolipidemic and hypocholesterolemic activities, Antispermatogenic activity. [14]

2.1.3. Bibhitaka fruit i.e, Terminalia bellerica Ab.

Biological source: The drug consists of fruits obtained from the plant known as *Terminalia bellerica* (Family: Combritaceae), growing wild in India, Sri lanka, and S.E. Asia. [16]

Synonym: Beleric Myrobalan. [16]

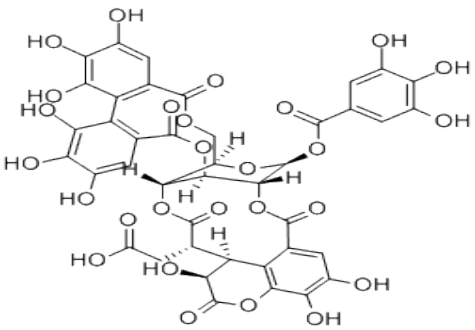
Vernacular names: Bibhitaki (Sanskrit), Bahera, Bahira, Bahura (Hindi). [16]

Morphology: It is a large deciduous tree with petiolate, broadly elliptic leaves, which are clustered towards the end of branches. The fruits are green and inflated when young and yellowish and shrink (nearly seen as ribbed) when mature. The nut is stony. [16]

Figure 2.5. Morphology of (a) Terminalia *bellerica tree,* (b) *Terminalia bellerica fruit*

Chemical constituents:

(a) (b)



(c)

Figure 2.6. Chemical structure of (a) Gallic acid, (b) Phenylembellin and (c) Chebulaginic acid

Traditional uses: In Catarrhal condition, congestion, cough, bronchitis, asthma, sore throats, laxative action, in treating piles, diarrhoea, dysentery, parasitic worms, it has liver protecting action, used in jaundice and gall bladder stones, to lower cholesterol level, as Antiseptic, anti-inflammatory, antimicrobial, for cuts, wounds, skin diseases, corneal ulcers. [16]

Pharmacological activities: In Acute and Sub-acute Toxicities, Antioxidant, Antimicrobial and Toxicity Studies, Invitro glucoamylase activity, Antidiarrhoeal activity, Activities of Accessory reproductive ducts in male rats, Streptozotocin induced Antidiabetic activity, Analgesic activity, Alloxan induced hyperglycemic and antioxidant activity, Immune response Invitro, Antihypertensive Effect, Anti salmonella activity, Anti-Spasmodic and Bronchodialatory Properties, Hepatoprotective activity, Antimicrobial activity. [16]

2.1.4. Vasa leaf i.e, Adhatoda vasica Ab.

Biological source: The drug consists of leaves obtained from the plants known as *Adhatoda vasia Nees* (Family: Acanthaceace), grown throughout Indo-Malayan region, Sri Lanka, Upper and Lower Myanmar, Southern China, Laos, and the Malay- Peninsular and Indonesian Archipelago. [17]

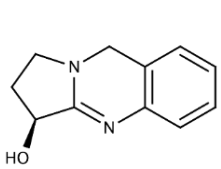
Synonym: Beleric Myrobalan. [17]

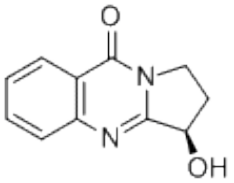
Vernacular name: Malabar Nut, Adulsa, Adhatoda, Vasa, Vasaka (English), Bahera (Hindi), Bhomora (Assamese), Bahedan (Gujarati). [17]

Morphology: It is a shrub with lance-shaped leaves 10 to 15 centimetres in length by 4 centimetres wide. The leaves are oppositely arranged, smooth-edged, and borne on short petioles. When dry, they are dull brownish-green coloured, and are bitter in taste. [18]

Figure 2.7. Morphology of *Adhatoda vasica plant*

Chemical constituents:





(a)Vasicine (b)Vasicinone

Figure 2.8. Chemical structure of (a) Vasicine and (b) Vasicinone

Traditional uses: In Asthma, fever, Bronchitis, Chronic coughs, as an Expectorant, in Skin conditons, in Tuberculosis, Malaria, in Sparain, in constipation, stomach ache and Ulcers.[18]

Pharmacological activities: Antibacterial Activity, Bronchodilatory Activity, Anti-Allergic, Anti-Asthmatic Activities, Radioprotective Effects, Antimutagenic Activity, Anti-Tubercular Activity, Anti-Ulcer Activity, Allopathic Activity, Anti-feedant and Toxic Activity, Sucrase Inhibitory Activity, Anti-inflammatory Activity, Abortifacient Activity. [18]

2.1.5. Bhunimba herb i.e, Andrographis paniculata

Biological source: The drug consists of the whole plant known as *Andrographis paniculata (Burm. f. Wall.) ex Nees* (Family: Acanthaceae), found throughout the Indian plains from Himachal Pradesh to Assam and Mizoram and all over South India. [19]

Synonym: Swertia chirata Bunch Ham., Swertia chirayayita (Roxb .ex flem) Karst. [18]

Vernacular names: Chriayata (Hindi), Chitretta (English), Gujarati name- Kariyatum. [18]

Morphology: It is an annual plant. Stem is erect, multi-branched, green coloured, 0.3-1.0 m in height, 2- 6 mm in diameter, with wings on the angles of the younger parts, slightly inflated at the nodes. [19]

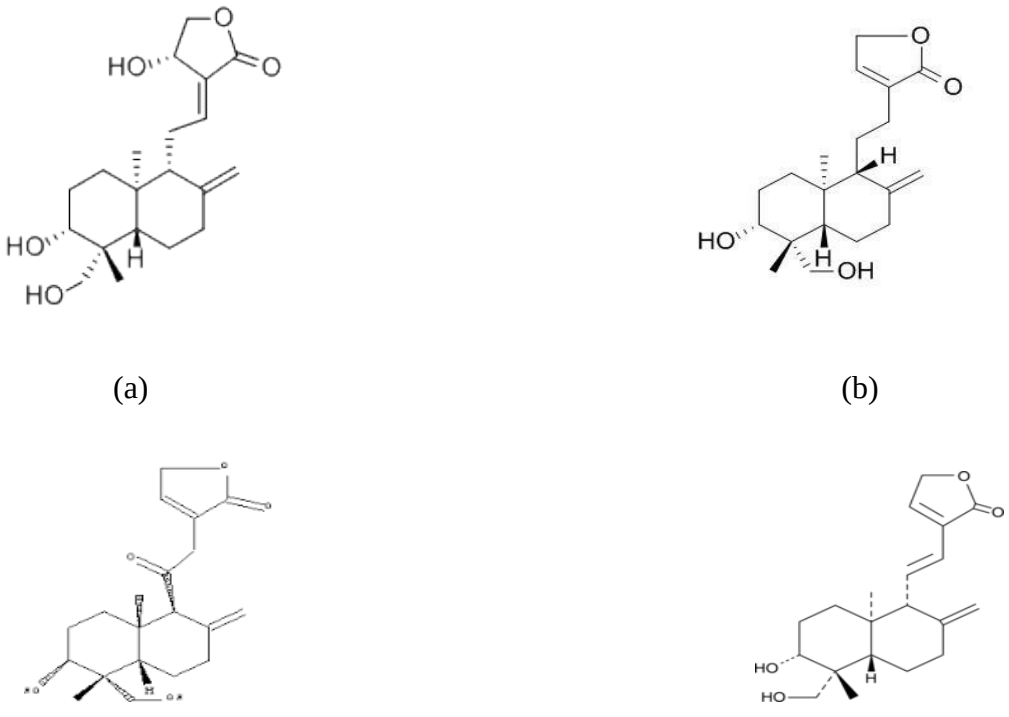
Leaves are opposite; leaf lanceolate shaped, 2-11 cm in length, 0.5-2.5cm apex, glaborous surfaces, dark green from above and gray- green from below; petiole is short or subsessile.

Flowers: terminal and axillary racemes, integrated big panicles, bracts and bracteolates are small, lanceolate, corolla is purplish white, two lipped.

Fruit is capsule, flat, oblong, 1cm in length, slightly glandular. Seeds are 12 small, red in colour, square. [20]

Figure 2.9. Morphology of *Andrographis paniculata herb*

Chemical constituent:



(a) (b)

(c) (d)

Figure 2.10. Chemical structures of (a) Andrographolide, b)14- deoxyandrographolide, (c) 14-deoxy-11-oxoandrographolide, (d)14-deoxy -11, 12-didehydroandrographolide

Traditional use: Antibacterial, antifungal, antiviral, choleretic, hypoglycemic, hypocholesterolemic, adaptogenic, anti-inflammatory, emollient, astringent, diuretic, emmenagogue, gastric and liver tonic, carminative, antihelmintic, and antipyretic, in leprosy, gonorrhea, scabies, boils, skin eruptions, chronic and seasonal fevers, to relieve griping, irregular bowel habits, and loss of appetite, for dyspepsia associated with gaseous distension, in chronic dysentery, it is also an antipyretic, detoxicant, anti-inflammatory, in pharyngolaryngitis, diarrhea, dysentery, cough with thick sputum, carbuncle, sores, and snake bites epidemic encephalitis B, suppurative otitis media, neonatal subcutaneous annular ulcer, vaginitis, cervical erosion, pelvic inflammation, herpes zoster, chicken pox, mumps, neurodermatitis, eczema, and burns. [21]

Pharmacological activities: Anti-malarial activity, anticancer activity, anti-diabetic activity, Liver protection, anti-inflammatory, antithrombotic activity, in cardiovascular diseases, anti- cancer and anti- HIV activity. [22]

2.1.6. Katuka root i.e, Picrorrhiza kurroa

Biological source: The drug consists of the roots obtained from the plant known as *Picrorhiza kurroa* (Family: Scrophulariaceae**), that** grows in the hilly regions of the North-Western Himalayan region from Kashmir to Sikkim of India and Nepal. [23]

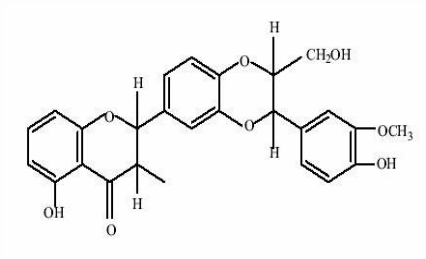
Synonym: Titka kul (Ayurvedic). [23]

Vernacular names: Tikta, Tikta rohini, Katurohini¸ Sutiktaka, Kauka (Sanskrit), Black Hellebore, Hellebore, Yellow gentian (English), Kutki (Hindi), Kadu, Katu (Gujarat). [23]

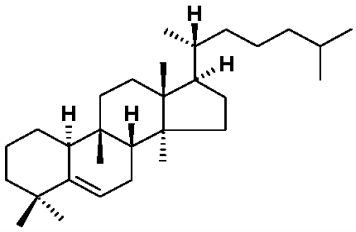
Morphology: The bitter-tasting roots are hard, about 6-10 inches in length, and creeping. The roots are invariably wrinkled in the longitudinal fashion having transverse cracks. They are grayish brown in coloured, while the fracture is tough. Root stacks are irregularly curved as thick as the little finger. [24]

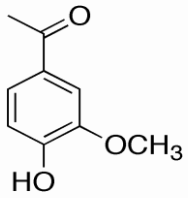
Figure 2.11. Morphology of (a) *Picrorrhiza kurroa plant*, (b) *Picrorrhiza kurroa root*

Chemical constituents:



(a)





(b) (c)

Figure 2.12. Chemical structures of (a) Kutkins (Kutkoside and Picroside), (b) Cucurbitacin and (c)Apocynin

Traditional uses: To treat liver and upper respiratory conditions, treatment of a wide range of conditions, including fevers, chronic diarrhea, constipation, dyspepsia and jaundice. [23]

Pharmacological activities: Antibacterial, antiperiodic, cathartic (in large doses), laxative (in smaller doses) stomachic and bitter tonic, hepatoprotective, anticholestatic (relieves obstruction of bile salts), anti-inflammatory, anti-allergy, antioxidant; modulates the immune system and liver enzyme levels. [24]

2.1.7. Nimba stem bark i.e, Azadirachts indica Ab.

Biological source: The drug consists of stem bark obtained from the plant called *Azadirachta indica* (Family: Maliaceae), cultivated in various parts of the Indian sub-continent. [25]

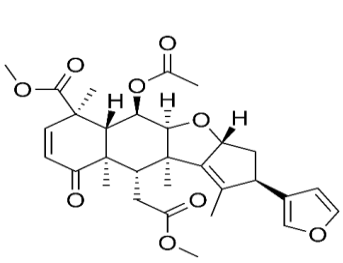
Synonym: Melia azadirachta. A. Juss (Indian Neem/ Indian lilac), M. azedarach (Persian lilac). [25]

Vernacular names: Neem, margosa (English), nimb, nim (Hindi); limbdo (Gujarati), bevu, bevina mana, olle (Kannada). [25]

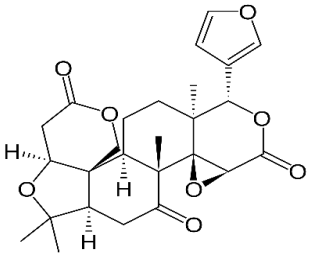
Morphology: A medium to large tree, 15–20 m in height with a clear bole of up to 7 m, bark is tubercled, grayish to dark grey. [25]

Figure 2.13. Morphology of (a) *Azadirachta indica tree*, (b) *Azadirachta indica stem bark*

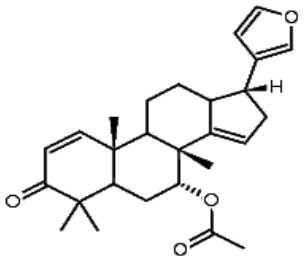
Chemical constituents:

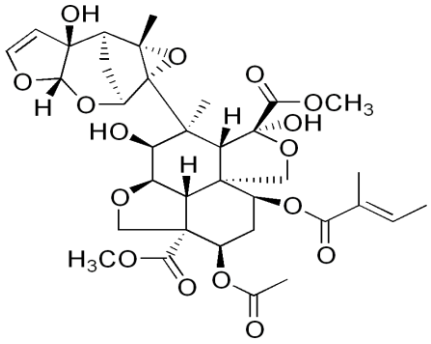


(a)



(b)





(b) (d)

Figure 2.14. Chemical structures of (a)Nimbin, (b)Limonoid, (c)Azadirone, (d) Azadirachtin

Traditional uses: in pest control, cosmetics, medicines, soil amendment, in storage of grains, to treat chickenpox and warts, to increase immunity, to reduce fever caused by malaria, for treating various foot fungi, to work against termites, in curing neuromuscular pains, Twigs of neem as toothbrushes, in toothpastes, in the healing of cuts, burns, earache, sprain and headache, fever, to control fleas & ticks on pets, against skin infections such as acne, psoriasis, scabies, eczema, etc., in treating diabetes, AIDS, cancer, heart disease, herpes, allergies, ulcers, hepatitis and several other diseases. [25]

Pharmacological activities: Anti-inflammatory, antipyretic and analgesic activities, Immunostimulant activity, Hypoglycemic activity, Antiulcer effect, Antifertility effect, Antimalarial activity, Antifungal activity, Antibacterial activity, Antiviral activity, Anticarcinogenic activity, Hepatoprotective activity and Antioxidant activity.

2.1.8. Amruta stem i.e, Tinospora cordifolia

Biological source: The drug consists of the stem obtained from the plant known as *Tinospora cordifolia* (Family: Menispermaceae), indigenous to India, Myanmar, Sri Lanka and China.[26]

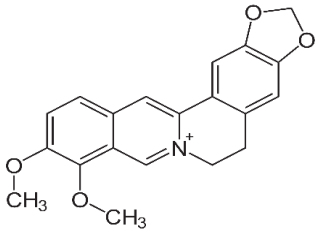
Synonym: Tinospora Gulancha, Guduchi, Amrita, Giloy, Gurcha. [26]

Vernacular names: *Guduchi*, *Amrita*, *Amritavalli*, *Madhuparn* (Sanskrit), *Giloe*, *Gurcha* (Hindi), *Garo, Galac* (Gujarati). [26]

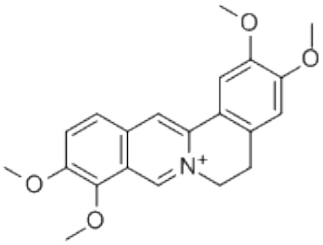
Morphology: The plant is an herbaceous vine, the stems are succulent with long filiform fleshy aerial roots from the branches. Stem appears in varying thicknesses, ranging from 0.6 to 5 cm in diameter; young stems are green with smooth surfaces, swelling at nodes; the older ones are light brown with warty protuberances due to circular lenticels; tastes bitter.[26]

Figure 2.15. Morphology of (a) *Tinospora cordifolia plant*, (b) *Tinospora cordifolia stem*

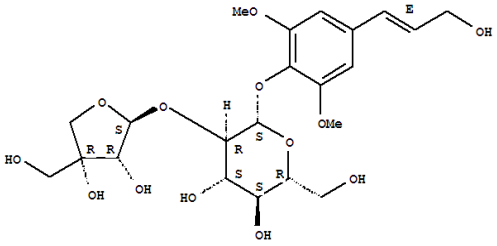
Chemical constituents:



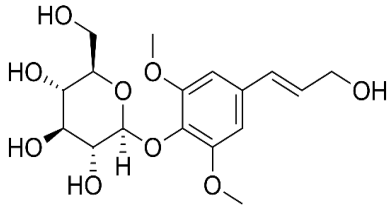
(a)



(b)



(c)



(d)

Figure 2.16. Chemical structures of (a)Berberine, (b)Palmatine, (c)Cordifolioside, (d)Syringin

Traditional uses: For the treatment of different ailments because of its anti-periodic, anti-spasmodic, anti-microbial, anti-osteoporotic, anti-inflammatory, anti-arthritic, anti-allergic, and anti-diabetic properties. [26]

Pharmacological activities: Anti-inflammatory, Anti- arthritic, Anti-osteoporotic activities, Anti-allergic activity, Antioxidant activity, Antineoplastic and Radio-protective activity, Antipyretic and Anti-infective activity, Hepato-protective activity, Anti-Hyperglycemic activity, Immunomodulatory activity, Diuretic activity, Cardioprotective activity, Anti-leprotic activity, Gastro-intestinal and Anti-ulcer activity, Antifertility activity, Osteoprotective activity. [26]

2.1.9. Sarpankha herb i.e, Tephrosia purpurea Ab

Biological source: The drug consists of the whole herb known as *Tephrosia purpurea* (Family: Fabaceae), found throughout India, widely distributed in tropical, sub-tropical and arid regions of the world. [27]

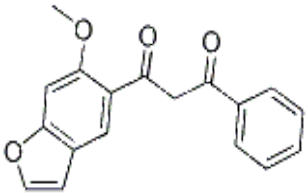
Synonym: *Tephrosia purpurea var. angustata Miq., Tephrosia purpurea var. angustissima (Shuttlew. Ex Chapm.) B.L. Rob.* [27]

Vernacular names: Indian Indigo, Wild Indigo, Purple tephrosia, Common Tephrosia (English), Sarpankha, Dhamasia (Sanskrit), Sarpankh (Hindi), Unnali (Gujarati), Sarpankho, Satavar (Punjabi) [27]

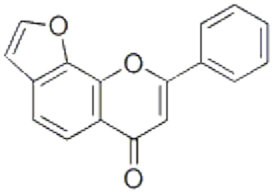
Morphology: Self-generating, erect or spreading perennial herb. It is a small shrub that grows up to 1.5 meters tall. It has bi-pinnate leaves (7cm in length) with 4-9 pairs, obovate, 0.8 – 2 × 0.3 – 0.7 cm; petiole to 1 cm. Pseudo racemes leaf-opposed, 8 cm in length; bract up to 2mm, pubescent; lobes lanceolate; upper lobes 2.5 mm, equal to lower one. Corolla bluish-pink to purple, 8.5 × 8mm, wings 7.5 × 3 mm; staminal sheath 5 mm; filaments 2 mm. Ovary 5 mm, apprised – pubescent; style 3 mm, glabrous. Pod 4 × 0.4 cm; seeds ovoid, 3.5 mm, strophiole in the middle of seed. [27]

Figure 2.17. Morphology of *Tephrosia purpurea herb*

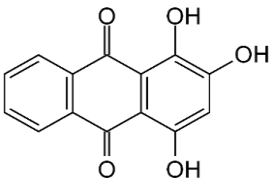
Chemical constituents:



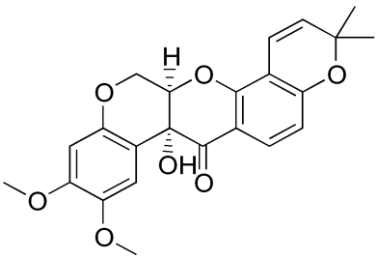
(a)



(b)



(c)



(d)

Figure 2.18. Chemical structures of (a) Pongamol, (b)Lanceolatin, (c) Purpurin, (d) Tephrosin

Traditional uses: To cure wounds, gastro-duodenal disorders, in kidney, liver, spleen, heart and blood related disorders, in bronchitis, boils, pimples and bleeding piles, in vomiting, in dyspepsia, chronic diarrhea, impotency, asthma, diarrhea, gonorrhea, rheumatism, ulcer,

urinary disorders, to cure tumors, ulcers, leprosy, allergic and inflammatory conditions such as rheumatism, asthma and bronchitis. [27]

Pharmacological activities: Roots: Anti-ulcer activity, Anti- carcinogenic and anti-lipid per oxidative activity, Anti-microbial activity, Anti-inflammatory and analgesic activity, Anti-oxidant activity, Hepatoprotective activity, CNS depressant and analgesic activity. Leaves: In cutaneous Toxicity, Spasmolytic activity, Anti-hyperglycemic, Anti-lipid peroxidative activity, Anti-oxidant activity, Anti-Pyretic activity, Anti-hyperlipidemic activity, Anthelmintic activity. Whole plant: Renal oxidative stress, Anti-leishminal activity, Anti-epileptic activity, Anti-carcinogenic activity, Anti-hypercholesterolemic, Anxiolytic activity, Diuretic activity, Anti- diarrheal activity. [27]

A brief introduction to the nine Herbal constituents of Hepasave formulation is given in Table 2.1.

Table 2.1.Components of Hepasave syrup and their brief description

Sr. No.	Component	Biological Source	Chemical component	Use	Quantity (in 10ml)
1.	Amalaki fruit	*Phyllanthus emblica*	Gallic acid, Tannic acid	Astringent, Antioxidant, Bitter, Hepato-protective	130.0 mg
2.	Haritaki fruit	*Terminalia chebula AB*	Gallic acid, Tannic acid	Astringent, Antioxidant, Bitter, Hepato-protective	130.0 mg
3	Bibhitaka f Ruit	*Terminalia bellerica AB*	Gallic acid, Tannic acid	Astringent, Antioxidant, Bitter, Hepato-protective	130.0 mg
4	Vasa leaves	*Adhatoda vasica AB*	Vasicine	Antioxidant, Antiapoptotic, Antiinflammatory	130.0 mg
5	Bhunimba herb	*Andrographis paniculata BHP*	Andrographolide	Hepato-protective, Anti-inflammatory, Anti-apoptotic	130.0 mg
6	Katuka root	*Picrorrhiza kurroa AB*	Kutkin	Antiinflammatory, Hepatoprotective	130.0 mg
7	Nimba stem bark	*Azadirachta indica AB*	Azadirachtin, nimbidin	Antiinflammatory, Antioxidant, Hepatoprotective	130.0 mg
8	Amruta stem	*Tinospora cordifolia AB*	Tinosporic acid, Cordifoliside A to E, Berberine	Hepatoprotective, Antioxidant, Immune amphoteric	130.0 mg
9	Sarpankh herb	*Tephrosia purpurea AB*	Pongamol, lanceolatin A, B	Antioxidant, Antiinflammatory	2.1 mg

2.2. DRUG PROFILE OF BIOMARKERS:

2.2.1. Andrographolide

Andrographolide (3-[2-[decahydro-6-hydroxy-5- (hydroxymethyl)-5,8a- dimethyl-2-methylene-1- napthalenyl]ethylidene]dihydro- 4-hydroxy-2(3H)-furanone), is a labdane diterpenoi of plant origin. [29]

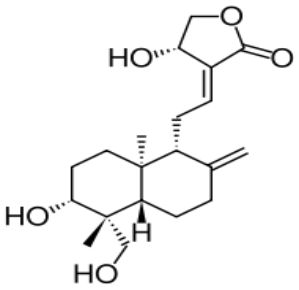


Figure 2.19. Chemical structure of Andrographolide

The different physicochemical properties of Andrographolide are given in Table 2.2.

Table 2.2. Physicochemical properties of Andrographolide

Sr. No.	Physical properties	Result
1	Molecular formula	$C_{20}H_{30}O_5$ [29]
2	IUPAC name	(3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5,8a- dimethyl-2-methylene-1-napthalenyl]ethylidene]dihydro- 4-hydroxy-2(3H)-furanone) [28]
3	Molecular weight	350.45 gm/mol [29]
4	Category	Phytochemical- Labdane diterpenoid (Diterpene lactone) [29] Pharmacological- Anti-inflammatory, Anti-protozoal [29]
5	Appearance	Light greenish or white crystalline powder
6	Solubility [30] i.Dimethyl formamide ii.Methanol	 Highly soluble Highly soluble

	iii. DMSO iv.Ethanol	Soluble Soluble
7	Optical rotation	[α]20/D −126°, c = 1.5 in acetic acid [29]
8	Refractive Index	n^{20}_{D} 1.57 [28]
9	Melting point	229-232 ºC [28]

Category: antioxidant, hepatoprotective, antimicrobial, anticancer, antivenom, anti HIV, antimalarial, antipyretic, antifertility, antidiarrhoeal, antidiabetic, antihyperlipidemic. [28]

Official status: Drug is certified under the USP Reference Standards Committee, as an official Reference Standard (USP 2010), and is also official in Indian Herbal Pharmacopoeia, 1998. [29]

Storage condition: Store in an air-tight container, protected from light at 2ºC to 8 ºC.

Mechanism of action:

Andrographolide is an active constituent extracted and isolated from Andrographis paniculata. Ex vivo, the compound illustrates a considerable dose dependent protective activity against paracetamol-induced toxicity on isolated rat hepatocytes upon administration of andrograoholide. Tryptan blue exclusion and oxygen uptake tests clearly indicate an augmented percent viability of the hepatocytes. The bioactive constitutient also antagonizes toxic effects of CCl4 and acetaminophen on certain enzymes (GOT, GPT and alkaline phosphates) in serum as well as in isolated hepatic cells.[12] evaluated the hepatoprotective efficacy of AP-extract against CCl4 and acetaminophen-induced toxicities on HepG2 cell lines. The results clearly depicted APextract to exert a choleretic effect that reduces the cholestasis and diminishes retention as well as increase the excretion of toxic xenobiotics from liver. Further, it also stimulated immune system to fight against inflammation, is mediated from the release of cytokinin from immunomodulators. [30]

2.2.2. Gallic acid

Gallic acid (3,4,5-trihydroxybenzoic acid) is a trihydroxybenzoic acid (an organic acid), of plant origin. [50]

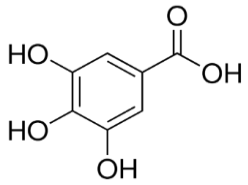


Figure 2.20. Chemical structure of Gallic acid

The different physicochemical properties of Gallic acid are given in Table 2.3.

Table 2.3. Physicochemical properties of Gallic acid

Sr. No.	Physical properties	Result
1	Molecular formula	$C_7H_6O_5$ [50]
2	IUPAC name	3,4,5-trihydroxybenzoic acid [51]
3	Molecular weight	170.120 g/mol [53]
4	Category	Phytochemical- Organic acid [50] Pharmacological- Astringent, Antioxidant, Hepatoprotective [50]
5	Appearance	White crystalline powder
6	Solubility [55] i.Methanol ii.Ethanol iii.Acetone iii.Water	 Soluble Soluble Soluble Sparingly soluble
7	Refractive Index	n^{20}_D 1.69

8	Melting point	260 °C
9	Nature	Acidic

Category: Gastroprtective, Antiulcerogenic, Anti-inflammatory, Hypoglycemic, Hypolipidaemic, Antianaemic, Antibacterial, Antioxidant, Radio-protective. [49]

Official status: Drug is not official in USP, or any other Pharmacopoeia. [52]

Storage condition: Store at -20°C in an air-tight container.

Mechanism of action: Liver is the target organ of Sodium fluoride (NaF) toxicity, and the leakage of hepatic enzymes such as ALT, AST and ALP are commonly used as an indirect biochemical index of hepatocellular damage. NaF intoxication causes a significant increase in the activities of Amino transferases (ALT, AST) and Alkaline phosphatase (ALP), probably resulting from hepatocyte membrane damage. If the liver is injured, its cells spill out the enzymes into blood. The rise of the enzymes activities is probably due to the enhancement of cytoplasmic and/or mitochondrial membranes permeability, it could be expected to occur associated with pathology involving damage or necrosis of hepatocytes on the other hand. [48] Significant decrease in plasma proteins and albumin levels were recorded. The decrease in the proteins levels of fluoride-treated rats might be due to changes in protein synthesis and/or metabolism. The administration of GA with NaF has protected the liver function from NaF intoxication as indicated by the significant restoration of plasma biochemical indicators such as AST, ALT, ALP, proteins and albumin levels. [50]

2.2.3. Kutkin

Kutkin (2-Methoxy-4-(1,1,2-trihydroxyethyl)phenyl beta-D-glucopyranoside 1-cinnamate) is a Bitter glucoside of plant origin. [58]

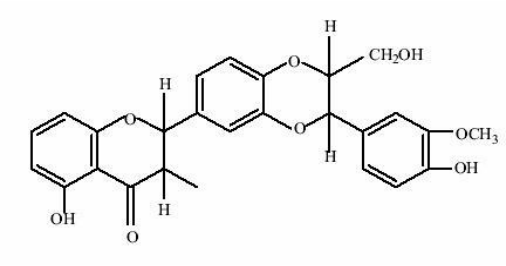


Figure 2.21. Chemical structure of Kutkin

The different physicochemical properties of Kutkin are given in Table 2.4.

Table 2.4. Physicochemical properties of Kutkin

Sr. No.	Physical properties	Result
1	Molecular formula	$C_{22}H_{28}O_{12}$ [58]
2	IUPAC name	2-Methoxy-4-(1,1,2-trihydroxyethyl)phenyl beta-D-glucopyranoside 1-cinnamate [58]
3	Molecular weight	484.153 Da [59]
4	Category	Phytochemical- Bitter glucoside [59] Pharmacological- Hepatoprotective, Antioxidant, Anti-inflammatory [58]
5	Appearance	Yellowish brown powder
6	Solubility [57] i.Methanol ii.Ethanol iii. Ethyl acetate iii.Water	 Soluble Soluble Slightly soluble Sparingly soluble

7	Melting point	214-215 °C

Category: Antibacterial, antiperiodic, cathartic (in large doses), laxative (in smaller doses), stomachic and bitter tonic, hepatoprotective, anticholestatic (relieves obstruction of bile salts), anti-inflammatory, anti-allergy, antioxidant. [58]

Official status: Drug is not official in USP or any other Pharmacopoeia. [54]

Storage condition: In a dry area, protected from direct sunlight at 8ºC to 20ºC.

Mechanism of action: Kutkin, the active principal of Picrorhiza kurroa is comprise of kutkoside and iridoid glycosides like picrosides I, II, and III. The hepatoprotective action of Picrorhiza kurroa may be attributed to its ability to inhibit the generation of oxygen anions and to scavenge free radicals. Picrorhiza's antioxidant effect has been shown to be similar to that of superoxide dismutase, metal-ion chelators, and xanthine oxidase inhibitors. Animal studies indicate that Picrorhiza's constituents exhibit a strong anticholestatic activity against a variety of liver-toxic substances, appearing to be even more potent than silymarin. Picrorhiza also exhibits a dose-dependent choleretic activity, evidenced by an increase in bile salts and acids, and bile flow. Several animal studies, primarily in rats, have demonstrated that the active constituents of Picrorhiza kurroa are effective at preventing liver toxicity and the subsequent biochemical changes caused by numerous toxic agents. Hepatocytes damaged by exposure to galactosamine, thiocetamide and carbon tetrachloride were incubated with Picrorhiza constituents. [54]

2.2.4. Vasicine

Vasicine (1,2,3,9-Tetrahydropyrrolo[2,1-b]quinazolin-3-ol) is a Quinazoline alkaloid of plant origin. [67]

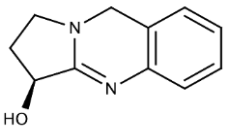


Figure 2.22. Chemical structure of Vasicine

The different physicochemical properties of Vasicine are given in Table 2.5.

Table 2.5. Physicochemical properties of Vasicine

Sr. No.	Physical properties	Result
1	Molecular formula	$C_{11}H_{12}N_2O$ [67]
2	IUPAC name	1,2,3,9-Tetrahydropyrrolo[2,1-b]quinazolin-3-ol
3	Molecular weight	188.23 g/mol [67]
4	Category	Phytochemical- Quinazoline alkaloid Pharmacological- Antioxidant, Anti-inflammatory
5	Appearance	White to yellow powder
6	Solubility [68] i. Methanol ii. Ethanol iii. Acetone iii. Chloroform	 Very soluble Very soluble Soluble Very soluble

7	Refractive Index	n^{20}_{D} 1.709
8	Melting point	209-211 °C

Category: Antibacterial, Bronchodilatory, Anti-Allergic, Anti-Asthmatic Activities, Radioprotective, Antimutagenic, Anti-Tubercular, Anti-Ulcer, Allopathic, Anti-feedant, Anti-inflammatory, Abortifacient. [65]

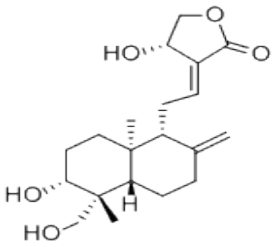
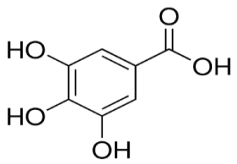
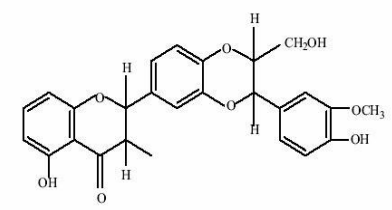
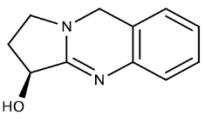
Official status: The drug is certified under the USP Reference Standards Committee, as an official Reference Standard.

Storage condition: Store below 4°C. Keep away from light

Mechanism of action: Vasicine treatment has effect on superoxide dismutase, catalase, glutathione peroxidase and "reduced glutathione". The extracts of Adhatoda vasica (AV), along with few other plants are known for their effect on reduced glutathione and lipid peroxidation in liver (antioxidant) and in 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays (radical-scavenging activities). All plant extracts showed antioxidant activity (A. vasica – 337 μg/ml). Among hexane, chloroform and methanolic extracts, the methanolic extract had shown to have maximum antioxidant activity. [67]

The brief description of the biomarkers under analysis is given in Table 2.6.

Table 2.6. Brief description of the Biomarkers under analysis

Name of Standard	Category	Molecular formula	Molecular weight	Structure
Andrographolide	Triterpen-oid	$C_{20}H_{30}O_5$	350.45 g/mol	
Gallic acid	Tannin	$C_7H_6O_5$	170.12 g/mol	
Kutkin	Bitter Glucoside	$C_{25}H_{17}O_9$	312.20 g/mol	
Vasicine	Quinazoli ne alkaloid	$C_{11}H_{12}N_2O$	188.226 g/mol	

CHAPTER- 3

LITERATURE REVIEW

3. LITERATURE REVIEW

A number of analytical methods of HPLC and HPTLC have been reported for Andrographolide and Gallic acid, more than those for Kutkin, including those of individual determination as well as of Simultaneous estimation with other Biomarkers in Polyherbal formulations. These methods are utilized for various purposes like quality control of pharmaceutical dosage forms, stability studies, study of pharmacokinetics of analyte and its metabolite.

Table 3.1.: Literature review of various Biomarkers under study

Author	Title	Details of Research	Reference
Andrographolide			
Mamatha A.	"Quantitative HPTLC analysis of Andrographolide in Andrographis paniculata obtained from different geographical sources (India)".	Mobile phase: Chloroform:Methanol (7:1 v/v). Chamber saturation time: 10 min Developing distance: 9cm. Scanning wavelength: 231nm Slit dimension: 5.0 x 0.45mm	36
Archana P. Raina, A Kumar, S. K. Pratik	"HPTLC analysis of hepatoprotective diterpenoid andrographolide from *Andrographis paniculata* nees (*kalmegh*)"	Mobile phase: chloroform: methanol (7:1 v/v), pre-saturation: 15 min Development distance: 8 cm Slit width: 5×0.45 mm, Scan wavelength: 232 nm	37
Monika jadhao	"Estimation of Andrographolide in Herbal powder and Polyherbal asava by HPTLC".	Mobile phase: Benzene: ethyl acetate (5:5 v/v) Rf value: 0.10 Λmax: 222 nm.	38
Punit K. Jain,	"High-performance thin layer chromatography method for estimation	Mobile phase: chloroform:toluene:methanol (66:26:8, v/v/v).	39

V. Ravichandran, Prateek K. Jain, Ram K. Agrawal,	of andrographolide in herbal extract and polyherbal formulations"	Chamber saturation time: 30 min. Chromatogram run length: 80 mm. Scanning wavelength: 190–400 nm. Λmax: 229 nm.	
Pawar R.K., Sharma Shivani, Singh K.C., Sharma Rajeev K.R	"Development and Validation of HPTLC method for the determination of Andrographolide from Andrographis paniculata (whole plant)".	Mobile phase: Toluene : Ethyl acetate : Formic acid (5:4.5:0.5 v/v/v/v) Saturation time: 30 min. Development distance: 8 cm Λmax: 254 nm.	40
Meenu Sharma, R. G. Sharma	"Identification, purification and quantification of Andrographolide from Andrographis paniculata (Burm. F.) Nees by HPTLC at different stages of Life cycle of Crop".	Mobile phase: Chloroform: Methanol (7 : 1 v/v). Chamber saturation time: 30 minutes Development distance: 8.5 cm. Scanning wavelength: 250 nm Λmax: 250 nm	41
Meenu Sharma, Aakansha Sharma, Sandeep Tyagi	"Quantitative HPLC analysis of Andrographolide in Andrographis paniculata at two different stages of Life cycle of plant".	Mobile phase: Methanol:Water (65: 35 v/v) C 18 column (250 nm x 4.6 nm) Flow rate: 1.5 mL/min. Scan wavelength: 223 nm	42
Katta Vijaykumar, Papolu B.S.Murthy, et al.	"Estimation of Adrographolide in Andrographis paniculata Herb, Extracts and Dosage forms"	Mobile phase: Toluene: ethyl acetate: formic acid (5.0: 3.5: 1.5 v/v/v/v) Chamber saturation time: 30 min Chromatogram run: 8 cm. Scanning wavelength: 223 nm	43

K. Senthil Kumaran, et al.	"An HPLC Method for the estimation of Andrographolide in Rabbit Serum"	Neuclosil C18 octadecyl silane (ODS) column (5 μ 250 x 4.6 mm) Mobile phase: Methanol: water (65:35 v/v). Flow rate: 1.0 ml/min. Scanning wavelength: 223 nm	44
Yong-Xi Song, Shi-Ping Liu, Zhao Jin, Jian-Fei Qin, and Zhi-Yuan Jiang	"Qualitative and Quantitative Analysis of Andrographis paniculata by Rapid Resolution Liquid Chromatography/Time-of-Flight Mass Spectrometry"	ACE C18 column Mobile phase: Acetonitrile: water (60:40 v/v) with 0.1% formic acid Flow rate: 0.2 mL/min	45
Tao Xu, Jian Pan, and Lingli Zhao	"Simultaneous Determination of Four Andrographolides in Andrographis paniculata Nees by Silver Ion Reversed Phase High Performance Liquid Chromatography"	Mobile phase: Methanol-water (55:45 v/v) C18 column (5 microm, 150 mm x 4.6 mm i.d.) Scanning wavelength: 205 nm	46
Chandana Majee, B. K. Gupta, R. Mazumder, G. S. Chakraborthy	"HPLC Method Development and Characterization of Bio-Active Molecule Isolated from Andrographis paniculata"	C18 column (250 nm x 4.6 nm). Isocratic elution with methanol Flow rate: 1 ml/min. Scan wavelength: 230 nm	47
Punit K. Jain, et al.	"High-performance thin layer chromatography method for estimation of andrographolide in	Mobile phase: Chloroform:toluene:methanol (66:26:8, v/v/v) R_f value: 0.49	48

	herbal extract and polyherbal formulations."		
Dilip Jadhao, Bhaskar Thorat	"Purification (crystallization) of Bioactive ingredient Andrographolide from Andrographis paniculata"	Zorbax Eclipse XDB–C18 column (ϕ4.6 mm×150 mm, 5 µm). Mobile phase: Methanol:0.1% v/v H3PO4 (70:30) Flow rate: 1 ml/min. Scan wavelength: 223 nm	49
Vivek K.R. Ghosh, Shrinivas G. Bhope, et al.	"Development and Validation of Dissolution test method for Andrographolide from Film- coated polyherbal formulation."	Inertsil ODS-4 (250 × 4.6 mm, i.d.; 5 µm) column Mobile phase: Orthophosphoric acid (0.1% v/v) in Milli-Q water (pH 2.2): Acetonitrile. Flow rate: 1 ml/min Scan wavelength: 226 nm	50
Gallic acid			
Md. Sarfaraj Hussain, et al.	"Validation of the method for the simultaneous estimation of bioactive marker gallic acid and quercetin in ***Abutilon indicum*** by HPTLC"	Mobile phase: Toluene-ethyl acetate-formic acid (5:4:1, v/v/v). Scan wavelength: 270nm	51
Rui song, Ying Cheng, Yuan Tian, Zun-Jian Zhang	"A validated solid-phase extraction HPLC meth od for the simultaneous determination of gallic acid and tannic acid in rhubarb decoction."	Agilent Zorbax SB-C $_{18}$ (250 mm × 4.6 mm, 5 m) Mobile phase: A mixture of methanol (0.06% formic acid) and water (0.1% formic acid)	52
Patel Madhavi G., Patel Vishal R., Patel Rakesh K.	"Development and Validation of Improved RP-HPLC method for Identification and Estimation of Ellagic and Gallic acid in Triphala churna".	HPLC column Rt value Mobile phase Run time	53
Kamal Kardani, et al	"RP-HPLC Method Development and Validation of Gallic	Mobile Phase: Water : Acetonitrile (80 :20 %v/v) pH 3.00 by OPA	54

	acid in Polyherbal Tablet Formulation".	Scan Wavelength: 272 nm Run time: 6 min Flow Rate: 1 mL/min	
Hafsa Deshmukh, Pradnya J. Prabhu	"Development of RP-HPLC method for Qualitative Analysis of Active Ingredient (Gallic acid) from Stem Bark of Dendrophthoe falcate Linn".	Scan wavelength: 271nm flow rate 1ml/min ThermoMOS 2 HYPERSIL C18 column (250 cm × 4.6 mm, 5µm ODS 3) Mobile phase: 0.1% Orthophosphoric acid : Acetonitrile (400: 600 v/v) Run time :13 min	55
Laxman Sawant, et al.	"Quantitative HPLC Analysis of Ascorbic Acid and Gallic Acid in Phyllanthus Emblica".	Zorbax SB RP-C18 column (4.6 mm × 250 mm, i.d., 5-µm pore size) Mobile phase: 0.1% (v/v) acetic acid in water: methanol (gradient) Flow rate was 0.9 mL min	56
Gupta Mradu1 et al.	"HPLC Profiles of Standard Phenolic Compounds Present in Medicinal Plants".	Symmetry C18 (5µm, 4.6*250mm) Flow rate: 1.0 ml/min. Scan wavelength: 280 360 nm Mobile phase: Methanol: Acetic acid (70:30)	57
Laxman Sawant1 et al.	"Development and Validation of HPLC Method for Quantification of Phytoconstituents in Phyllanthus emblica".	Scan wavelength: 272nm. Column: Thermo Scientific BDS HYPERSIL Phenyl reversed-phase column (250mm×4.6mm, 5µm). Mobile phase: 0.1% ortho-phosphoric acid: Methanol (95:05v/v) (A) and acetonitrile (B) Flow rate: 1.5 ml/min, gradient mode	58
Nikita R. Sawant, Abhijit R. Chavan	"Determination of Gallic acid from their Methanolic Extract of Punica granatum By HPLC Method".	Scan wavelength: 270 nm Column: SymmetryC-18 (4.6 ×250 mm, 5 µm) Mobile phase: Methanol: Ethyl acetate: Water (25:5:70 v/v) Flow rate: 0.7 l/min	59
Angshuman Biswas 1 et al.	"Quantitative Estimation of Gallic Acid in Amla Extract by Gradient RP-HPLC method".	C18 column (250 mm x 4.6 mm I.D., 5 µm) Mobile phase: Acetonitrile: water containing 0.01 % v/v ortho phosphoric acid (80: 20% v/v)	60

		Scan wavelength: 272 nm Flow rate: 1ml/min.	
Deepika Sharma, Yash Paul Singla	"Analysis of Gallic acid and 4-Hydroxy benzoic acid in Prosopsis cineraria leaf extract using High Performance Liquid Chromatography".	C18 (250*4.6mm 5µ) Shodex column. Mobile Phase A- 800 ml of water + 200 ml of buffer i.e 11.5 gm H3PO4 + 1000 ml water (pH2.5 with ammonia), Mobile Phase B- 250 ml Buffer+ 750 ml methanol. Flow-rate: 1 mL/ min Scan wavelength: 280 nm	61
Sachin U Rakesh et al.	"HPTLC Method for Quantitative Determination of Gallic Acid in Hydroalcoholic Extract of Dried Flowers of Nymphaea Stellata Willd".	Chromato development: 80 mm Mobile phase: Chloroform : ethyl acetate formicacid,7.5:6:0.5(v/v/v) Scan wavelength: 292 nm.	62
Vinitkumar Y. Thakker et al.	"Simultaneous estimation of Gallic acid, Curcumin and Quercetin by HPTLC Method".	Mobile phase: Toluene: ethyl acetate: forminc acid (4.5:3.0:0.2 v/v/v) Scan wavelength: 366 nm Rf: 0.40	63
Garg et al.	"Fingerprint Profile of Selected Ayurvedic Churnas/ Preparations: An Overview."	Mobile phase: Toluene: Ethyl acetate: Formic acid (5.0:3.5:1.0 v/v)	64
V. Leela, A. Saraswathy	"Quantification of Pharmacologically active markers Gallic acid, Quercetin and Lupeol from Acacia Leucophloea Willd flowers by HPTLC Method".	Mobile phase: Toluene:Ethyl acetate:Formic acid (6:4:0.8 v/v/v).	65
Kutkin			
Bhandari P, KumarN, Singh B, Ahuja PS	"Online HPLC-DPPH method for antioxidant activity of Picrorhiza kurroa Royle ex Benth. and characterization of kutkoside by ultra-performance LC-	Column: Zorbax Extend C-18 column (250 mm × 4.6 mm, 5 µm particle size) Mobile phase: MeOH/water (80:20) Flow rate: 1 ml/min.	66

	electrospray ionization quadrupole time-of-flight mass spectrometry.”	Scan wavelength: 517 nm	
Manisha Vite, Dr. Naresh Chugh, et al.	“Standardization of an Ayurvedic formulation Jatyadi Ghrita by RP-HPLC.”	Column: Inertsil ODS-3, C18 (250x4.6 mm,5µ) Mobile phase: 0.1%Orthophosphoric acid in Water: Acetonitrile (75:25v/v) Flow rate: 1.0 (ml/min) Scan wave length: 265 nm Run time: 15 min	67
Deepti Rathee, Permender Rathee, et al.	“Phytochemical screening and antimicrobial activity of *Picrorrhiza kurroa*, an Indian traditional plant used to treat chronic diarrhea”.	Mobile phase: Ethylacetate:formic acid:methanol (6:0.6:0.4 v/v/v) Scan wavelength: 290 nm	68
Vite Manisha H et al	“Standardization of Jatyadi Ghrita by HPTLC Method”.	Mobile phase: Ethyl acetate: methanol: glacial acetic acid: (18:5:0.2 v/v/v) Scan wavelength: 280 nm Rf value: 0.64	69
Milind S. Bagul, M. Rajani	“Quantification of Ellagic acid, Gallic acid and Picroside-I from Phalatrikadi kvatha churna by HPTLC”.	Mobile phase: Ethyl acetate: formic acid: methanol (6:0.6:0.4 v/v/v) Scan wavelength: 280 nm Rf value: 0.64	70
Vasicine			
Kamlesh D, Vaibhav M. Shinde and Kakasaheb R. Mahadik	“Optimization and validation of reverse phase HPLC and HPTLC method for simultaneous quantification of vasicine and vasicinone in Sida Species”.	HPLC: Mobile phase: of Acetonitrile: 0.1 M phosphate buffer - glacial acetic acid (15:85:1, v/v/v) with pH 4.0 Column: C18-ODS-Hypersil column Retention time: 5 min HPTLC: Mobile phase: Ethyl acetate: methanol: ammonia (8:2: 0.2, v/v)	71
Richa, Shikha Sharma et al.	“Phytochemical investigartions and Anatomical stuyd of three species of Sida.”	Phenomenex C18 column (250 mm X 4.6 mm, 5 µm Flow rate: 1.0 ml/min.	72

		Scan wavelength: 280 nm.	
A C Suthar, K V Katkar et al.	"Quantitative estimation of Vasicine and Vasicinone in Adhatoda vasica by HPTLC"	Mobile phase: Chloroform: Methanol (90:10) Saturation time: 20 mins Development length: 8 cm Scan wavelength: 280 nm	73

CHAPTER- 4

AIM OF PRESENT WORK

4. AIM OF PRESENT WORK

HEPASAVE tonic is labelled to contain plants like *Amalaki fruit i.e. Phyllanthus emblica Ab., Haritaki fruit i.e., Terminalia chebula Ab., Bibhitaka fruit i.e., Terminalia bellerica AB., Vasa leaf i.e., Adhatoda vasica Ab., Bhunimba herb i.e., Andrographis paniculata, Katuka root i.e., Picrorrhiza kurroa, Nimba stem bark i.e., Azadirachta indica Ab, Amruta stem i.e., Tinospora cordifolia, Sarpankha herbTephrosia purpurea Ab.,* and also a Flavored syrup base. It has been observed from literature survey that *Andrographis paniculata, Picrorrhiza kurroa* are hepatoprotective herbal plants which are used in liver disorders. Thus, Andrographolide from *Andrographis paniculata*, Kutkin *Picrorrhiza kurroa*, etc can serve the purpose of biomarkers used for detection of these plants, which are also official in the Pharmacopoeias. Andrographolide and Vasicine are certified under the USP Reference Standards Committee, as an official Reference Standard (USP, 2010), and Andrographolide is also official in Indian Herbal Pharmacopoeia, 1998.

Various reports are available which provides a number of developed analytical methods for the estimation of biomarkers like Andrographolide, Gallic acid, Kutkin and Vasicine. There are many HPTLC and RP- HPLC methods available, which includes estimation of Andrographolide and Gallic acid individually, and a few reports are available for the determination of Kutkin (Picroside-I, Picroside-II, Picroside-III, individually or in combination with each other, or even as a combination to other biomarkers). In case of HEPASAVE syrup, Andrographolides, Kutkin, Gallic acid and Vasicine have been utilised as the biomarker compounds under analysis for the quality control of their formulation. A couple of HPTLC and HPLC methods have been reported for the simultaneous estimation of Andrograpolide and Gallic acid in combination with other biomarkers, and a couple of such methods have been reported for the simultaneous estimation of Andrographolide and Kutkin, together with other biomarkers. However, no method, till date, has been reported for the simultaneous estimation of Andrographolide, Gallic acid, Kutkin and Vasicine, either by any HPLC or by HPTLC methods, and of course, not for the polyherbal formulation, HEPASAVE.

Thus, it is necessary to develop a simple and rapid analytical method like, HPTLC and RP-HPLC for the simultaneous estimation of Andrographolide (AG), Gallic acid (GA), Kutkin (KT) and Vasicine (VS) in the Polyherbal formulation i.e, HEPASAVE for a routine quality control study.

The objective of the present study can be depicted briefly, as follows

- To select the biomarkers for quality control of HEPASAVE formulation for the simultaneous estimation of the four Biomarkers, namely, AG, GA, KT and VS by HPTLC and RP- HPLC.
- To develop a specific, simple, rapid, sensitive and reproducible HPTLC method for the quantitative estimation of AG, GA, KT, VS in their combined polyherbal formulation.
- To validate the proposed HPTLC method for various Method validation parameters as per the ICH guidelines.
- To apply the developed HPTLC method for the quantification of selected biomarkers from their formulation, HEPASAVE.
- To develop a RP- HPLC method for the simultaneous estimation of the selected biomarkers in a Hepatoprotective Polyherbal formulation.
- To apply the developed RP- HPLC method for the quantification of the biomarkers from their formulation, HEPASAVE.

CHAPTER- 5

EVALUATION OF FORMULATION AND IDENTIFICATION OF BIOMARKERS

5. EVALUATION OF FORMULATION AND IDENTIFICATION OF BIOMARKERS

5.1. EXPERIMENTAL WORK

5.1.1. Procurement of Formulation

The formulation, Hepasave syrup (100ml), was received from Cadila Pharmaceuticals Limited, Trasad Road, Dholka-387810, Dist. Ahmedabad (Gujarat), India.

5.1.2. Organoleptic Characteristics of the Formulation

The organoleptic characters of the sample were carried out based on the method described in Wallis. To determine the odour of an innocuous material, small portion of the sample was placed in the beaker of suitable size, and examined by slow and repeated inhalation of the air over the material. If no distinct odour was perceptible, the sample was crushed between the thumb and index finger, between the palms of the hands, using gentle pressure or if the material was known to be dangerous, by other suitable means such as pouring a small quantity of boiling water onto the crushed sample placed in a beaker. First, the strength of the odour was determined (none, weak, distinct, strong) and then the odor sensation (aromatic, fruity, musty, mouldy, rancid, etc.) was studied. Taste was distinctively classified as aromatic, pungent, sweet, sour, astringent, mucilaginous, or bitter. The test for density was carried out by taking a known amount of liquid (volume in ml) in a Specific density glassware and then weighing its weight and then subtracting the weight of empty glassware from its final weight and then applying the formula of Density is equal to Mass of a sample divided by the volume of the same known amount of sample.

5.1.3. Identification of Biomarkers for the Quality control of Formulation

Identification of Andrographolide, Gallic acid and Kutkin was carried out by Melting point and UV-visible spectroscopy.

5.1.3.1. Melting point determination:

Instrumentation: Melting points of AG, GA and KT were determined using the melting point apparatus Model T0603160; manufactured by EIE Instruments Pvt. Ltd.

The melting points of the compounds were taken by Open capillary method.

5.1.3.2. U.V Spectroscopy:

Instrumentation: U.V spectra of AG, GA and KT were taken for identification of the drugs using UV-visible spectrophotometer, Model UV-2450 PC Series; manufactured by Shimadzu Inc; Japan. (Figure 4.1.1., 4.1.2., 4.1.3.; Table 4.1.2.).

Preparation of Standard solutions: A 10 µg/ml solution of Andrographolide, Gallic acid and Kutkin was prepared in Methanol and scanned in U.V- Visible Spectrometer in the range of 200- 400 nm and λ max was found to be 225 nm, 273 nm and 268 nm, respectively, having an absorbance of 1.495, 0.489 and 0.035, respectively.

The prepared standard solutions of each of the standards were estimated for their Wavelength maxima values.

CHAPTER- 6

HPTLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF AG, GA AND KT IN A POLYHERBAL FORMULATION

6. HPTLC METHOD DEVELOPMENT FOR THE SIMULTANEOUS ESTIMATION OF AG, GA AND KT IN A POLYHERBAL FORMULATION

6.1. INSTRUMENTS AND APPARATUS

TLC plate: Pre-coated silica gel aluminium plate $60F_{254}$ (20 × 10 cm); 200 µm thickness (E. Merk, Germany)

Applicator: CAMAG- Linomat V semi-automatic with Nitrogen flow

Applicator syringe: CAMAG 100 µl Applicator syringe (Hamilton, Bonaduz, Schweiz)

Development chamber: CAMAG Twin trough chamber (10cm × 20cm) with a stainless steel lid

Dryer: Philips hair dryer.

Scanner: CAMAG TLC Scanner 3 supported by the winCATS software (version 1.4.2.8121): For densitometry.

U.V. cabinet: UV cabinet with dual wavelength UV lamp (254 nm and 366 nm)

Weight balance: CITIZEN Scale (CX 220)

Ultra Sonicator: Trans-o-sonic, India

Hot air oven: EIE Instruments Pvt. Ltd.

Glasswares: (1) Amber coloured volumetric flask – 100 ml, 25 ml, 10 ml.

(2) 60 ml, 250 ml Seperating funnel.

6.2. REAGENTS AND MATERIALS

AG standard: procured from Cadilla Pharmaceuticals Limited, Trasad Road, Dholka-387810, Dist. Ahmedabad (Gujarat), India.

GA standard: procured from Cadilla Pharmaceuticals Limited, Trasad Road, Dholka-387810, Dist. Ahmedabad (Gujarat), India.

KT standard: procured from Cadilla Pharmaceuticals Limited, Trasad Road, Dholka-387810, Dist. Ahmedabad (Gujarat), India

All the other reagents used were of AR Grade.

6.3. EXPERIMENTAL WORK

6.3.1. Preparation of Standard Solutions

5 mg of Andrographolide, Gallic acid and Kutkin were weighed accurately and transferred in 10 ml amber-coloured vol. flasks separately. The volume was adjusted up to 10 ml separately, using Methanol (500 µg/ml each). From these stock solutions, 1 ml of Andrographolide and Kutkin solutions, and 0.4 ml of Gallic acid solution were transferred in 10 ml amber-coloured vol. flasks separately and volume was made up with Methanol and mixed to get the Standard solution of Andrographolide- 50 µg/ml, Gallic acid- 20 µg/ml and Kutkin- 50 µg/ml.

6.3.2. Preparation of Sample solution

5 ml Tonic was extracted with ethyl acetate (3 × 10 ml) by shaking extraction technique for 20 min. Ethyl acetate fractions were combined for further use followed by Filtration, concentration and it was transferred to a 25 ml amber-coloured vol flask, which was used for further chromatographic analysis.

Ethyl acetate extract of formulation was prepared as per the method described above, and 20 µl of the extract was applied on TLC plate along with 8 µl each of andrographolide, gallic acid and kutkin solutions separately of 50 µg/ml, 20 µg/ml and 50 µg/ml, respectively.

The plate was developed and analysed with the Chromatographic conditions in Section 6.3.3.

6.3.3. Trials of HPTLC for the Simultaneous estimation of Andrographolide, Gallic acid, Kutkin and Vasicine.

Different compositions of various mobile phases were tested along with the reported mobile phases for remarkably resolved and reproducible spots of AG, GA, KT and VS standards as well as the spots of these standards in the formulation extract.

The different mobile phases tried for estimation of the four biomarkers, AG, GA, KT and VS are:

TRIAL 1: Ethyl acetate: formic acid: methanol (6: 0.6: 0.4 v/v/v), 1% vanillin sulphuric acid for visualisation - Reported for Kutkin

TRIAL 2: Ethyl acetate: formic acid: methanol (3: 1: 3 v/v/v)

TRIAL 3: Benzene: ethyl acetate (5: 5 v/v) – Reported for Andrographolide

TRIAL 4: Methanol: ethyl acetate: acetic acid: formic acid (3: 2: 1: 0.5 v/v/v/v)

TRIAL 5: Toluene: Methanol (5: 5 v/v)

TRIAL 6: Toluene: Methanol: Formic acid (5: 5: 0.1 v/v/v)

TRIAL 7: Methanol: Water (7: 3 v/v)

TRIAL 8: Methanol: Water (5: 5 v/v)

TRIAL 9: Toluene: Ethyl acetate: Formic acid (5.0: 3.5: 1.0 v/v/v)

TRIAL 10: Toluene: ethyl acetate: formic acid: methanol (3: 3: 0.8: 0.4 v/v/v/v) – Reported for Gallic acid. It was tried for visualization with Anisaldehyde sulphuric acid reagent

TRIAL 11: Chloroform: Methanol (7: 1 v/v), 10% sulphuric acid as derivatizing agent – Reported for Andrographolide

TRIAL 12: Chloroform: toluene: methanol (66: 26: 8 v/v/v) - Reported for Andrographolide

TRIAL 13: Chloroform: toluene: methanol (80: 10: 15 v/v/v) - Reported for Andrographolide

TRIAL 14: Toluene: ethyl acetate: formic acid (6: 4: 0.8 v/v/v) – Reported for Gallic acid

TRIAL 15: Toluene: ethyl acetate: formic acid (4.5: 3: 0.2 v/v/v) – Reported for Gallic acid

TRIAL 16: Toluene: ethyl acetate: formic acid (7: 5: 1 v/v/v) – Reported for Gallic acid

TRIAL 17: Chloroform: methanol (8.5: 1.8 v/v) – Reported for Kutkin

TRIAL 18: Chloroform: methanol (80:20 v/v) – Reported for Kutkin

TRIAL 19: Ethyl acetate: chloroform: methanol: water (15: 8: 2: 0.5 v/v/v/v), Iodine spraying and then vanillin sulphuric acid as visualising agent - Reported for KT

TRIAL 20: Ethyl acetate: methanol: ammonia (8: 2: 0.2 v/v/v), Dragondorff's reagent – Reported for VS

TRIAL 21: Dioxane: toluene: ethyl acetate: methanol: ammonia (1.5: 2: 1: 1: 0.3 v/v/v/v/v) - Reported for VS

TRIAL 22: Chloroform: toluene: methanol (7.6: 0.95: 1.4 v/v/v), 5% sulphuric acid as derivatizing agent – Reported for AG

TRIAL 23: Toluene: ethyl acetate: formic acid: methanol (6: 3: 0.1: 0.6 v/v/v/v), 5% sulphuric acid in ethanol reagent as derivatizing agent – Reported for GA

TRIAL 24: Toluene: ethyl acetate: formic acid: methanol (3: 3: 1.2: 0.8 v/v/v/v)

TRIAL 25: Toluene: ethyl acetate: formic acid: methanol (3: 3: 0.8: 2.5 v/v/v/v)

TRIAL 26: Toluene: ethyl acetate: formic acid: methanol (3: 3: 0.8: 2 v/v/v/v)

6.3.4. Chromatographic conditions

The chromatographic conditions given in Table 6.1. were kept constant throughout the analysis.

Table 6.1. Optimized HPTLC conditions

Sr.no.	HPTLC Condition	HPTLC Condition Parameters
1.	**Stationary phase**	Pre-coated silica gel aluminium plate $60F_{254}$ (20 × 10 cm); 200 µm thickness (E. Merk, Germany)
2.	**Mobile phase**	Toluene:ethyl acetate:formic acid:methanol (3:3:0.8:0.4, v/v/v/v).
3.	**Chamber saturation**	35 min at room temperature (25 ± 2 °C and 40% RH)

4.	**Band dimensions**	6 mm in length, 10 mm from the bottom edge, 10 mm from the side edge.
5.	**Application rate**	0.1µL/s
6.	**Space between two bands**	6.6 mm
7.	**Run length and time**	55 mm for 25 min
8.	**Scanning parameters**	Densitometric scanning in the absorbance mode at 254 nm for Quantitative evaluation (CAMAG winCATS software). The scanning speed of 20 mm s^{-1} was employed
9.	**Slit dimension**	5 mm x 0.1 mm
10.	**Lamp**	Deuterium/Tungsten

The standard solutions of Andrographolide, Gallic acid and Kutkin as well as the final Ethyl acetate extract were injected to the TLC plate as per the above mentioned chromatographic conditions.

The chromatographic peaks of AG, GA and KT in formulation extract were compared with the Retention factor of the standards AG, GA and KT. Peak areas were recorded of the prepared standard solutions.

From the peak area of standard AG, GA and KT compared to the areas of these biomarkers in the Formulation, the amounts of these three Biomarkers in the extract as well as in the formulation were computed.

6.3.5. HPTLC Sequence of steps:

1) Activation of Silica gel plate

Analysis was performed on Pre-coated silica gel aluminium plate $60F_{254}$ (20 × 10 cm); 200 µm thickness (E. Merk, Germany). Plates were cleaned by pre-washing with methanol and dried in a hot air oven at 60ºC for 20 minutes. Before using, the plates were allowed to come to room temperature and used immediately.

2) Spotting

Standard zones were applied to the TLC plate as bands by the means of a CAMAG Linomat V semi-automated spray-on applicator with Nitrogen pressure flow, equipped with a 100-µl syringe and operated with the settings of band length 6 mm, 10 mm from the bottom edge, 10 mm from the side edge, application rate 0.1µL/s, distance between bands 6.6 mm.

3) Development

Spotted plates were developed in HPTLC twin-through chamber (10 x 20 cm) with a stainless steel lid, previously saturated with mobile phase for 35 minutes with the help of a filter paper. The plates were developed up to 55 mm solvent front. Development time was approx 25 minutes. After development, plates were removed immediately and dried using a Philips Hair dryer.

4) Scanning of TLC plates

The developed plates were scanned with the help of CAMAG TLC Scanner 3 at a λ_{max} of 254 nm with a deuterium and tungsten source, slit dimension was 5 × 0.1 mm, and a scanning speed of 20 mm/s using WINCATS-1.4.2.8121 software.

6.3.6. HPTLC Method Validation for Simultaneous Estimation of AG, GA and KT in Polyherbal Formulation.

The proposed HPTLC method was validated for linearity and range, specificity, precision, accuracy, LOD (limit of detection), LOQ (limit of quantification), sensitivity, robustness and system suitability testing as per the International Conference on Harmonization (ICH) guidelines.

6.3.6.1. Instrument Precision

Prior to method validation process, instrument precision was evaluated in terms of sample application, positioning of TLC scanner stage, and repeated scanning of the same spot. Standard solutions of andrographolide (50 µg/ml), gallic acid (20 µg/ml) and kutkin (50 µg/ml) were used.

(a) Precision of sample application:

A 14 µL of andrographolide solution (700 ng band^{-1}), 12 µl of gallic acid solution (240 ng band^{-1}) and 18 µL of kutkin solution (900 ng band^{-1}) was repeatedly applied on the plate to make six bands each for the three standards seperately.

(b) Reproducibility of scanning:

One same spot was scanned for andrographolide (200 ng/spot), gallic acid (200 ng/spot) and kutkin (2000ng/spot) individually six times without changing the plate position, and the results were analysed for each working standards. The relative standard deviation (%RSD) for peak area was calculated for the three working standards.

6.3.6.2. Linearity

A series of solutions of the working standard solution of Andrographolide (50 µg/ml) of 200-800 ng/spot, gallic acid (20 µg/ml) of 80-320 ng/spot and kutkin (50 µg/ml) of 2000-6000 ng/spot, seperately were used. For evaluation of linearity, peak area and concentration were subjected to least square regression analysis and the calibration equation and correlation coefficient were calculated.

6.3.6.3. Inter-day and Intra-day precision

Method precision was studied by analyzing the concentration levels of 400, 500, and 600 ng band–1 of Andrographolide and Kutkin, and 160, 200 and 240 ng band^{-1} for Gallic acid under the same analytical procedure and lab conditions. The intra- and inter-day studies were performed by injecting three different aliquots of the standard solution in triplicate in a day and on three different days, respectively; the results were expressed as % RSD.

6.3.6.4. Accuracy (Recovery analysis)

Accuracy of the present method was performed by conducting recovery studies using standard addition method. Three different concentrations of 400, 500, and 600 ng band–1 for Andrographolide and Kutkin, and 160, 200 and 240 ng band^{-1} for Gallic acid, with respect to a pre- analyzed sample of each, were added to the pre- analyzed sample, and the mixtures were reanalysed. The % recovery and % average recovery of each standard were calculated.

6.3.6.5. Limit of detection (LOD)

The calibration curve is repeated six times and the standard deviation of intercepts is calculated, from which the Limit of detection is calculated using the following equation:

$$LOD = 3.3\ \sigma / S$$

Where, σ = Standard deviation of intercept

S = Slope of the linearity curve

6.3.6.6. Limit of quantification (LOQ)

The calibration curve is repeated six times and the standard deviation of intercepts is calculated, from which the Limit of quantitation is calculated using the following equation:

$$LOQ = 10\ \sigma / S$$

Where, σ = Standard deviation of intercept

S = Slope of the linearity curve

6.3.7. Quantification of Andrographolide, Gallic acid and Kutkin in Formulation extract by HPTLC

20 µl of the Ethyl acetate exract was applied on TLC plate along with 8 µleach of andrographolide, gallic acid and kutkin solutions separately of 50 µg/ml, 20 µg/ml and 50 µg/ml, respectively. The plate was developed and analysed.

The amount of Biomarkers (andrographolide, gallic acid and kutkin) content were estimated in the formulation by the proposed method. The plate was developed and analysed.

6.2.4. Conclusion

HEPASAVE contains various Biomarkers as its constituents, including Andrographolide, Gallic acid and Kutkin, which have been estimated for their amount in the Polyherbal formulation. The method has been validated as per the ICH guidelines for Linearity, Accuracy and Precision. The Limit of detection and Limit of quantitation have also been carried out for the biomarkers. The literature survey revealed the need of a simple and rapid analytical method for quantitative estimation of AG, GA and KT in Hepasave, a polyherbal formulation. Thus, the developed and validated HPTLC method for the formulation can be said to be one of a kind, being the first method to describe a set of HPTLC conditions simultaneously quantitating AG, GA and KT in the Polyherbal formulation.

CHAPTER- 7

RP- HPLC METHOD DEVELOPMENT FOR THE SIMULTANEOUS ESTIMATION OF AG, GA AND KT IN A POLYHERBAL FORMULATION

7. RP- HPLC METHOD DEVELOPMENT FOR THE SIMULTANEOUS ESTIMATION OF AG, GA AND KT IN A POLYHERBAL FORMULATION

7.1. INSTRUMENTS AND APPARATUS

HPLC: JASCO 200 Series HPLC model (JASCO, Inc. JAPAN) with PU-2080 plus pump, MX- 2080-31 mixer, Rheodyne model 7125 with 20 µl fixed loop injector and Photo Diode Array detector was used. The software used was Borwin version 1.50.

pH Meter: pH meter 111E/101E (Analabs Scientific Instruments Ltd.) having resolution of ±0.01 pH and accuracy of ±0.01 pH was used.

Analytical Balance: Model CX 220 analytical balance (CITIZEN, India) was used.

Sonicator: Trans-O Sonic; D compact () having capacity of 2 liter was used.

Vacuum pump: Rocker vacuum pump- 600

Volumetric flask: Amber coloured volumetric flasks of 10 ml, 25 ml and 100 ml.

7.2. REAGENTS AND MATERIALS

AG standard: procured from Cadilla Pharmaceuticals Limited, Trasad Road, Dholka-387810, Dist. Ahmedabad (Gujarat), India.

GA standard: procured from Cadilla Pharmaceuticals Limited, Trasad Road, Dholka-387810, Dist. Ahmedabad (Gujarat), India.

KT standard: procured from Cadilla Pharmaceuticals Limited, Trasad Road, Dholka-387810, Dist. Ahmedabad (Gujarat), India

All the other reagents used were of AR Grade.

7.3. EXPERIMENTAL WORK

7.3.1. Preparation of Standard Solutions

Preparation- 1: The Standard solutions prepared were the same as those described in Section 6.3.1. in the HPTLC method.

Preparation- 2: 5 mg of Andrographolide, Gallic acid and Kutkin were weighed accurately and transferred in 10 ml amber-coloured vol. flasks separately. The volume was adjusted up to 10 ml separately, using Methanol (500 µg/ml each). From these Stock solutions, 1 ml of the Andrographolide, Gallic acid and Kutkin solutions were transferred in 10 ml amber-coloured vol. flasks separately and volume was made up with Methanol and mixed to produce solutions of Andrographolide- 50 µg/ml, Gallic acid- 50 µg/ml and Kutkin- 50 µg/ml.

7.3.2. Preparation of Sample solution

Preparation- 1: An accurately measured 5 ml of syrup was extracted with ethyl acetate (3 × 10 ml) by shaking extraction technique for 20 min. The combined extracts were filtered, concentrated, and transferred to a 25 ml volumetric flask. The volume was made up by ethyl acetate, which was used for further chromatographic analysis.

Preparation- 2: An accurately measured 50 ml of formulation was extracted with ethyl acetate (5 × 75 ml) by shaking extraction technique for 20 min. The combined extracts were heated on a water bath up to complete evaporation and then diluted with 50ml Mobile phase [Water: ACN (75: 25); pH 3.45 (0.1% Ortho Phosphoric acid)]. It was filtered, Ultra-sonicated and transferred to a 50 ml volumetric flask, which was used for further chromatographic analysis.

7.3.3. Trials of RP- HPLC for the Simultaneous estimation of Andrographolide, Gallic acid and Kutkin

Different compositions of some mobile phases were tested along with the reported mobile phases for resolved and reproducible peaks of AG, GA and KT standards as well as the spots of these three standards in the formulation extract are:

TRIAL 1: Methanol: Water (65: 35): of Andrographolide
The other chromatographic conditions employed are given as shown in the Table 7.1.

Prepared standard AG, GA and KT solutions (as per Preparation-1 of Section 7.3.1.) as well as the final Ethyl acetate extract (as per Preparation-1 of Section 7.3.2.) were injected to HPLC column as per the above mentioned chromatographic conditions.

TRIAL 2: ACN: Water (20:80); pH - 3 (0.1%Ortho Phosphoric acid): of Gallic acid
The other chromatographic conditions employed are given in Table 7.2.

Prepared standard AG, GA and KT solutions (as per Preparation-1 of Section 7.3.1.) as well as the final Ethyl acetate extract (as per Preparation-1 of Section 7.3.2.) were injected to HPLC column as per the above mentioned chromatographic conditions.

TRIAL 3: ACN: Water (25: 75); pH- 3.45 (0.1% Ortho Phosphoric acid): of Kutkin.
The other chromatographic conditions employed are given in Table 7.3.

Prepared standard AG, GA and KT solutions (as per Preparation-2 of Section 7.3.1.) as well as the final Ethyl acetate extract (as per Preparation-2 of Section 7.3.2.) were injected to HPLC column as per the above mentioned chromatographic conditions.

7.3.4. Chromatographic conditions

The chromatographic conditions kept constant throughout the analysis are shown in the Table 7.1.

Table 7.1. The optimized Chromatographic conditions for RP- HPLC i.e., for Trial- III

Sr. no.	Chromatographic condition	Value of Chromatographic condition
1.	Diluent	ACN: Water (25: 75); pH- 3.45 (0.1% Ortho Phosphoric acid)
2.	Column	C18 PUROSPHERE STAR Hyber 250 × 4.5 mm i.d., with 5 μm particle size
3.	Column temperature	25ºC
4.	Sample temperature	25ºC
5.	Mobile phase	ACN: Water (25: 75); pH- 3.45 (0.1% Ortho Phosphoric acid)
6.	Flow rate	1.0 mL/min
7.	Detection wavelength	254 nm
8.	Injection volume	20 Ml
9.	Blank	Methanol

Prepared standard Andrographolide, Gallic acid and Kutkin solutions as well as the Ethyl acetate extract were injected to HPLC column as per the above mentioned chromatographic conditions.

The chromatographic peaks of AG, GA and KT in formulation extract were compared with retention time of standard AG, GA and KT. Peak areas were recorded of prepared standard solutions.

From the peak area of standard AG, GA and KT, compared to the areas of these biomarkers in the Formulation, the amounts of these three Biomarkers in extract were computed.

7.4.3. Conclusion

The formulation, HEPASAVE contains Andrographolide, Gallic acid and Kutkin, which have been estimated for their amount in the formulation. The information obtained from the literature survey revealed the need of a simple and rapid RP- HPLC method for quantitative estimation of AG, GA and KT in HEPASAVE. Thus, the HPLC method developed for the formulation can be said to be one of its kind with the first method to describe a set of RP- HPLC conditions which could give the Quantification of AG, GA and KT in the Polyherbal formulation simultaneously.

CHAPTER- 8

SUMMARY

8. SUMMARY

The purpose of my study is to analyse a new Polyherbal formulation, Hepasave Syrup, of Cadila Pharmaceuticals Ltd., which can be used as an a powerful Hepato-protective, an Antioxidant, and a Bitter tonic. The formulation was evaluated for its Organoleptic characteristics. The drug was a homogenous brown coloured liquid, having a honey- like odour, sweet tasting, and a viscous syrup with 1.15 gm/ml density. The biomarkers selected for the study were Andrographolide, Gallic acid, Kutkin and Vasicine, which were selected based on the fact that the main chemical constituents responsible for the Hepatoprotective activity of the formulation were these four biomarkers, which has been supported by the reported studies carried out on medicinal plants containing the concerned biomarkers. The biomarkers were identified to be AG, GA, KT and VS, by Melting point determination and by U.V. Spectroscopy, when compared to the reported values. The trials carried out under the HPTLC method development showed that AG, GA and KT were present in the sample. However, the peak of VS in the formulation did not appear to support the quantitation of VS in the sample. Based on the trials, a set of HPTLC conditions was optimized for the quantitation of the biomarkers in the formulation and in the sample extract. The optimized method was validated and was found to be linear, precise and accurate. The LOD and LOQ of the Biomarkers was also carried out under the optimized set of conditions. The validated method was then applied for the Quantitation of selected biomarkers in the formulation as well as in the formulation extract. The amount of Andrographolide in the sample was found to be greater than that of Gallic acid and Kutkin, which supports the fact that HEPASAVE is a powerful hepatoprotective as AG has been reported in many studies for its hepatoprotective activity, followed by Gallic acid, which is a chemical constituent of almost all the renowned and reported antioxidant medicinal plants, which helps HEPASAVE to protect the liver against toxicity and inflammation.

Similarly, the trials carried out under RP- HPLC method development showed that AG, GA and KT were present in the sample, and the peak of VS in the formulation did not appear to support the purpose of quantitation of VS in the sample. Based on the trials, a set of HPTLC conditions was optimized for the quantitation of the biomarkers in the formulation and in the sample extract. The developed method was applied for the Quantitation of selected biomarkers in the formulation as well as in the formulation extract. The amount of Andrographolide in the sample was found to be greater than that of Gallic acid and Kutkin, similar to the HPTLC data. The calculated amounts of biomarkers in the formulation by HPTLC and HPLC method provided an information about the reliability of the method developed for the simultaneous estimation of AG, GA and KT in the formulation, the information of which can be applied to any study requiring a simultaneous estimation of AG, GA and KT in a combination for HPTLC and/or HPLC method.

The Future scope to the study includes the HPLC method employed in the current study can be validated as per Official guidelines, i.e ICH guidelines or U.S. Pharmacopoeia, for the estimation of Andrographolide, Gallic acid and Kutkin in Hepasave Tonic, or a new method can also be validated for the same. Various HPTLC and HPLC simultaneous

estimation methods can be developed and Validated for the estimation of Andrographolide, Gallic acid and Kutkin in Hepasave Tonic. The current HPTLC and HPLC methods can be further modified to develop a simultaneous estimation method for the estimation of Andrographolide, Gallic acid, Kutkin as well as Vasicine, which can be applied to other formulations containing Andrographolide, Gallic acid, Kutkin and Vasicine.

CHAPTER- 9

FUTURE SCOPE

9. FUTURE SCOPE

The Future scope to the study includes the HPLC method employed in the current study can be validated as per Official guidelines, i.e ICH guidelines or U.S. Pharmacopoeia, for the estimation of Andrographolide, Gallic acid and Kutkin in Hepasave Tonic, or a new method can also be validated for the same. Various HPTLC and HPLC simultaneous estimation methods can be developed and Validated for the estimation of Andrographolide, Gallic acid and Kutkin in Hepasave Tonic. The current HPTLC and HPLC methods can be further modified to develop a simultaneous estimation method for the estimation of Andrographolide, Gallic acid, Kutkin as well as Vasicine, which can be applied to other formulations containing Andrographolide, Gallic acid, Kutkin and Vasicine.

CHAPTER- 10

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