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STABILITY INDICATING RP-HPLC METHOD DEVELOPMENT AND VALIDATION OF EFONIDIPINE HYDROCHLORIDE ETHANOLATE AND CHLORTHALIDONE

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INTRODUCTION [1]

1.1 Analytical Method Development and Validation

➢Every year, a greater number of medications are added to the market. These medications could be brand-new or a partial structural alteration of an existing medicament. There is frequently a time lag between the date of a drug's introduction to the market and its inclusion in pharmacopoeias. This occurs due to possible uncertainties in the continued and wider use of these drugs, reports of new toxicities, the development of patient resistance, and the introduction of better drugs by competitors. In these circumstances, standard and analytical procedures for these drugs may not be available in the pharmacopoeias. As a result, innovative analysis procedures for such medications have become necessary.

➢Analytical method development and validation are the continuous and interdependent task associated with the research and development, quality control and quality assurance department. Analytical procedure plays a critical role in equivalence and risk assessment, management.

➢Analytical method could be spectral, chromatographic, electrochemical, hyphenated or miscellaneous. Analytical method development is the process of selecting an accurate assay procedure to determine the composition of a formulation. It is the process of proving that an analytical method is acceptable for use in laboratory to measure the concentration of subsequent samples Analytical methods should be used within GMP and GLP environments and must be developed using the protocols and acceptance criteria set out in the ICH guidelines Q2(R1).

➢Analytical Chemistry is the branch of science that uses advance technologies in determining the composition by analytical technique. We can achieve both qualitative as well as quantitative results. Analytical instruments play a major role in the process to achieve high quality and reliable analytical data. Thus, everyone in the analytical laboratory should be concerned about the quality assurance of equipment. Qualitative analysis means to identify the presence of molecule whereas quantitative analysis means to find out the exact concentration of drug in the formulation.

➢Analytical instruments play a major role in the process to achieve high quality and reliable analytical data, Analytical chemistry may be defined as the science and art of determining the composition of materials in terms of elements of composition contained. Pharmaceutical analysis is a bench of science that deals with the analytical procedures used to determine the purity, safety and quality of drugs and chemicals.

➢It contains procedures to determine the identity, strength, quality and purity of new compounds. It also involves procedures for separating, identifying, and determining the relative amount of the components in sample of matter.

1.1.1. Method development [2, 3]

➢The purpose of analytical method development is to establish the identity, purity, physical characteristics, and potency of drugs, including the drug's bioavailability and stability. Analytical method development and validation can be understood as the process of showing that analytical procedures are adequate for the purpose of assessing drugs, and particularly the active pharmaceutical ingredient (API). Analytical procedures are developed to test specific characteristics of the substances against the predefined acceptance criteria for such characteristics.
➢Thus, analytical method development involves the evaluation and selection of the most precise assay procedures to determine the composition of a drug. The prerequisite for method development are as follows:

1) Qualified and calibrated instruments
2) Documented methods
3) Reliable reference standard
4) Qualified analyst
5) Sample selection and integrity
6) Change control

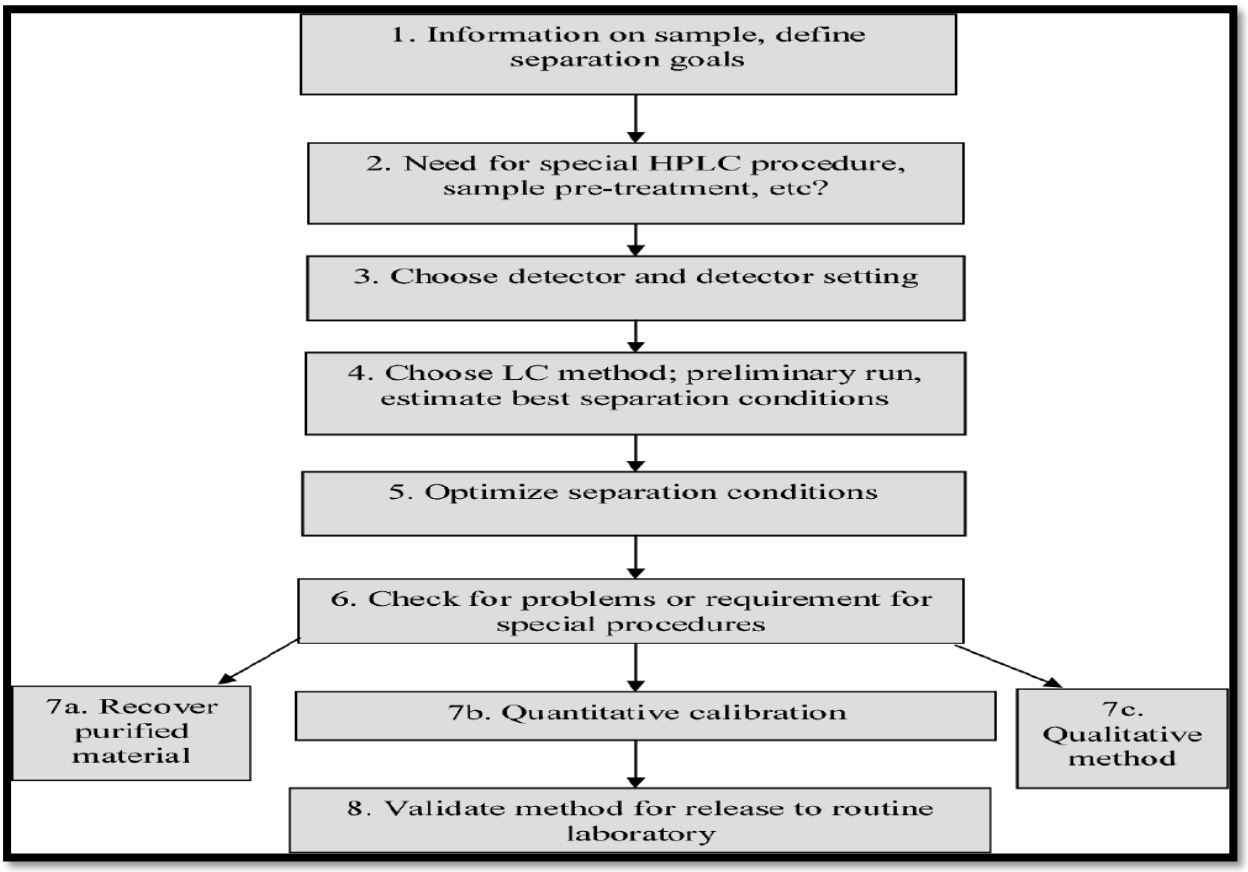
1.1.2. Steps involved in method development [4]

➢Analyte standard characterization: all known information about the analyte and its structure is collected i.e., physical and chemical properties
➢Method requirement: requirements of the analytical method that need to be developed are considered, the required detection limit, selectivity, linearity, accuracy and precision are defined
➢Literature search and prior methodology: The literature for all types of information related to the analyte is surveyed
➢Choosing a method: using the information in the literature and prints, methodology is adapted, if there are no prior methods for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out
➢Instrumental setup and initial studies: The required instrumentation is to be setup. Installation, operational and performance qualification of instrumentation using laboratory standard operating procedures (SOPs) are verified
➢Optimization: During optimization one parameter is changed at a time and set of condition are isolated, rather than using a trial-and-error approach.
➢Documentation of analytical figures of merits: the originally determined analytical figures of merits are limit of detection (LOD), limit of quantification (LOQ), linearity
➢Evaluation of method development with actual samples: the sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.
➢Determination of percent recovery of actual sample and demonstration of quantitative sample analysis: percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery from sample to sample and whether recovery has been optimized or not has been shown. It is not necessary to obtain 100% recovery as long as the results are reproducibility and known with a high degree of certainty.

HPLC METHOD DEVELOPMENT AND VALIDATION

1.2. Method validation [3, 4]

Method validation is the process of ensuring that a test procedure performs within acceptable standard of reliability, accuracy and precision for its intended purpose. In short, validation is the act of confirming that a method does what it is intended to do. A successful validation guarantees that both the technical and regulatory objective of an analytical method have been fulfilled.



Validation of analytical procedure is to demonstrate that it is suitable for its intended purposes, any developed method may be influenced by variables like different elapsed assay times, different days, reagents lots, instrument, equipment, environmental condition like temperature, etc so it is expected that after the method has been developed and before it is communicated or transferred from one lab to another, it is properly validated.

Analytical method have been validated in pursuance of ICH guideline of Q2(R1). Validation parameter are:

System suitability

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

1.2.1. System suitability

In step with the USP, machine suitability tests are an critical part of chromatographic strategies. Those assessments are used to verify that the resolution and reproducibility of the gadget are adequate for the evaluation to be executed. Gadget suitability exams are primarily based at idea that the gadget, electronics, analytical operations, and samples constitute an integral device that may be evaluated as a whole.

System suitability is the checking of a system to make certain system performance before or in the course of the evaluation of unknowns. Parameter such as plate rely, tailing factors, resolution and reproducibility (%RSD retention time and region of repetitive injection) are decided and in comparison, against the specs set for the approach.

1.2.2. Specificity

Definition: specificity is the ability to evaluate unequivocally the analyte within the presence of additives which may be expected to be present. Usually these might consist of impurities, degradants, matrix, and so on. Lack of specificity of an individual analytical procedure may be compensated via other assisting analytical methods.

ICH divides the term specificity into two separate categories

Identity: to ensure the identification of analyte.

Impurity exams: to make sure that each one the analytical tactics accomplished allow an accurate declaration of the content material of impurities of an analyte, i.e. associated checks, heavy metals, residual solvents content, and many others

Assay (content material or potency): to offer an exact end results this lets in an correct statements on the content material or potency of analyte in a sample. Analytical strategies which can measure the analyte response inside the presence of all capacity sample additives need to be used for specificity validation. Specificity in liquid chromatography is received by choosing most efficient columns and putting chromatographic situations, along with mobile section composition, column temperature and detector wavelength.

1.2.3. Linearity

The linearity of a method is a measure of how well a calibration plot of response vs. concentrations approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data is then processed using a linear least- square regression. The resulting plot slope, intercept and correlation coefficient provide the desired information on linearity.

1.2.4. Precision

Definition: it expresses closeness of settlement between a sequence of measurement obtained from multiple sampling of the same homogenous sample under the prescribed conditions.

Precision can be taken into consideration at three tiers: repeatability, intermediate precision and reproducibility. Repeatability is also called intra-assay precision. Repeatability is the precision of a method under the same operating condition over a short period of time. ICH recommends at least nine determination covering the desired variety for the technique (e.g., three concentration / 3 replicates as in the accuracy test), or not less than six determination at a hundred % of the check concentration for evaluation of repeatability which ought to be stated as popular deviation, relative general deviation (coefficient of variant). Intermediate precision is the agreement of complete measurements (including standard) when the same method is applied many times within the same laboratory. ICH defines intermediate precision as long- term variability of the dimension method and is determined by means of comparing the outcomes of a method run within a single laboratory over a number of weeks. It's also referred to as intraday precision. Reproducibility examines the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

1.2.5. Accuracy

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with a high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value. Typically, accuracy is represented and determined by recovery studies. There are three ways to determine accuracy:

1) Comparison to reference standard
2) Recovery of the analyte spiked into blank matrix
3) Standard addition of the analyte.

It should be clear how the individual or total impurities are to be determined. e.g. weight/weight or area percent in all cases with respect to the major analyte.

1.2.6. Range

The range of analytical procedure is the interval between the upper and lower concentration (amount) of analyte in the sample.

It is established by conforming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to the samples containing amount of analyte within or extremes of the specified range of the analytical procedure.

For assay – 80 to 120% of test concentration
Content uniformity – 70 to 130% of test concentration
Dissolution – Q-20% To 120%
Impurities – reporting level – 120% of impurity specification limit
Assay & impurities – reporting level to 120% of assay specific

1.2.7. Limit of detection

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be detected, but not necessarily quantified, under the stated experimental condition. With UV detectors, it is difficult to assure the detection precision of low level compound due to potential gradual loss of sensitivity of detector manufacturer. At low levels assurance is needed that the LOD and LOQ limits are achievable with the test method each time. With no reference standard for a given impurity or means to assure detect ability, extraneous peak could “disappear / appear”. Several approaches for determining the LOD are possible, depending on whether the procedure is a non-instrumental or instrumental.

- Based on visual evaluation
- Based on signal-to-noise
- Based on the standard deviation of the response and the slope The LOD may be expressed as: LOD = 3.3 σ/S

Where, σ = standard deviation of intercepts of calibration curves
S = mean of slopes of the calibration curves
The slope S may be estimated from the calibration curves of the analyte.

1.2.8. Limit of Quantification

Limit of quantification (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental condition. Several approaches for determining the LOQ are possible depending on whether the procedure is a non-instrumental or instrumental.

Based on visual evaluation
Based on signal-to-noise approach
Based on the standard deviation of the response and the slope The LOQ may be expressed as: LOQ = 10σ/S
Where, σ = standard deviation of intercepts of calibration curves S = mean of slope of the calibration curves. The slope S may be estimated from the calibration curve of the analyte

1.2.9. Robustness

The robustness of an analytical system is a degree of its ability to stay unaffected by small but deliberate variations in method parameter and affords an indication of its reliability during regular utilization. Within the case of liquid chromatography, example of normal variations are:

Influence of variations of pH in a mobile section. Have an impact on of variations in cellular segment composition
Unique columns (special lots)
Temperature Glide price

1.2.10. Ruggedness

The ruggedness of an analytical techniques is the degree of reproducibility of test outcomes received by means of the analysis of the equal samples beneath a diffusion of regular test conditions consisting of distinct laboratories, distinct analysts, using operational and environmental condition which could vary but are still inside the unique parameters of the assay.

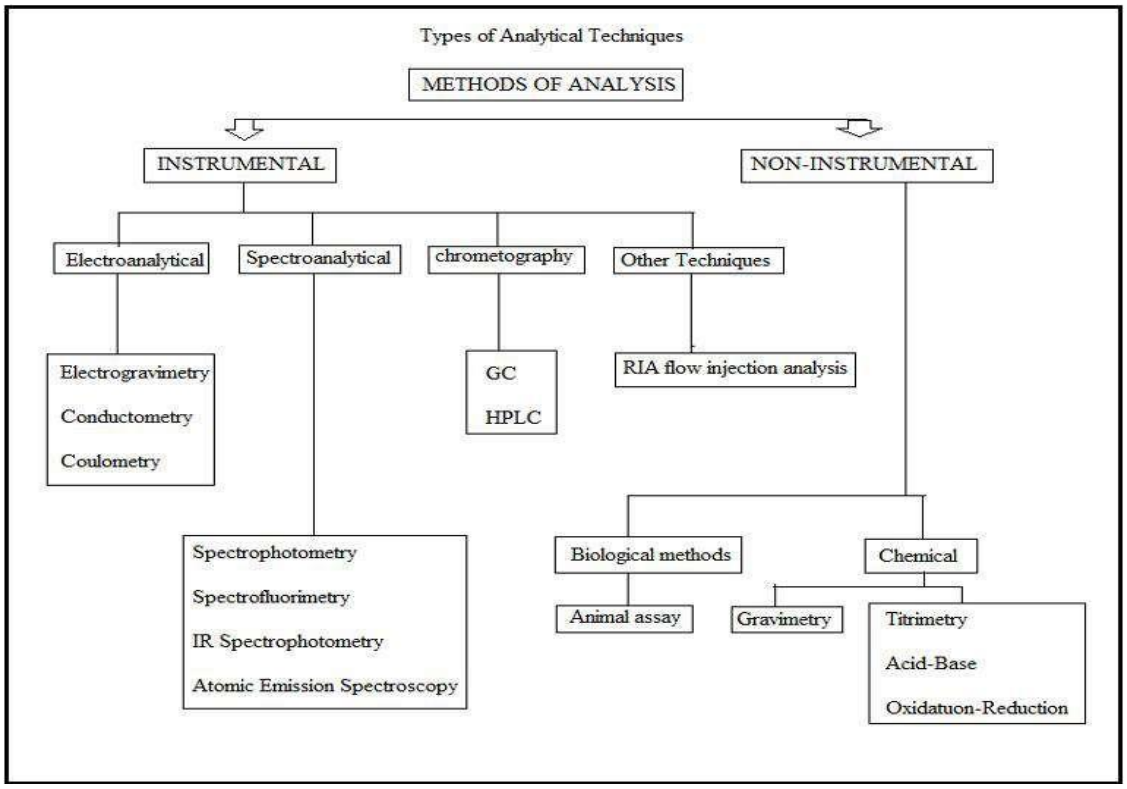
1.3. Need of analytical method development [3-4]

The need of validation of the analytical method development and validation emerged due to international competition, maintaining the standard of products in high commercial & market value and ethical reasons. Various international regulatory agencies have set the standard and fixed the protocol to match the reference for granting approval, authentication and registration. Some of the famous organization governing the quality standard are:

1) United states food and drug administration (USFDA)
2) Current good manufacturing practice (cGMP)
3) Good laboratory practice (GLP)
4) The pharmaceutical inspection cooperation scheme's (PIC/S)
5) The international conference for harmonization (ICH)
6) ISO/IEC 17025

When some changes are made in the validated nonstandard methods, the influence of such changes should be documented and a new validation should be carried out. If standard methods are available for a specific sample test, the most recent edition should be used. Validation includes specification of requirements, determination of method characteristics, a check that the requirement can be fulfilled by using the method, a statement on validity.

1.3.1. Classification of analytical methods.



Analytical methods are generally classified into two categories:

- **Instrumental**
- **Non-instrumental**

Instrumental method: A physical property is made to determine the contents of composition of a substance. E.g. electrochemical method- change in the electrical properties of the system. Example are electrogravimetry, potentiometric, conductometry.

Advantages:

- Small samples can be used
- High sensitivity can be obtained
- Determination is very fast
- Even complex samples can be handled easily

Non-instrumental method: in these methods chemical reaction are involved. These are volumetric and gravimetric methods. These are also known as classical method of analysis.

Advantages:

- Methods are based on absolute measurement
- Specialized training is usually not required
- Equipment needed is cheap

Method of analysis

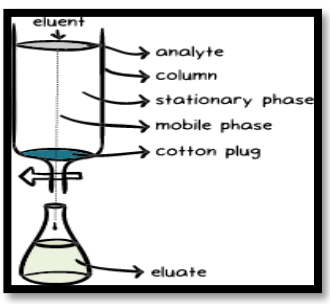
The instrumental separative techniques are divided into three categories

1) Chromatography
2) Electro analytical
3) Spectroanalytical

1.4. Chromatography [6-10]

Chromatography is a technique by which a mixture is separated into its components as a result of the ability of each component to be eluted along or through the stationary phase by mobile phase. The sample is placed on edge of the stationary phase (a solid or liquid) and a mobile phase is allowed to flow over the stationary phase to sweep the sample along the length of the stationary phase. Component which are strongly adsorbed to the stationary phase are swept less rapidly along the length of the stationary phase than those components that are less strongly adsorbed to stationary phase.
The word chromatography is derived from the Greek letters' chromos meaning colour and the graphy means colour writing. The initial use of the terms is attributed to T swett, who separated colour band of plant pigments on a chromatography column that consist of an adsorbent powder that was washed with a liquid solvent termed as mobile phase. This is carried down the length of the tube that contains an immobile solid or liquid phase i.e. stationary phase.
Each component is retained to a different degree in the system and retention is based on various attraction forces. A chromatogram is a graphical representation of the compound eluting from a chromatographic system. Chromatography can be used for qualitative and quantitative determination of various food constituents.
Paper chromatography, thin layer chromatography, gas liquid chromatography, high performance liquid chromatography are the available choices for assay involving sophisticated equipment, which are highly sensitive, accurate and consume very tiny number of samples for analysis.

1.4.1. Principles of chromatography [6-10]

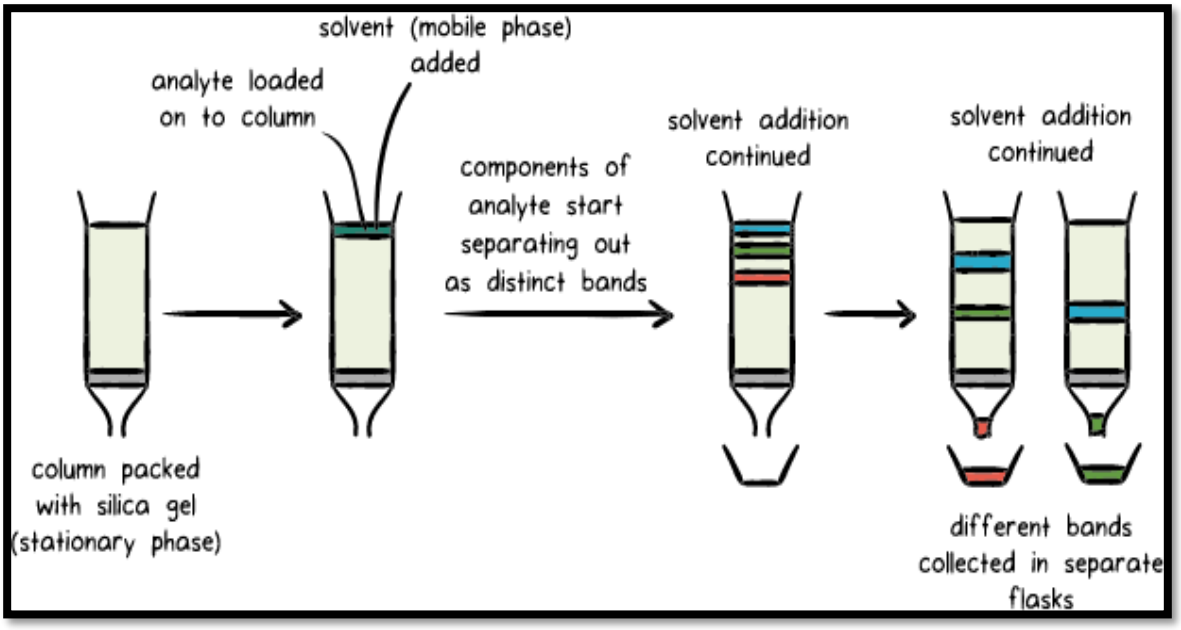


Chromatography is based on the principle where molecule in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase. The factor effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-

liquid) and affinity or differences among their molecular weight. Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatographic system, while others pass rapidly into mobile phase, and leave the system faster.

Terms that are commonly used in the context of chromatography:

- Mobile phase or carrier: solvent moving through the column
- Stationary phase : substance that stays fixed inside the column
- Eluent : fluid entering the column
- Eluate : fluid exiting the column (that is collected in flask)
- Elution : The process of washing out a compound through a column using a suitable solvent
- Analyte : mixtures whose individual components have to be separated



1.5. HPLC (High Performance Liquid chromatography) [6-10]

The techniques of high performance liquid chromatography (HPLC) was developed in the later 1960s and early 1970s from knowledge of the theoretical principles that already had been established for earlier chromatographic techniques in particular for column chromatography. HPLC differ from column chromatography in that mobile phase is pumped through the packed column under high pressure.

High performance liquid chromatography (HPLC) is basically a highly improved form of column liquid chromatography, instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressure of up to 400 atmosphere. That makes it much faster.

All chromatographic separations, including HPLC operate under the same basic principle; separation of a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation.

1.5.1. Basic principle of HPLC

High performance liquid chromatography (HPLC) is a separation technique utilizing differences in distribution of compound in phases called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles and the mobile phase designates the liquid flowing over the particles. As a results, the component move at different speeds over the stationary phase and there by separated by each other. The column is a stainless steel (or resin) tube, which is packed with spherical solid particles. Mobile phase constantly fed into the column inlet at a constant rate by a liquid pump. Sample is injected from sample injector, located near the column inlet, the injected sample enters the column with mobile phase and the components in these samples migrates through it, passing between the stationary phase and mobile phase.

A detector connected to the outlet of the column detects each compound eluting from the column, the recorder starts at the time when sample is injected and monitors the separation process and a graph is obtained. This graph is called

chromatogram. The time required for a compound to elute (called retention time) and the relationship between the compound concentration (amount) and peak area on the characteristics of the compound. Depending on the composition of the mobile phase, two different modes are generally applicable.

If the composition of the mobile phase remains constant during the separation process, the HPLC system is defined as an isocratic elution system.

When the composition of mobile phase is changed during separation, the HPLC system is defined as a gradient elution system.

1.5.2. Types of HPLC

There are following variants of HPLC, depending upon the phases system (stationary) in the process

1) Normal phase HPLC

This method separate analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase. Therefore, the stationary phase is usually silica and typical mobile phases are Hexane, Methylene chloride, Chloroform, Diethyl ether, and mixtures of these.

Polar samples are thus retained on the polar surfaces of the column packing longer than less polar materials.

2) Reversed phase HPLC

The stationary phase is no polar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and Methanol or Acetonitrile. it works on the principle of hydrophobic interaction hence the more nonpolar the material is, the longer it will be retained.

3) Size-exclusion HPLC

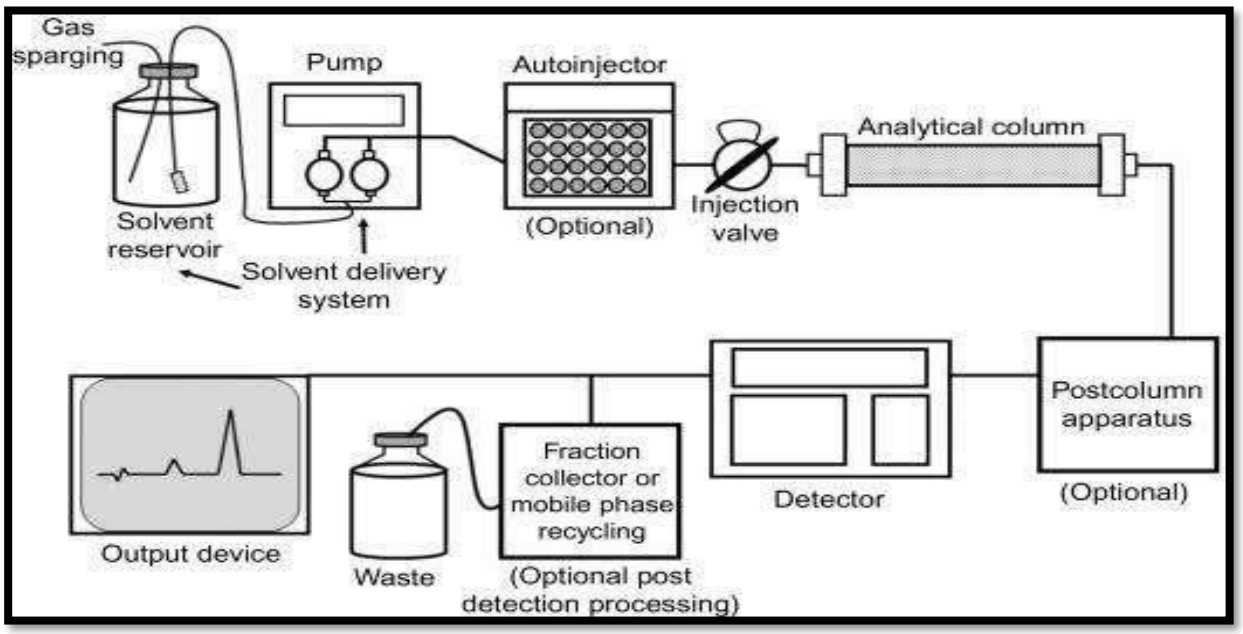
The column is filled with material having precisely controlled pore sizes, and the particles are separated according to its their molecular size. Larger molecule are rapidly washed through the column; smaller molecules penetrates inside the porous of the packing particles and elute later.

4) Ion-Exchange HPLC

The stationary phase has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

INSTRUMENTATION -

- A HPLC consist of Solvent storage bottle
- Gradient controlled and mixing unit
- De-gassing solvents
- Pump
- Pressure gauge
- Pre-column
- Sample introduction system
- Column
- Detector
- Recorder



Typical HPLC system consist of

I.Solvent reservoir

Mobile phase contents are contained in a glass reservoir. The mobile phase, or solvent, in HPLC is usually a mixture of polar and non-polar liquid components whose respective concentration is varied depending on the composition of the sample. Stainless steel should be avoided when solvents containing to 1 liter mobile phase (pure organic solvents or aqueous solutions of salts and buffers).

II.Pump

Most modern pumps allow controlled mixing of different solvents from different reservoirs. A pump aspirates the mobile phase from the solvents reservoir and forces it through the system's column and detector. Depending on a number of factors including column dimensions, particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressure of up to 6000psi can be generated.

III.Sample injector

This allows an injection of the sample of analyte mixtures into the stream of the mobile phase before it enters the column. The injector can be a single injection or an automated injection system. an injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100ml of volume with high reproducibility and under high pressure (up to 4000psi).

IV.Column

This is most important tool of HPLC systems; it actually produces a separation of the analyte in the mixture. A column is the place where the mobile phase is contact with the stationary phase, forming an interface with enormous surface. Most of the chromatography development in recent years went towards the design of many different ways to increase this interfacial contact.

Column are usually made of polished stainless steel, are between 50 and 30mm long and have internal diameter of between 2 and 5mm. they are commonly filled with a stationary phase with a particle size of 3-10µm. ideally the temperature of the mobile phase and the column should be kept constant during an analysis.

V.Detector

The HPLC detector, located at the end of the column detect the analytes as they elute from the chromatographic column. UV (ultraviolet) is the most common detector used in pharmaceutical analysis, which allow monitoring the continuous registration of the UV absorbance at a selected wavelength or over a span of wavelength.

- **Detectors used in HPLC**

a)UV visible Detector
b) Refractive index Detector
c)Fluorescence Detector
d) Radioactive Detector
e)Conductivity Detector
f) Electrochemical Detector
g) Mass spectrometer Detector

VI.Data collection devices and control system

This is computer-based system that controls all parameter of HPLC instrument (eluent composition (mixing of different solvents) temperature, injection sequence etc.) signals from the detector may be collected on chart recorder or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret.

VII.System suitability specification and tests

System suitability testing is essential for the assurance of the quality performance of the chromatographic system. The system suitability testing (SST) is used to verify that an analytical method was suitable for its intended purpose the day of analysis was done. It is an essential parameter to ensure the quality of the method for correct measurements. An SST is run each time an analysis is performed and each SST is specific to an individual method with pre-defined acceptance criteria for certain parameter e.g. absorbance criteria values being between 0.2 and 1.0 for photometric content determination method.

The parameters used in system suitability test reports are as follows:

Theoretical plate (N)

Numbers of theoretical plates determine the efficiency of the column. A column in chromatography is assumed to be divided into N number of theoretical plates. This can be calculated in various ways, for example the USP uses the peak width at the base and the BP uses the peak width at half the height.

BP N = 5.545(t/Wh) ^{2}

Or

USP N = 16(t/Wb) ^{2}

Therefore the plates number is high the column is more efficient. The plate number depends on column length i.e. the longer the column the larger the plate number.

Capacity factors (K)

K = tR – t0/t0 Where,

tR: Retention time

t0: Void volume / Dead volume.

The capacity factor is a measure of where the peak of interest is located with respect to the void volume, i.e., elution time of the non retained components. K indicates its retention behavior in a column. if K value is small then the compound is eluted near void volume as it is poorly retained. If K values is large then it takes longer analysis time with peak broadening and decrease in sensitivity, but better separation is achieved.

Recommendation: between 1 to 5

This value gives an indication of how long each component is retained on the column.

Tailing factor (T)

T = W0.05/2f

Where,

W0.05: peak width at 5% height

F : Distance from the peak maximum to the leading edge of the peak height from the baseline.

Tailing factor is defined as measuring the symmetry of the peak.

The value of tailing factor increases as tailing become more pronounced. As the peak tailing increases, quantization accuracy decreases.

Recommendation: T must be < 2

Relative retention (ά)

ά = K'1/K'2

Where,

K'1 : first capacity factor K'2 : second capacity factor

This describe the relative position of two adjacent peaks. Ideally, it is calculated using the capacity factor because the peaks separation depends on the components interaction with the stationary phase. If the capacity factor is used then the separation factor should be consistent for a given column, mobile phase composition, specified temperature and regardless of the instrument used.

Resolution (Rs)

Rs = 2(t2-t1)/W1+W2

Where,

t1&t2: Retention time first & second adjustment band W1&W2 : baseline bandwidth for first & second adjustment band Resolution provides a quantitative measure of ability of the column to separate the two analytes. This measurement is obtained by retention time and peak width which is easily obtained from the chromatogram. Resolution must be at least 0.5 for two peaks to remain separate. Completely separated peaks obtained if resolution "R" > 1.5. if length of column is increases resolution will be improved but the analysis time will also be increased.

1.5.3. Application of HPLC

HPLC helps in identification and quantification of compound. HPLC analysis provides purification and separation of compound, high resolution and precision.
There are various other application of HPLC mentioned:.

- Application in flavor and food industry
- Water and soft drinks quality
- Analysis of preservative Fruit juices – sugar analysis Environmental application Bio monitoring
- Drinking water – phenol compounds Clinical application
- Antibiotics analysis Analysis of urine Analysis of blood substance Pharmaceutical application
- Quality control and Quality Assurance Shelf life determination Dissolution study of dosage formulation.

Limitation of HPLC

- For complex mixtures limited resolution is obtained
- Interfacing HPLC with mass spectrometry is required for qualitative analysis

1.6. INTRODUCTION TO DISEASE [12]

Hypertension (HTN or HT), also known as high blood pressure, is a long-term medical condition in which the blood pressure in the arteries is persistently elevated. High blood pressure typically does not cause symptoms. Long term high blood pressure, however, is a major risk factor for coronary artery disease, stroke, heart failure, atrial fibrillation, chronic kidney disease.
Hypertension can be divided into-

1) Borderline hypertension 140/190-95 mm of Hg
2) Mild hypertension 140-160/90-95 mm of Hg
3) Moderate hypertension >140/105-120 mm of Hg
4) Severe hypertension > 140/>120 mm of Hg

Hypertension is classified into two groups

- **Essential or primary hypertension**
- **Secondary hypertension**

I.Essential or primary hypertension

Primary hypertension is a most common form of hypertension, due to nonspecific lifestyle and genetic factor. Lifestyle factors that increase the risk include excess salt in the diet, excess body weight, smoking and alcohol.

II.Secondary hypertension

Secondary hypertension occurs due to definable causes like renal disease, disease of adrenal gland, endocrine disorder, and pregnancy.
Blood pressure is expressed by two measurements, the systolic and diastolic pressures. BP is commonly expressed as the ratio of systolic BP (that is, the pressure that the blood exerts on the arterial walls when the heart contracts) and the diastolic BP (the pressure when heart relaxes). Atrial blood pressure is "normal" when the systolic pressure is 90-119 mm Hg and the diastolic pressure is 60-79 mm Hg (normal BP- 120/80 mmHg). Blood pressure <90/60 mmHg are considered abnormally low (hypotension).
Lifestyle changes and medication can lower blood pressure and decreases the risk of health complications. Lifestyle

changes include weight loss, physical exercise, decreased salt intake, reducing alcohol intake, and a healthy diet. If lifestyle changes are not sufficient then blood pressure medication are used.
Hypertension is commonly associated with other cardiovascular risk factors, such as obesity, diabetes and Dyslipidaemia.

1.6.1. ANTIHYPERTENSIVE AGENTS are categorized as:

Diuretics
Thiazide: Hydrochlorothiazide, Chlorthalidone, Indapamide
High ceiling: Furosemide, Torsemide, Ethacrynic acid.
K+ Sparing: Spironolactone, Amiloride
ACE Inhibitor
Captopril, Enalpril, Lisinopril, Perindopril, Ramipril, Fosinopril, etc.
Angiotensin (AT1 receptor) blockers
Losartan, Candesartan, Irbesartan, Valsartan, Telmisartan, Fimasartan
Direct Renin inhibitor
Aliskiren
β Adrenergic blocker
Propranolol, Metoprolol, Atenolol, etc.
Calcium channel blocker
Verapamil, Diltiazem, Nifidipine, Felodipine, Amlodipine, Nitrendipine Lacidipine, Efonidipine etc.
β+ɑ Adrenergic blocker
Labetolol, Carvedilol
ɑ Adrenergic blocker
Prazosin, Terazosin, Doxazosin, Phentolamine, Phenoxybenzamine
Central sympatholytics
Clonidine, methyldopa
Vasodilators
Arteriolar : Hydralazine, Minoxidil, Diazoxide
Arteriolar+venous : sodium nitroprusside
Others (Adrenergic neurone blocker)
Reserpine, Guanethidine, etc
Ganglionic blockers
Pentolinium,etc

STABILITY INDICATING METHOD DEVELOPMENT [17-18]

Validated quantitative test methods that can detect changes with time in the chemical, physical, or microbiological properties of drug substances or drug products. They are specific so that the quantity of the active ingredient, degradation products and other components of interest may be accurately measured without interference in the material being tested. Purposeful degradation studies of the drug substance include appropriate solution and solid-state stress conditions (e.g., acid/base hydrolysis, heat, humidity, oxidation, and light exposure, in accordance with ICH guidelines).

1.7.1 Need of Stability Indicating Analytical Methods

For marketing applications, current FDA and ICH guidance recommends inclusion of the results, including chromatograms of stressed samples, demonstration of the stability-indicating nature of the analytical procedures, and the degradation pathways of the drug substance in solution, solid state, and drug product. The structures of significant degradation products and the associated procedures for their isolation and/or characterization also are expected to be included in the filing.

1.7.2 Objective

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, and enables recommended storage conditions, re-test periods and shelf lives to be established.

Specified stress conditions should result in approximately 10–20% degradation of the drug substance or represent a reasonable maximum condition achievable for the drug substance. If no degradation is observed under the conditions that will be detailed in this section, it is recommended that stress testing be stopped.

1.8 FORCED DEGRADATION STUDY: [19]

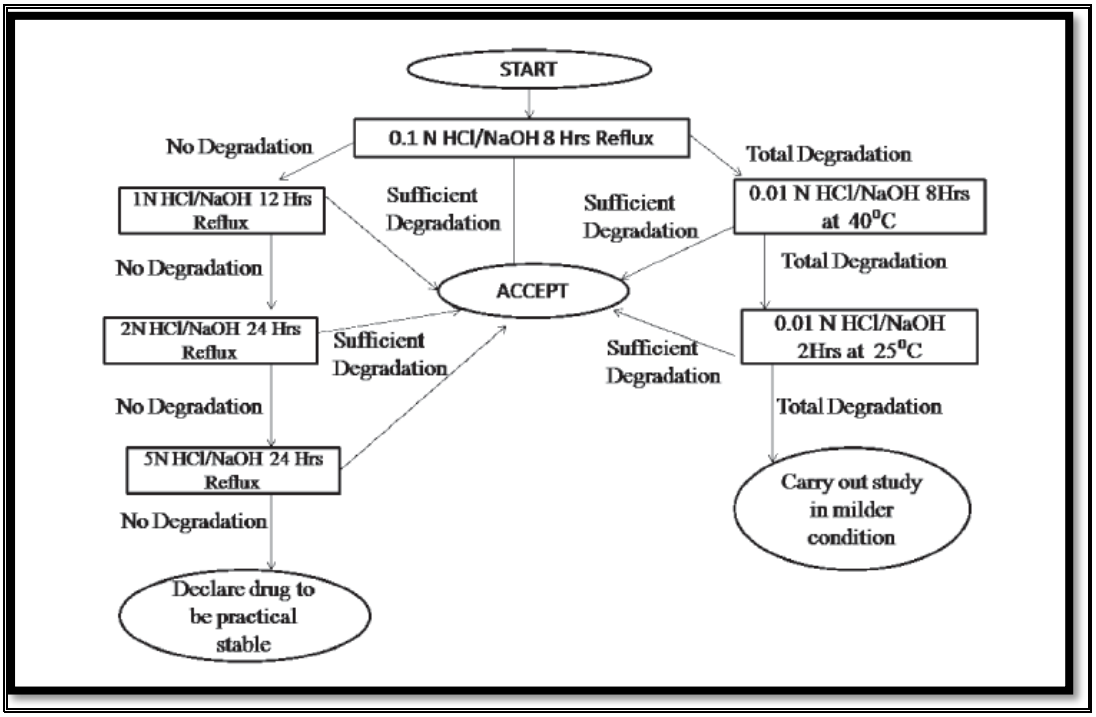
Forced degradation (FD) study process in which the natural degradation rate of a pharmaceutical product is increased by the application of an additional stress. FD studies (i) help to identify reactions that cause degradation of pharmaceutical product, (ii) are part of the development strategy and an integral component of validating analytical methods that indicate stability and detect impurities which are formed during manufacture, storage, or use and their properties are different from the desired product with respect to activity, efficiency and safety (iii) and designed to generate product-related variants and develop analytical methods to determine the degradation products formed during accelerated and long-term stability studies.

Origin of Degradation product/Degradation Related Impurities (DRIs)

The main cause of appearance of impurities in drug substance or product is due to its degradation. The chemical instability of the drug substance under the conditions of heat, humidity, solvent, pH, and light encountered during manufacture, isolation, purification, drying, storage, transportation, and/or formulation is main cause of its degradation. It is governed by inherent chemical stability of the drug substance. The major routes of degradation of any drug substance include hydrolysis, oxidation, heat and photolysis. The stress testing helps in generation all possible degradation products that may form under different conditions:

1.8.1. Hydrolytic condition

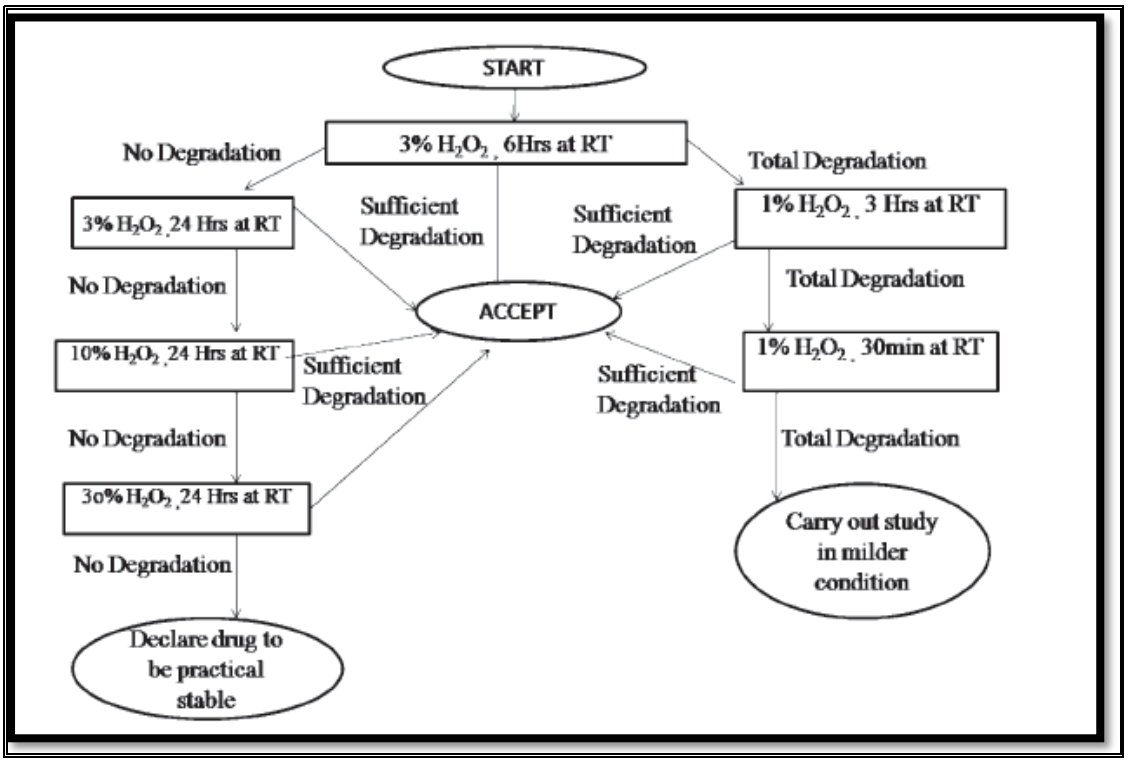
Hydrolysis is one of the most common degradation chemical reactions over wide range of pH. Hydrolysis is a solvolytic process in which drug reacts with water to yield breakdown products of different chemical compositions. Water either as a solvent or as moisture in the air comes in contact with pharmaceuical dosage forms is responsible for degradation most of the drugs.



1.8.2 Oxidative condition

Many drug substances undergo autoxidation i.e., oxidation under normal storage condition and involving ground state elemental oxygen. Therefore it is an important degradation pathway of many drugs. Autoxidation is a free radical reaction that requires free radical initiator to begin the chain reaction. Hydrogen peroxide, metal ions, or trace level of impurities in a drug substance act as initiators for autoxidation.

Hydrogen peroxide is very common oxidant to produce oxidative degradation products which may arise as minor impurities during long term stability studies. It can be used in the concentration range of 3-30% at a temperature not exceeding 40 ˚C for 2-8 days. The oxidative stress testing is initially carried out in 3% H_2O_2 at room temperature for 6 hr and it can be increased/ decreased to achieve sufficient degradation. The time can also be increased up to 24 hr with 30% or decreased up to 30 min with 1% of H_2O_2



1.8.3 Thermal condition

In general, rate of a reaction increase with increase in temperature. Hence, the drugs are susceptible to degradation at higher temperature. Many APIs are sensitive to heat or tropical temperatures. For example, vitamins, peptides, etc. Thermal degradation involves different reactions like pyrolysis, hydrolysis, decarboxylation, isomerization, rearrangement and polymerization. Effect of temperature on thermal degradation of a substance is studied through Arrhenius equation:

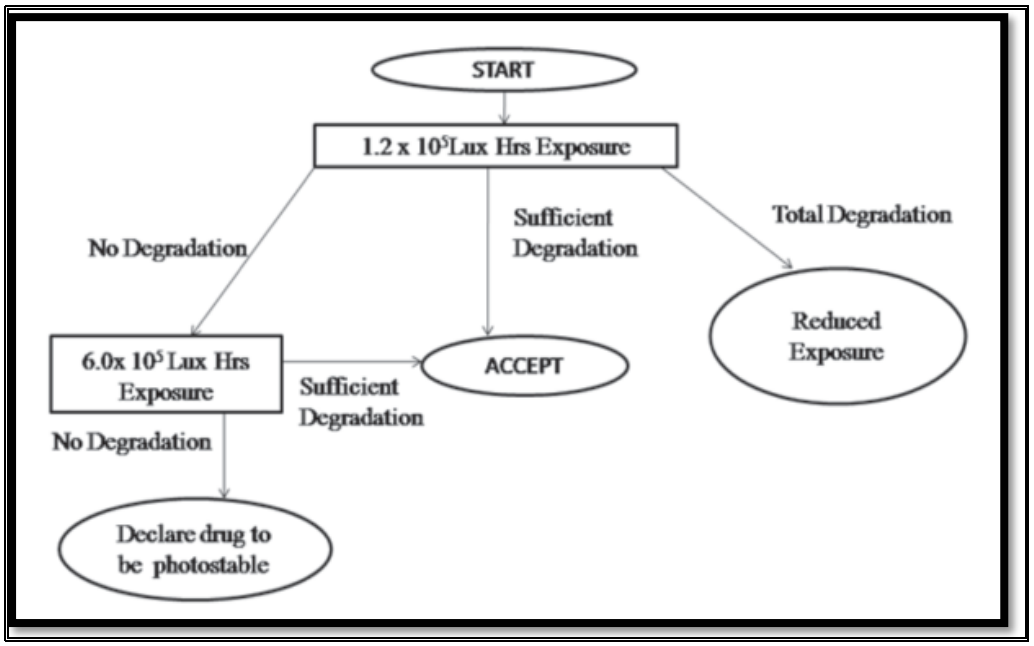
K= Ae-Ea/RT

Where,
k is specific reaction rate, A is frequency factor,
Ea is energy of activation, R is gas constant (1.987 cal/deg mole) and T is absolute temperature1.
Thermal degradation study is carried out at 40˚C to 80˚C. The most widely accepted temperature is 70˚C at low and high humidity for 1-2 months. High temperature (>80˚C) may not produce predictive degradation pathway9,27. The use of high-temperatures in predictive degradation studies assumes that the drug molecule will follow the same pathway of decomposition at all temperatures. This assumption may not hold true for all drug molecules, and therefore great care must be taken in using the extreme temperatures easily accessible in a sealed-vessel microwave experiment for predictive degradation studies.

1.8.4. Photolytic condition

Exposure of drug molecules may produce photolytic degraded products. The rate of photo degradation depends upon the intensity of incident light and quantity of light absorbed by the drug molecule. Photolytic degradation is carried out by exposing the drug substance (in solid as well as in the solution form) or drug product to a combination of visible and UV light. The most commonly accepted wavelength of light is in the range of 300-800 nm to cause the photolytic degradation.

The photolytic degradation can occur through non oxidative or oxidative photolytic reaction. The nonoxidative photolytic reaction include isomerization, dimerization, cyclization, rearrangements, decarboxylation and haemolytic cleavage of X-C hetero bonds, N-alkyl bond(dealkylation and deamination), SO2-C bonds etc and while oxidative photolytic reaction occur through either singlet oxygen(1O2) or triplet oxygen(3O2) mechanism. The overall illumination showed not be less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 W-h/m2. However, illumination is decreased or increased to achieve sufficient degradation. The maximum illumination recommended is 6 million lux h33



PRELIMINARY WORK:

Procurements of drug:

1.Efonidipine HCl Ethanolate sAPI was received as gift sample from Zuventus Pharma Ltd., Mumbai, Maharashtra

2.Chlorthalidone API was received as gift sample from (Zota Health Care Ltd., Surat, Gujarat)

3.Identification of API

4.The identification of API was carried out by performing melting point, IR- study andsolubility study

Melting point determination:

Melting point at which the substances change in physical state from start from solid to liquid at a constant temperature. The sample of Efonidipine HCl Ethanolate and Chlorthalidone was filled in capillary and both placed into the melting point apparatus compared observedmelting point with reference melting point.

Melting point determination

Drugs	Reported Melting Point (°C)	Observed Melting Point (°C)
Efonidipine Hydrochloride Ethanolate	151 °C [25]	153-155 °C
Chlorthalidone	239 °C [40]	236-240 °C

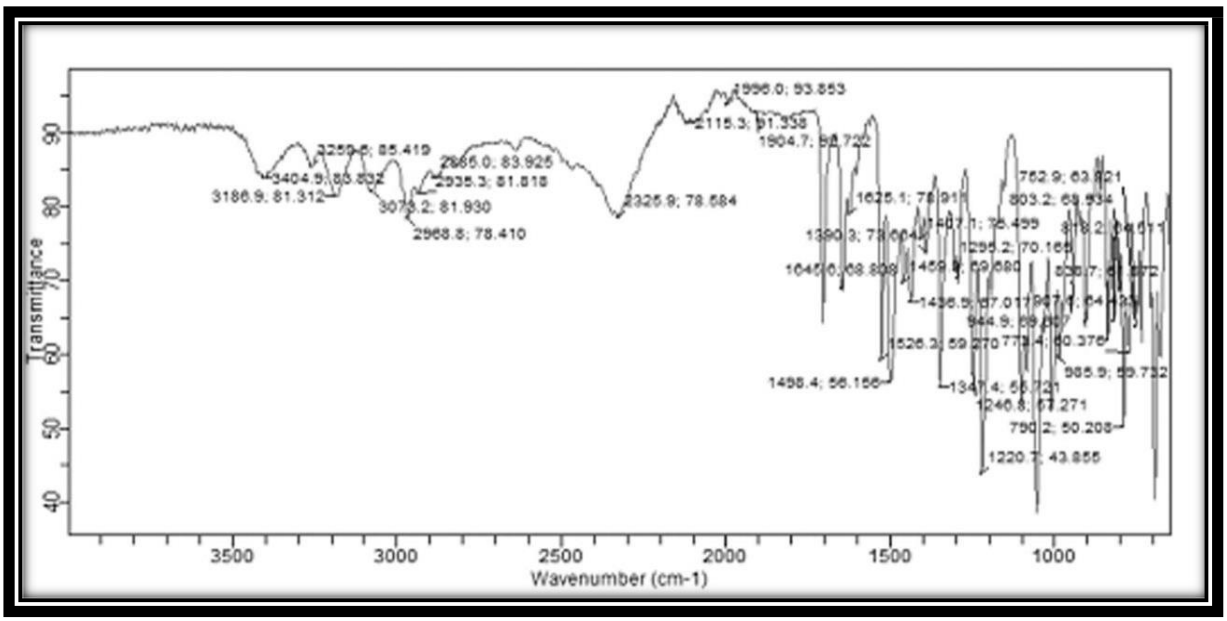
Solubility study

Drugs	Efonidipine Hydrochloride Ethanolate	Chlorthalidone
Water	Insoluble	Soluble
Methanol	Soluble	Soluble
Acetonitrile	Slightly soluble	Slightly soluble

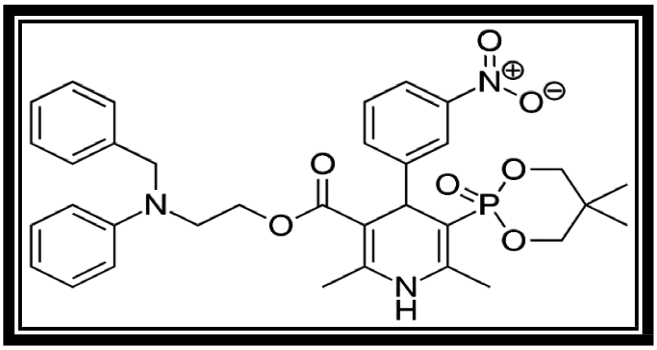
IR spectra study:

- The IR spectrum of EFO and CHLO obtain by using FT-IR spectrophotometer (Bruker Alfa FTIR-ATR instrument equipped with OPUS software)

- The reference IR spectrum of EFO and CHLO (from literature review) and sample IR spectrum of EFO and CHLO are shown in Fig..The finger region of the obtained IR spectrum of the EFO and CHLO was compared with the reference spectrum of both finger print region.



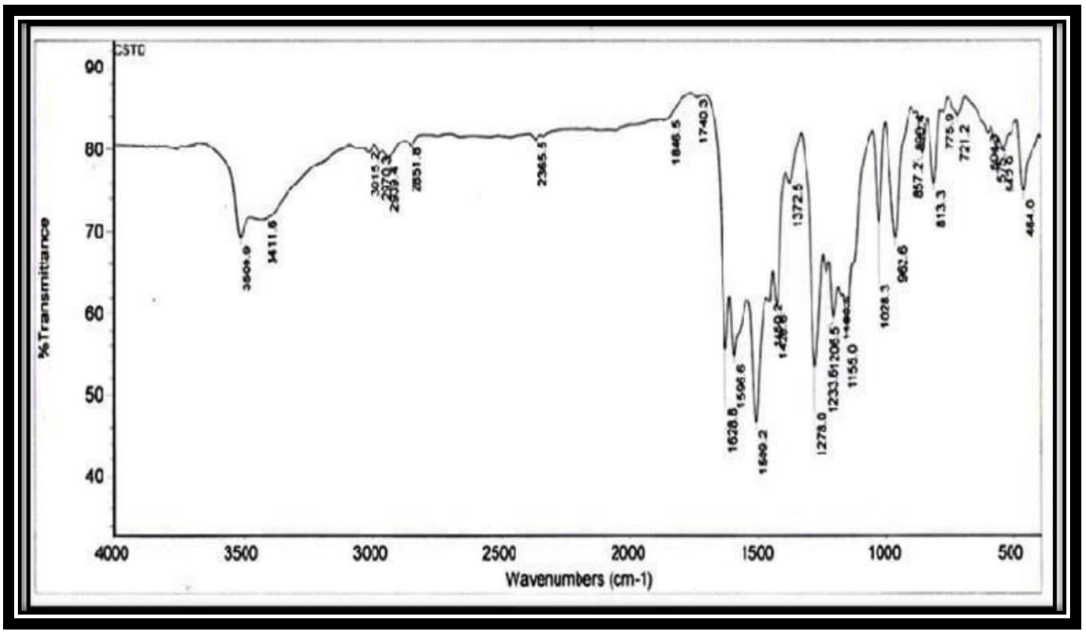
IR Spectra of Efoni (Sample)



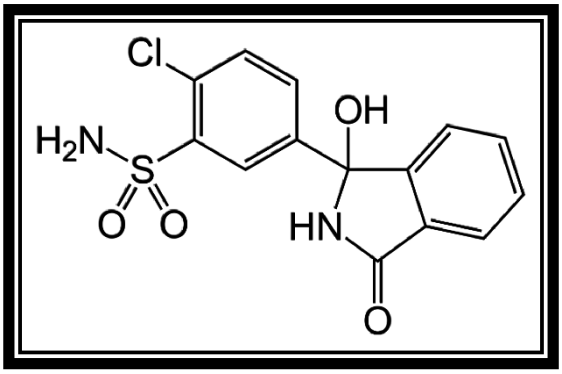
Structure of EFO

IR value for Efonidipine Hydrochloride Ethanolate

Sr. No.	Functional Group	Reported Wavenumber (cm^{-1})	Observed Wavenumber (cm^{-1})
1.	N-H	3450-3300	3404.9
2.	C=O (Ester)	1650-1630	1645
3.	C-O	1300-1000	1246
4.	P=O	1210-1140	1220.7
5.	N=O	1550-1350	1526.3 & 1347.4



IR Spectrum of Chlorthalidone



Structure of Chlorthalidone

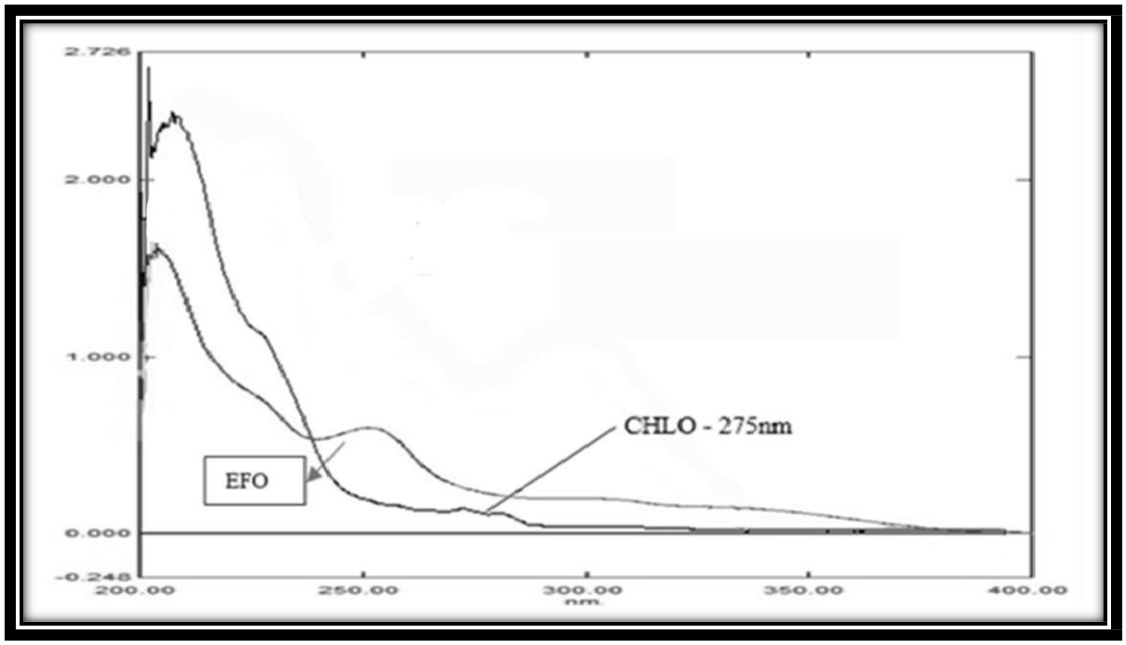
IR Value for Chlorthalidone

Sr. No.	Functional Group	Reported Wave number (cm^{-1})	Observed Wave number (cm^{-1})
1.	S=O Stretching	1375-1300	1372.5
2.	C–Cl	785-540	775.9
3.	N-H Stretching	1650-1580	1594.6
4.	C=O Stretching for amide	1680-1630	1638.8
5.	C=C Stretching	1662-1626	1614
6.	Alcohol OH Stretch	3600-3400	3509.9
7.	C-H Stretching	3150-3000	3015

UV Absorption Study

UV spectra of drugs in methanol depicted that the wavelength maxima of EFO and CHLO were at 254 nm and 275 nm respectively as shown in Figure 6.1.

For High Performance Liquid Chromatography 234 nm was selected wavelength.



Overlain UV Spectrum in methanol

DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD:

Development and validation of stability indicating RP-HPLC Method for Simultaneous Estimation of chlorthalidone and Efonidipine HCl Ethanolate in synthetic mixture.

➢ Materials and Methodology:

➢ Instruments and Apparatus:

- On Shimadzu P series integrated HPLC was equipped with a quaternary gradient unit, a LC-20 AD solvent delivery unit, DGU-20AR degassing unit, a CTO-10ASVP column oven, SPD-M40 PDA detector and a SIL-20AC programmable auto sampler controlled by LAB SOLUTION software.
- The Shimpack ODS C18 column 25 cm (4.6 mm x 250mm, 5 um) was used as a stationary phase. For filtration of solution, a Nylon-66 membrane filter was used.
- Weighing was done with an electronic analytical balance (Shimadzu AUW-220D).
- For twice distilled water, a water distillation equipment is utilised.
- For filtering, a Sonicator (D 120/1H, Trans-O-Sonic) with Nylon membrane filters (0.45m, 47 mm D) was employed.
- All tools and glasswares were calibrated before use, including volumetric flasks and pipettes (Borosil).

➢ Reagents and Material

- Chlorthalidone (CHL) API (Zota Health Care Ltd., Surat, Gujarat)
- Efonidipine HCl Ethanolate (EFO) (Zuventus Pharma Ltd., Mumbai, Maharashtra)
- Methanol, Acetonitrile HPLC grade (Rankem) was used.
- Double distilled Water

Development of RP-HPLC method:

- The various stages of sample preparation, chromatographic separation, detection, and quantification are evaluated and optimised during method development.

Selection of analytical wavelength:

The RP-HPLC method uses with PDA detector for detection of chlorthalidone (CHLO) and Efonidipine HCl Ethanolate (EFO). A suitable wavelength for detection is one that produces a favourable response

• Preparation of standard solutions:

✓ Stock solution of CHLO and EFO (100µg/ml)

Weigh accurately 10 mg of CHLO and EFO transferred to a 100 ml volumetric flask, where they were dissolved in a small amount of methanol. The amount was then diluted up to the mark with methanol to obtain the final CHLO and EFO concentrations (100 µg/ml).

• Preparation of Linearity standard solution:

Pipette out 0.5, 1, 1.5, 2 and 2.5ml of CHLO working standard solution (100µg/ml) and 1.5, 3, 4.5, 6 and 7.5ml of EFO stock solution (1000 µg/ml) into a series of 10ml volumetric flasks and adjust the volume to mark with methanol to give solution strength 5, 10, 15, 20 and 25µg/ml for CHLO and 15, 30, 45, 60 and 75µg/ml for EFO.

• Preparation of test solution:

In a 100ml volumetric flask, combine 12.5mg CHLO and 40mg EFO, add methanol to reach the desired solution strength (125, 400µg/ml) and sonicate for 10 minutes. 1 mL of the aforementioned solution was transferred to a 10 ml volumetric flask and diluted with mobile phase to the mark CHLO ultimate concentration was 12.5µg/ml, while EFO was 40µg/ml.

• Preparation of mobile phase:

Phosphate Buffer pH 3: Weigh accurately and transfer about 1.36 g of potassium dihydrogen orthophosphate and 2 ml of triethylamines in 800 ml of water, adjust the pH 3 with orthophosphoric acid and add water sufficient

The Phosphate Buffer pH 3, Acetonitrile, Methanol in the volume ratio 30:40:30 was used as a mobile phase. The mobile phase was filtered through a 0.45m membrane filter and degassed for 15 minutes in a sonicator.

• Chromatographic conditions:

A variety of mobile phases were used in the chromatographic separation. Every day, the mobile phase was filtered through a 0.45m membrane filter and degassed in a sonicator for ten minutes.

- **Column:** Shimpack ODS C18 (250 mm x 4.6 mm, 5µm)
- **Mobile Phase:** Phosphate Buffer pH 3 adjusted with o-phosphoric acid, Acetonitrile, Methanol (30:40:30 v/v/v)
- **Detector:** SPD M-40 PDA
- **Flow rate:** 1.0 ml/min
- **Injection volume:** 10 µl, SIL-20AC programmable auto sampler
- **Detection Wavelength:** PDA detector
- **Mode:** Isocratic

The chromatogram was performed until full separation was achieved. LAB-Solution HPLC software was used to record data such as peak area, height, retention time, resolution, tailing factor, and so on.

• Optimization of mobile phase composition:

Following a review of the literature, several mobile phases were chosen based on the solubility of the medication in the solvents. Methanol, acetonitrile, and water were used to test various solvents and mixtures of solvents. Using mobile phase mixture of Phosphate Buffer pH 3 adjusted with o-phosphoric acid, acetonitrile, methanol (30:40:30 v/v/v) produced a decent peak shape, good resolution, theoretical plate more than 2000 and acceptable results. As a result, mobile phase optimization for HPLC methods entails a number of experiments, as

❖ VALIDATION OF RP-HPLC METHOD:

The new method was validated according to ICH recommendations by determining several analytical method validation parameters.

• System Suitability studies:

The appropriateness of the system was determined by examining six replicates of CHLO and EFO in mixture concentrations of 15µg/ml and 45µg/ml. For peak area and retention time, the acceptance criteria are less than 2% R.S.D., theoretical plates higher than 2000, tailing factor less than 2.0, and capacity factor greater than 3.0. The results of the system suitability analysis met the required standards.

• FORCED DEGRADATION STUDIES

The International Conference on Harmonization (ICH) guideline entitled stability testing new drug substances and products requires that stress testing be carried out to elucidate the inherent stability characteristics of the active substance. The aim of this work was to perform the stress degradation studies on the CHLO and EFO using the proposed method.

(1) Preparation of stock

The stock solution was prepared by dissolving 10mg of CHLO and 30 mg of EFO methanol as diluents in 100 ml volumetric flask and then sonicated for 10 min and finally made up to the volume (Stock solution).

Acid Hydrolysis

The acid hydrolysis was done by pipetted out 3 ml of solution along with 3 ml of 0.5 N HCl into 10 ml volumetric flask. This was kept at 60°C for 3 hours and then neutralized with 0.5 N NaOH, followed by filtration with 0.45 µm syringe filter and placement in vials.

Alkaline Hydrolysis

The alkaline hydrolysis was carried out by pipetted out 3 ml of solution along with 3 ml of 0.5 N NaOH into 10 ml volumetric flask. This was kept at 60°C for 3 hours and then neutralized with 0.5 N HCl, followed by filtration with 0.45 µm syringe filter and placed vials.

Oxidative degradation

The oxidative degradation was carried out by pipetted out 3 ml of solution along with 3 ml of 3% w/v of hydrogen peroxide into a 10 ml volumetric flask. This was then kept at room temperature for 60 min, followed by filtration with 0.45 µm syringe filter and placed in vials.

Thermal degradation

Thermal degradation was carried out by placing solid samples in a Petri dish and keeping these in a hot air oven at 110°C for 3 hrs, followed by filtration with 0.45 µm syringe filter and placed in vials.

Photo degradation

The photolytic degradation was carried out by taking solid samples spread out as a thin layer on a Petri plate. It subsequently exposed to UV light in a chamber for 48 hrs. The stressed sample was filtered through 0.45 µm syringe filter before its analysis.

(2) Specificity:

The method's specificity was determined by evaluating reference pharmaceuticals as well as CHLO and EFO samples. The presence of an excipient in the synthetic combination has no effect on the outcome. As a result of the findings, it appears that the proposed method is inimitable.

(3) Linearity and Range:

For CHLO and EFO, the linearity response was determined by evaluating 5 separate levels of calibration curve in the range of 5-25µg/ml and 15-75µg/ml (n=6). The correlation coefficient is determined by plotting the calibration curve of peak area vs. concentration.

(4) Precision:

- **Repeatability:**

The repeatability was determined by analysing solutions containing 15µg/ml and 45µg/ml for CHLO and EFO, respectively, and analysing the identical solutions and calculating the percent R.S.D.

- **Intra-day precision:**

The new method's intra-day precision was evaluated by analysing solutions comprising concentrations of 15, 45, 75µg/ml for EFO and 5, 15, 25µg/ml for CHLO and three replicates (n=3) each on the same day.

- **Inter-day Precision:**

The new method's inter-day precision was tested by analysing sample solutions comprising concentrations of 15, 45, 75µg/ml for EFO and 5, 15, 25µg/ml for CHLO on three different days.

(5) Accuracy:

The method's accuracy was determined by calculating percent recovery of medication using a standard addition procedure to a pre-quantified sample solution of synthetic combination at three different levels: 50%, 100%, and 150%. In nine distinct 10 ml volumetric flasks, pre-quantified sample solution of synthetic combination taken 10µg/ml and 30µg/ml of CHLO and EFO, respectively. In this pre-quantified sample, a standard solution of CHLO and EFO API was spiked at three levels (50, 100, and 150%) in respective flasks (three flasks for each level) and volume was made up to mark with mobile phase. Each solution was examined, and percent recoveries were estimated using data from nine determinations over three concentration levels encompassing the necessary range.

RESULT AND DISCUSSION:

Selection of analytical wavelength:

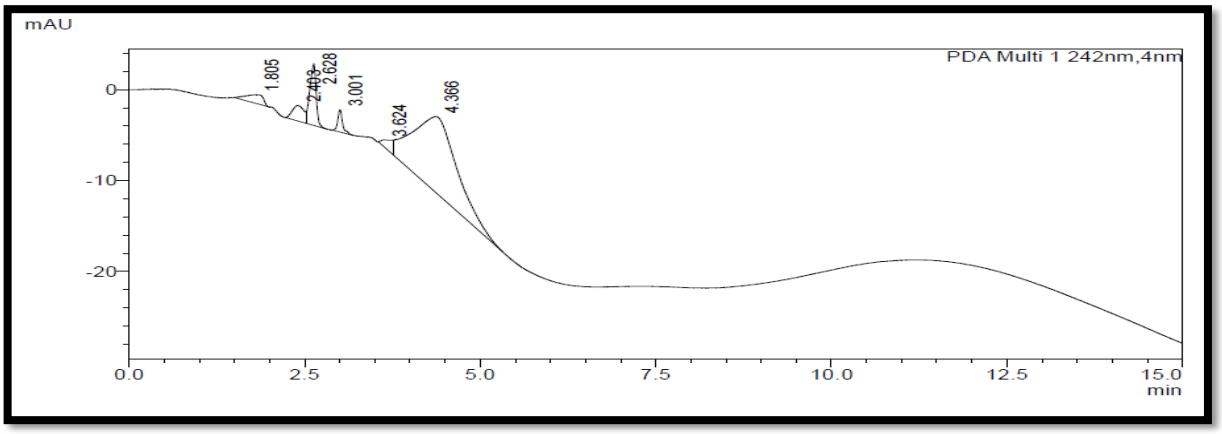
The RP-HPLC method uses with PDA detector for detection of chlorthalidone (CHLO) and Efonidipine HCl Ethanolate (EFO). A suitable wavelength for detection is one that produces a favourable response.

Optimization of Chromatographic condition:

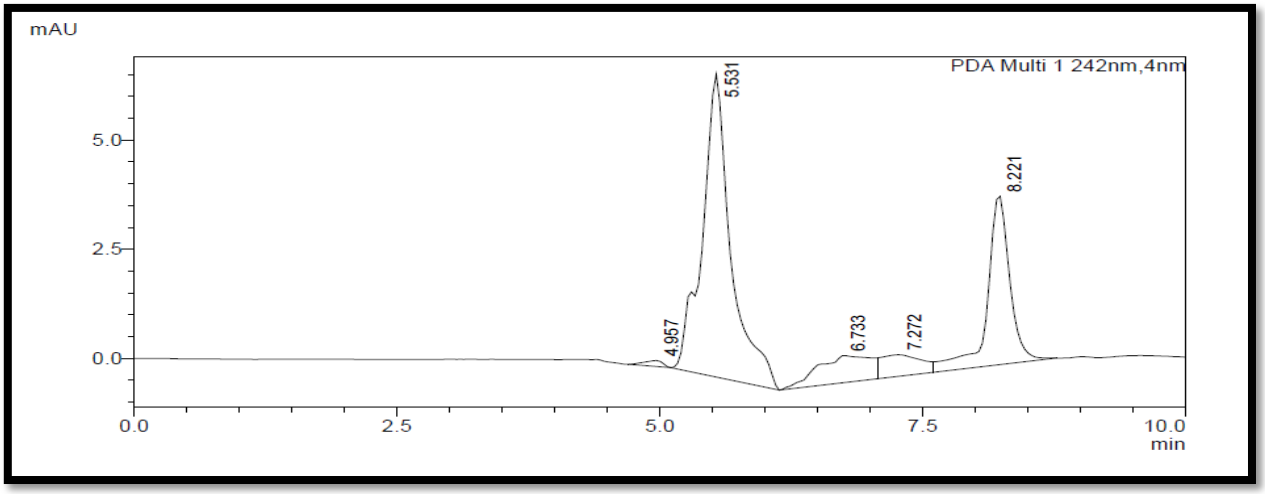
Preliminary trials for the optimization of the mobile phase were conducted based on a literature review, utilising solvents such as methanol, acetonitrile, and water in various quantities, with the results described in Table 7.3. The chromatographic method was optimized by varying parameters, such as flow rate, mobile phase, column temperature and detection wavelength. The method was performed with various columns such as the C18 column (4.6×250 mm, 5 mm) were found to be ideal as it gives good peak shape and resolution at 1.0 ml per min flow. The method was optimized with mobile phase composition of Phosphate Buffer pH 3 adjusted with o-phosphoric acid, acetonitrile, methanol (30:40:30 v/v/v) at a flow rate of 1 mL/min and at detection using PDA detector at 254nm using a C18 column (4.6×250 mm, 5 mm) column. The mobile phase experiments and the chromatogram are shown below.

HPLC Trials for optimization of mobile phase

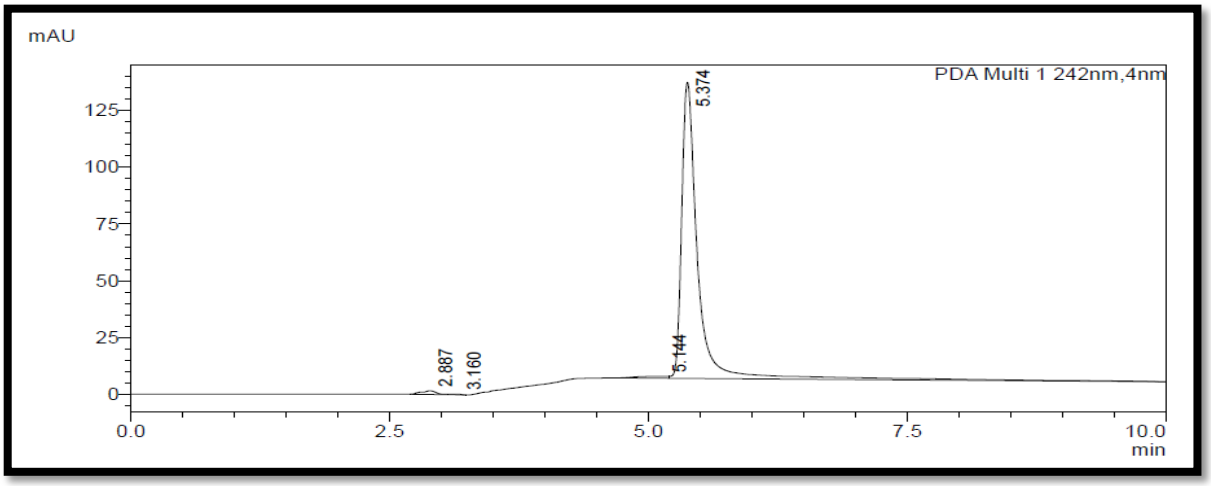
TRIALS	Mobile phase	Drug	Inference
1.	Acetonitrile: Water (50:50 v/v)	EFO and CHLO	No drug peak shape found to be
2.	Water: Methanol (50:50 v/v)	EFO and CHLO	Only One drug EFO observed and CHLO peak not observed
3.	Acetonitrile: Water: Methanol (35:35:30 v/v/v)	EFO and CHLO	Both drug peak observed but peak splitting and resolution is poor
4.	Acetonitrile: Water: Methanol (35:40:25 v/v/v)	EFO and CHLO	Both drug peak observed but peak splitting and resolution is poor
5.	Phosphate Buffer pH 3 adjusted with o-phosphoric acid, acetonitrile, methanol (30:40:30 v/v/v)	EFO and CHLO	EFO eluted at Rt = 5.119 min and CHLO at 7.131 with proper shape, theoretical plate greater than 2000 and resolution is greater than 2



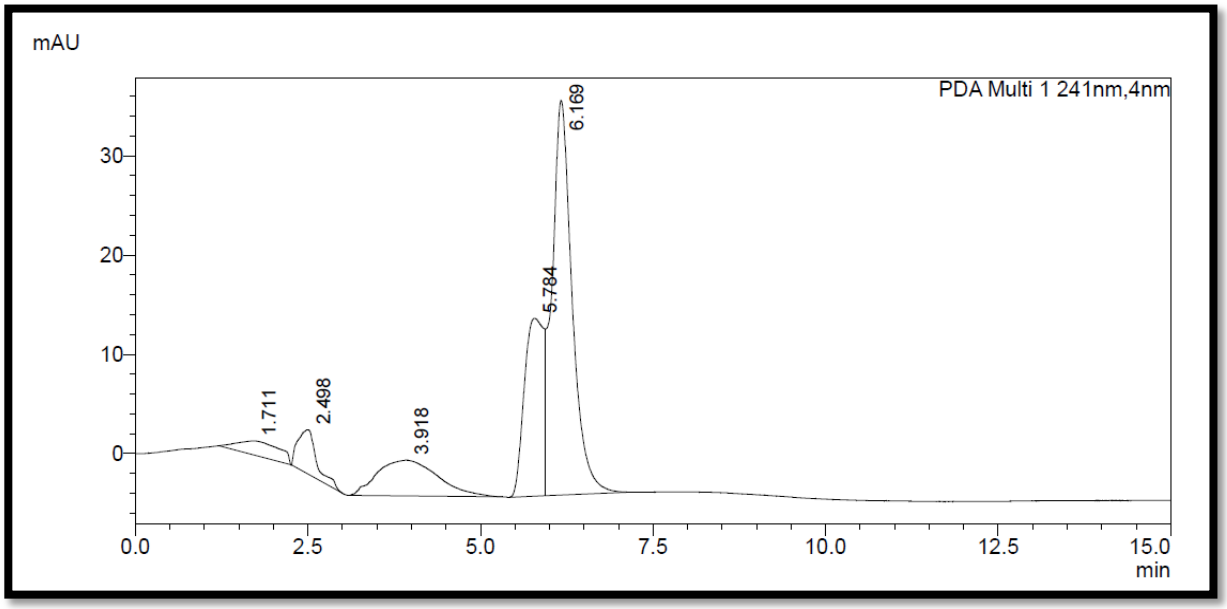
Chromatogram of mixture using Acetonitrile: Water (50:50 %v/v)



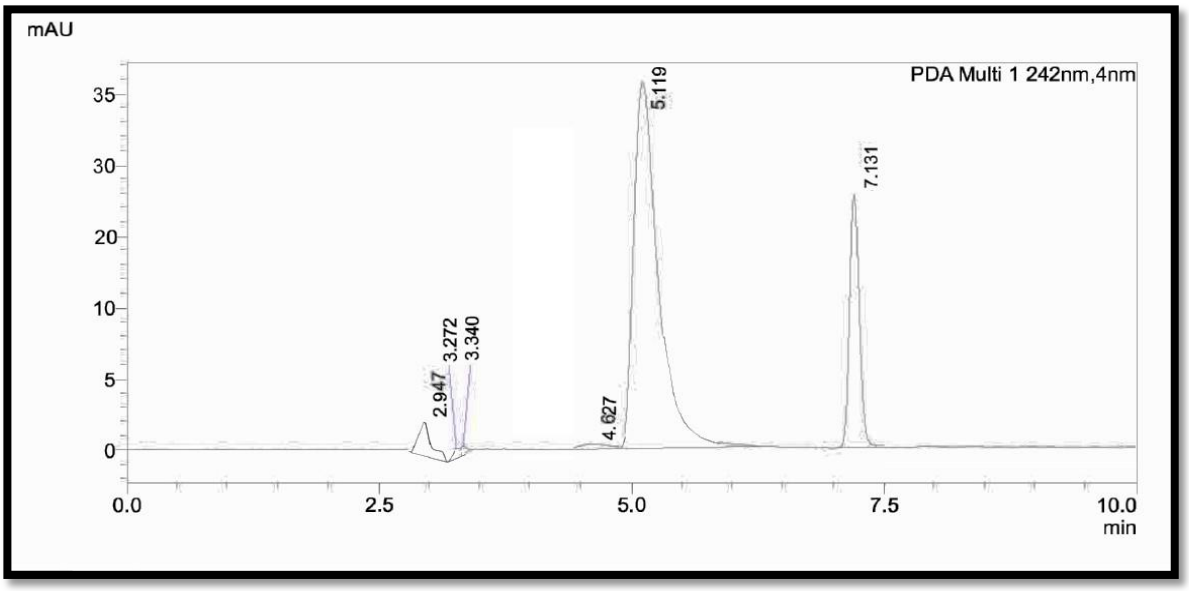
hromatogram of mixture using Water: Methanol (50:50 v/v)



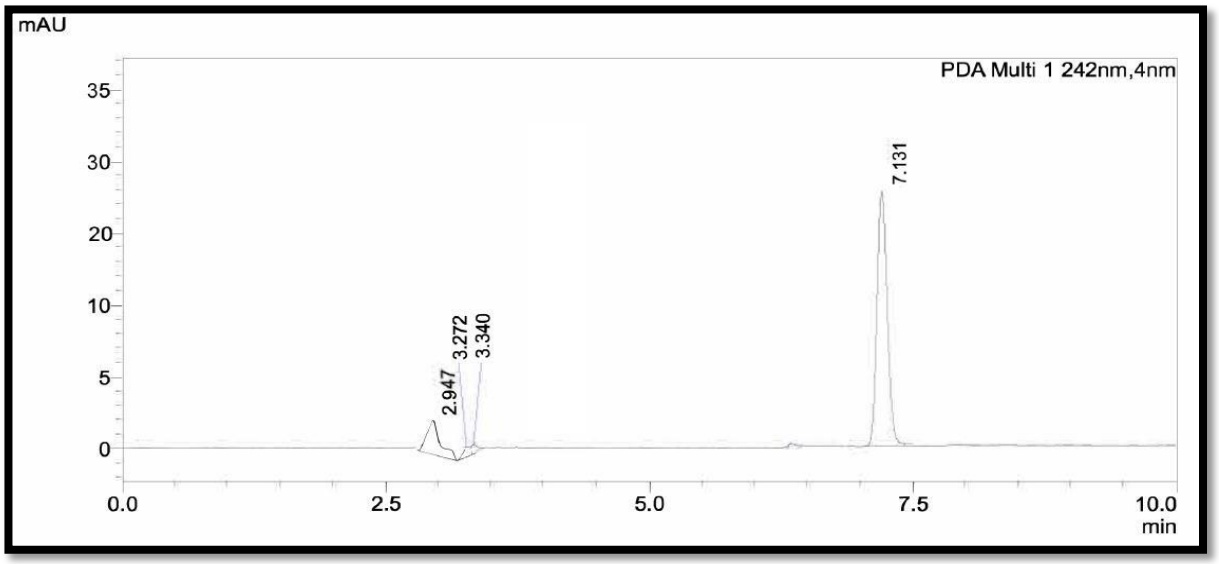
Chromatogram of mixture using Acetonitrile: Water: Methanol (35:35:30 v/v/v)



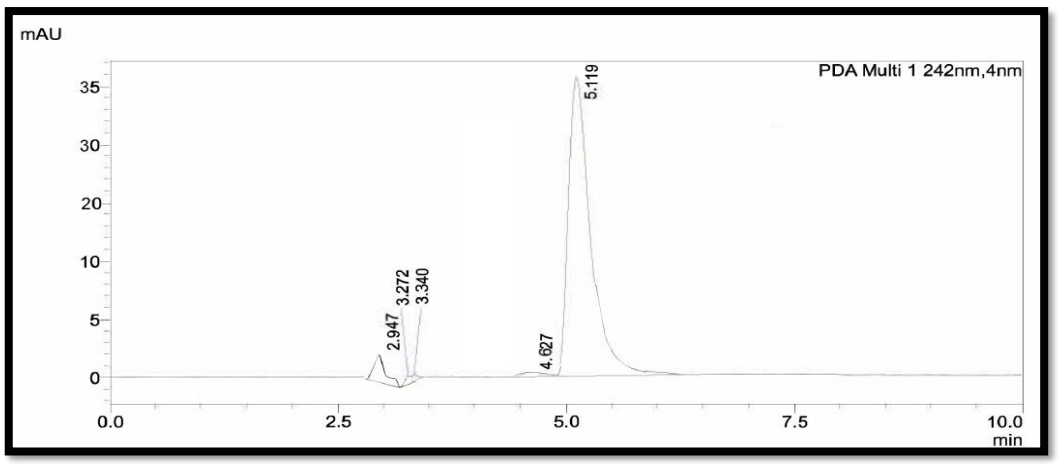
Chromatogram using Acetonitrile: Methanol : Water (35:40:25 v/v/v)



Chromatogram using Phosphate Buffer pH 3 adjusted with o-phosphoric acid, acetonitrile, methanol (30:40:30 v/v/v) showed peak of EFO eluted at Rt = 5.119 min and CHLO at 7.131 with proper shape, theoretical plate greater than 2000 and resolution is greater than 2.



Chromatogram using of CHLO at 7.131 min with optimized chromatographic condition.



Chromatogram using of EFO at 5.121 min with optimized chromatographic condition.

OPTIMIZE CHROMATOGRAPHIC CONDITION:

The final optimised mobile phase is shown in table after optimization of the mobile phase.

Optimized mobile phase:

Sr.no	Parameter	Condition
1	Mobile phase	Phosphate Buffer pH 3 adjusted with o-phosphoric acid, acetonitrile, methanol (30:40:30 v/v/v)
2	Flow Rate	1 ml/min
3	Run Time	10 min
4	Volume of Injection	10 µl
5	Detection of Wavelength	PDA detector at 242 nm
6	Column	Shimpack ODS C18 (250 mm x 4.6 mm, 5µm)
7	Column Temperature	40 ° C

➢METHOD VALIDATION:

(1) System suitability:

Analyzing CHLO and EFO at mixture concentrations of 15µg/ml and 45µg/ml was used to determine system applicability. For peak area and retention time, the acceptance criteria are less than 2% R.S.D., theoretical plates higher than 2000, tailing factor less than 2.0, and resolution greater than 2.0. The results of the system suitability analysis met the required standards.

System suitability analysis:

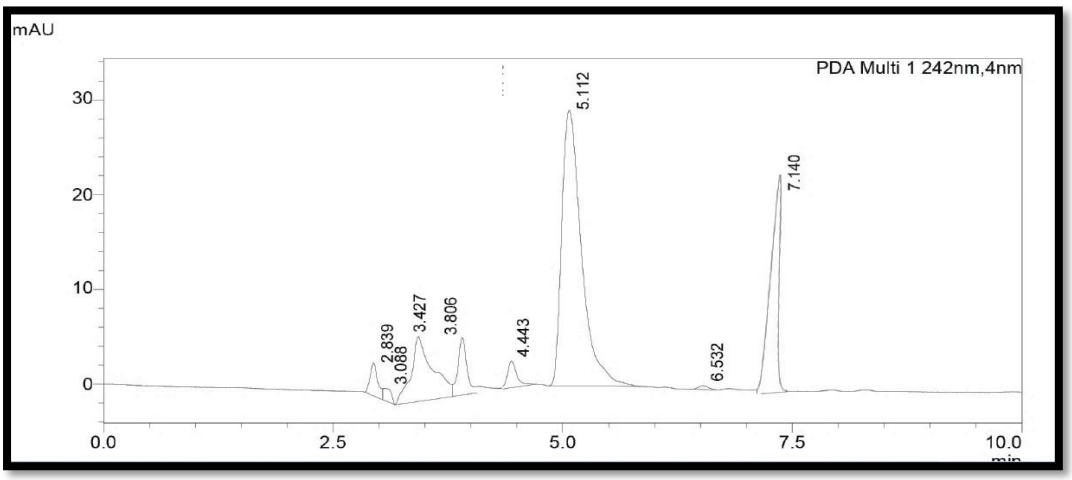
Drugs	Parameters	Mean ± S.D. (n=6)	% R.S.D.
EFO	Retention Time	5.131 ± 0.01	0.19
	Theoretical Plate	16248 ± 213.7	1.32
	Tailing Factor	1.14 ± 0.021	1.80
CHLO	Retention Time	7.124 ± 0.01	0.11
	Theoretical Plate	57929 ± 970.97	1.68
	Tailing Factor	1.118 ± 0.01	0.74
	Resolution	7.20 ± 0.048	0.67

FORCED DEGRADATION STUDIES

Forced degradations were performed to show the stability indicating properties of the analytical method, particularly when there is no information available about the potential degradation products**.**

Acid Hydrolysis

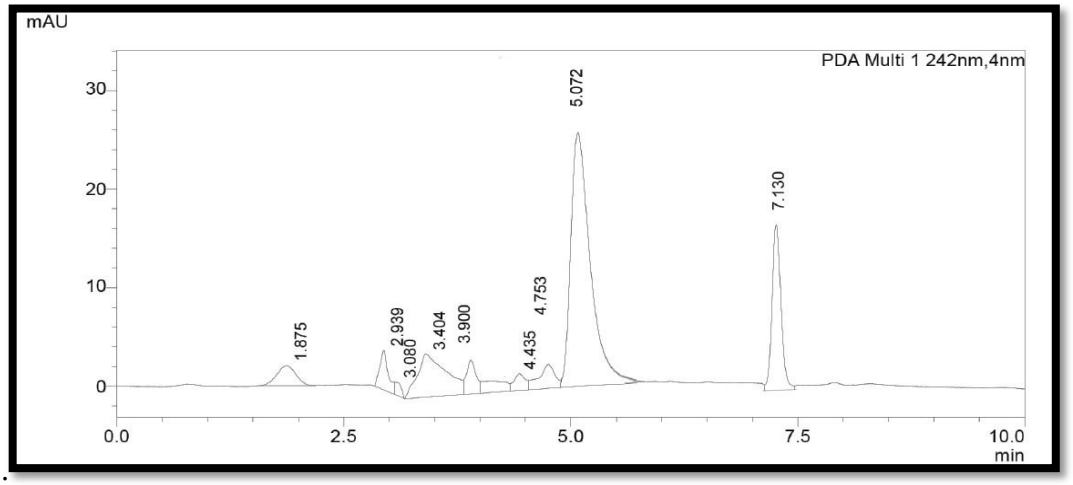
The acid hydrolysis was done by pipetted out 3 ml of solution along with 3 ml of 0.5 N HCl into 10 ml volumetric flask. This was kept at 60°C for 3 hours and then neutralized with 0.5 N NaOH, followed by filtration with 0.45 µm syringe filter and placement in vials. The acidic condition applied on the CHLO and EFO for above condition induced the hydrolysis of CHLO causing assay loss of about 5.95 % and degradative peak observed at 2.83 and 3.41 min for CHLO and EFO causing assay loss of about 4.08 % and degradative peak observed at 3.806 and 4.443 min for EFO.



Acid Hydrolysis chromatogram of EFO and CHLO mixtures

Alkaline Hydrolysis

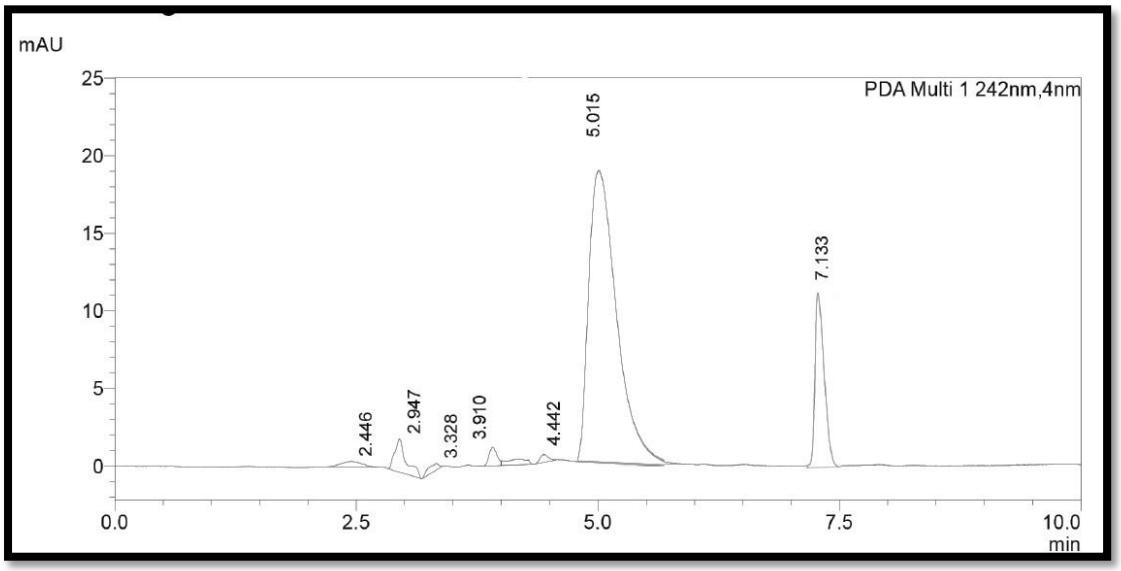
The alkaline hydrolysis was carried out by pipetted out 3 ml of solution along with 3 ml of 0.1 N NaOH into 10 ml volumetric flask. This was kept at 60°C for 2 hours and then neutralized with 0.1 N HCl, followed by filtration with 0.45 µm syringe filter and placed in vials. The alkaline condition applied on the CHLO and EFO for above condition induced the hydrolysis of CHLO causing assay loss of about 7.13 % and degradative peak observed at 2.939 and
3.404 min for CHLO and EFO causing assay loss of about 6.28 % and degradative peak observed at 1.875 and 4.753 min for EFO.



Alkaline Hydrolysis chromatogram of EFO and CHLO mixtures

Oxidative degradation

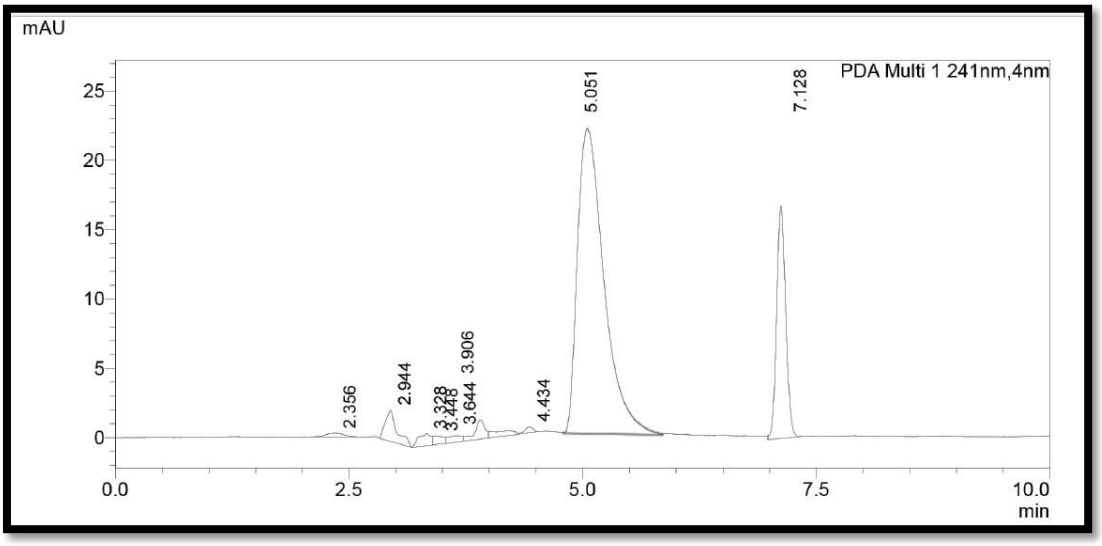
The oxidative degradation was carried out by pipetted out 3 ml of solution along with 3 ml of 3% w/v of hydrogen peroxide into a 10 ml volumetric flask. This was then kept at room temperature for 30 min, followed by filtration with 0.45 µm syringe filter and placed in vials. The oxidative degradation condition applied on the CHLO and EFO for above condition induced the hydrolysis of CHLO causing assay loss of about 3.48%, degradative peak observed at 2.947 and 3.910 min for CHLO and EFO causing assay loss of about 5.09 % and degradative peak observed at 4.442 min for EFO.



Oxidative degradation chromatogram of EFO and CHLO mixtures

Thermal degradation

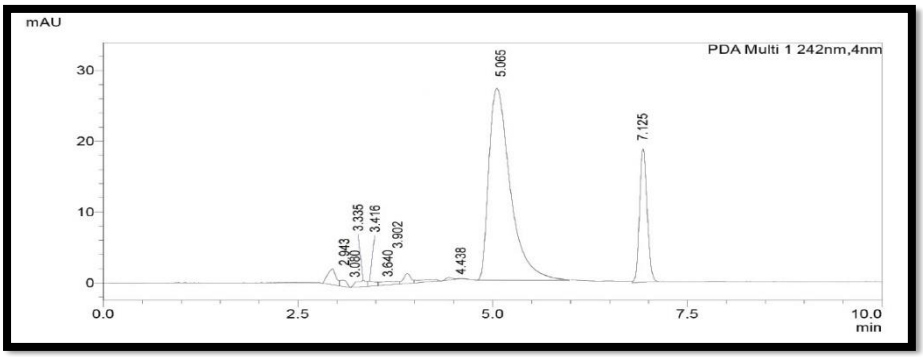
Thermal degradation was carried out by placing solid samples in a Petri dish and keeping these in a hot air oven at 110°C for 3 hrs, followed by filtration with 0.45 µm syringe filter and placed in vials. The thermal degradation condition applied on the CHLO and EFO for above condition, show both drugs were stable no degradative peak observed for CHLO and EFO. CHLO causing assay loss of about 2.09% and EFO causing assay loss of about 1.55 %.



Thermal degradation chromatogram of EFO and CHLO mixtures

Photo degradation

The photolytic degradation was carried out by taking solid samples spread out as a thin layer on a Petri plate. It subsequently exposed to UV light in a chamber for 48 hrs. The stressed sample was filtered through 0.45 µm syringe filter before its analysis. The photolytic degradation condition applied on the CHLO and EFO for above condition, show both drugs were stable no degradative peak observed for CHLO and EFO. CHLO causing assay loss of about 1.55% and EFO causing assay loss of about 1.72 %.



Photolytic Degradation chromatogram of EFO and CHLO mixtures

Forced degradation data*(±SD) of the method

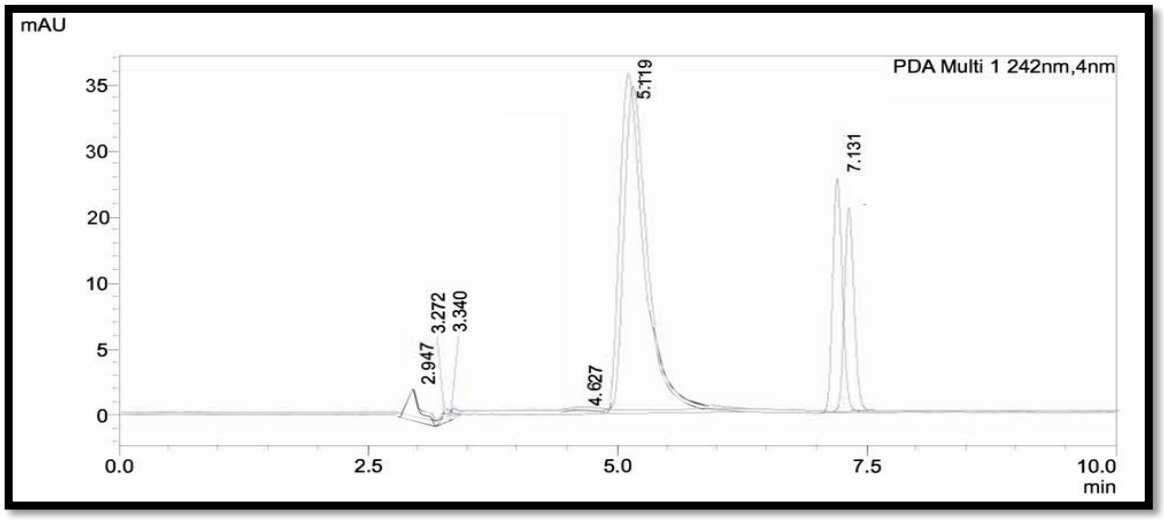
Stress condition	Amount of CHLO degraded (%)	Amount of CHLO recovered (%)	Amount of EFO degraded (%)	Amount of EFO recovered (%)
Acidic	5.95	94.05	4.08	95.92
Alkali	7.13	92.87	6.28	93.72
Oxidative	3.48	96.52	5.09	94.91
Photo stability	2.09	97.91	2.18	97.82
Dry Heat	1.55	98.45	1.72	98.28

*Average of three determinations (each condition); SD; Standard deviation

(2) Specificity:

The method's specificity was determined by evaluating reference pharmaceuticals as well as EFO and CHLO samples. The presence of an excipient in the synthetic combination has no effect on the outcome. As a result of the findings, it appears that the proposed method is unique.

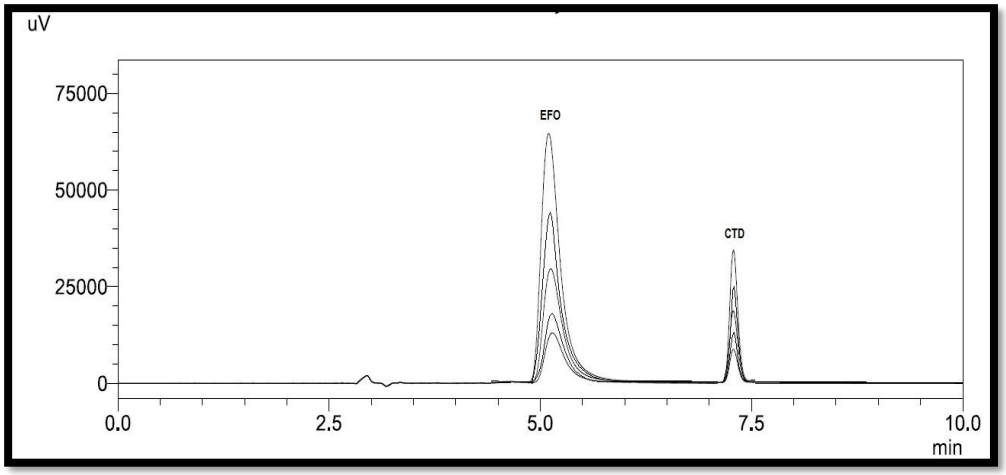
Chromatogram of EFO and CHLO using Phosphate Buffer pH 3 adjusted with o-phosphoric acid,



acetonitrile, methanol (30:40:30 v/v/v) as mobile phase in API

(3) Linearity and Range:

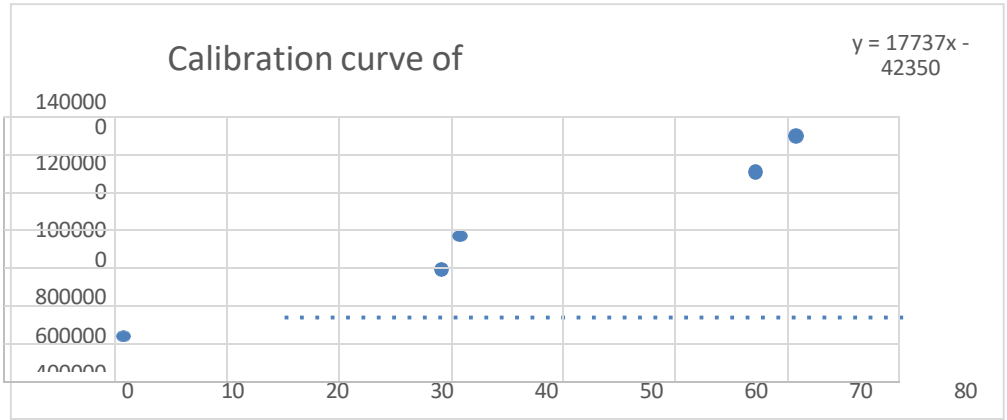
Plotting the mean peak area of EFO and CHLO against concentration over the ranges of 15-75 µg/ml for EFO and 5-25 µg/ml for CHLO, respectively, yielded a representative calibration curve (Figure 7.14 and 7.15). In the above conc. range, responses were found to be linear, with correlation values of 0.9994 for EFO and 0.9949 for CHLO. The linearity results for EFO and CHLO are reported in Tables 7.6 and 7.7, respectively. Figure 7.13 shows an overlay chromatogram of 15-75 µg/ml for EFO and 5-25 µg/ml for CHLO at 242 nm. The calibration range was designed so that the combination ratio was maintained throughout the simultaneous estimation of both bulk and synthetic mixture medicines. Table
7.7 shows the calibration curve's outcome as well as a regression analysis of the calibration curve.



Overlay Chromatogram of EFO (15-75 µg/ml) and CHLO (5-25 µg/ml),

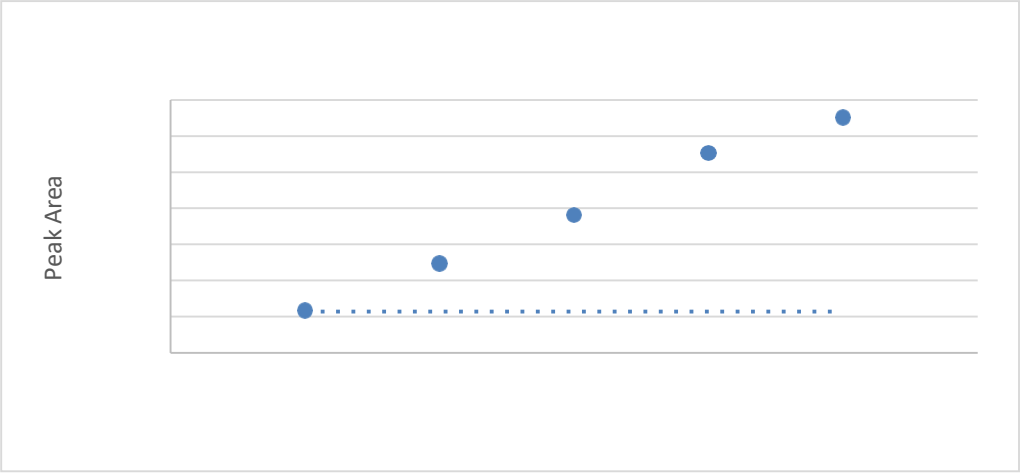
Linearity data of EFO

Concentration (µg/ml)	Peak Area Mean ± SD (n=6)	% RSD
15	221682 ± 3580.50	1.62
30	483789 ± 7432.47	1.54
45	772997 ± 7840.07	1.01
60	1013358 ± 7171.95	0.71
75	1287136 ± 15023.66	1.17



Calibration curve for Efonidipine HCl Ethanolate (EFO)Linearity data of CHLO

Concentration (µg/ml)	Area Mean ± SD (n=6)	% RSD
5	58284 ± 1098.58	1.88
10	123611 ± 1200.69	0.97
15	190320 ± 1665.83	0.88
20	276696 ± 1497.22	0.54
25	325655 ± 4324.35	1.33



Calibration curve for Chlorthalidone (CHLO)

Regression Analysis of calibration curve:

Parameter	EFO	CHLO
Concentration range (µg/ml)	15-75 µg/ml	100-500 µg/ml
Regression equation	y = 17737x - 42350	y = 13757x - 11435
Regression coefficient (r^2)	0.9994	0.9949
Standard deviation of slope	120.78	181.47
Standard deviation of Intercept	4958.40	2014.87
Limit of detection (µg/ml)	0.925	0.483
Limit of quantification (µg/ml)	2.80	1.46

(4) Precision:

Repeatability:

EFO and CHLO solutions containing 45µg/ml and 15µg/ml, respectively, and the same solution were examined seven times. EFO had a %RSD of 1.370% and CHLO had a
%RSD of 0.82%. The fact that the %RSD value was less than 2.0 suggested that the approach was precise

Repeatability data for EFO and CHLO

Drug	Concentration (µg/ml)	Mean peak area ± S.D. (n=7)	% R.S.D.
EFO	45	766330 ± 10462.63	1.37
CHLO	15	190487 ± 1562.58	0.82

Intraday precision:

The 15, 45, 75µg/ml for EFO and 5, 15, 25µg/ml for CHLO were examined three times on the same day using a devised HPLC method, and %RSD was calculated. EFO had a %RSD of 0.59- 1.29 %, while CHLO had a % RSD of 0.30 – 1.91 %. The fact that the %RSD value was less than 2.0 suggested that the approach was precise. (Figure 7.10)

Intraday precision data for estimation of EFO and CHLO (n=3)

Conc. (µg/ml)		Mean peak area ± SD EFO	% RSD	Mean peakarea ±SD CHLO	% RSD
EFO	**CHLO**				
15	5	219915.33 ± 2843.12	1.29	57918.00 ± 529.15	0.91
45	15	776441.11 ± 5091.75	0.66	191403.33 ± 577.35	0.30
75	25	1269290.83 ± 7476.30	0.59	327822.00 ± 2645.75	0.81

Inter-day precision:

The 15, 45, 75µg/ml for EFO and 5, 15, 25µg/ml for CHLO were examined three times on the same day using a devised HPLC method, and %RSD was calculated. EFO had a %RSD of 0.67- 1.73 %, while CHLO had a % RSD of 0.69 –
1.33 %. The fact that the %RSD value was less than 2.0 suggested that the approach was precise.

Inter-day precision data for estimation of EFO and CHLO (n=3)

Conc. (µg/ml)		Mean peak area ± SD EFO	% RSD	Mean peakarea ±SD CHLO	% RSD
EFO	CHLO				
15	5	227415.33 ± 1527.53	0.67	57483.67 ± 765.07	1.33
45	15	763663.33 ± 10503.97	1.38	190570.00 ± 1322.88	0.69
75	25	1294980.33 ± 22390.75	1.73	329155.33 ± 4163.33	1.26

(5) Accuracy:

Recovery studies from synthetic mixtures at three levels of standard addition (50%, 100%, and 150%) validated the method's accuracy. The data in Tables 7.12 and 7.13 show that the developed procedure is reliable. EFO and CHLO were reported to have %recovery rates of 98.39 – 101.86 % and 98.22 – 101.78 %, respectively.

Accuracy data of EFO (n=3)

Level	Conc. of EFO from Synthetic mixture (µg/ml)	Amount of Std. EFO added (µg/ml)	Total amount of EFO (µg/ml)	Total amount ofEFO Recovered (µg/ml) Mean ± SD	% Recovery
0	30	0	30	29.84 ± 0.32	99.46 %
50	30	15	45	45.84 ± 0.36	101.86 %
100	30	30	60	59.68 ± 0.31	99.46 %
150	30	45	75	73.79 ± 0.62	98.39 %

Accuracy data of CHLO (n=3)

Level	Conc. of CHLO from Synthetic mixture (µg/ml)	Amount of Std. CHLO added (µg/ml)	Total amount ofCHLO (µg/ml)	Total amount ofCHLO Recovered (µg/ml) Mean ± SD	% Recovery
0	10	0	10	9.92 0.11	99.19 %
50	10	5	15	14.78 ± 0.17	98.54 %
100	10	10	20	20.36 ± 0.15	101.78 %
150	10	15	25	24.56 ± 0.20	98.22 %

(6) Robustness:

To test robustness, a slight purposeful adjustment in HPLC conditions was performed. For both EFO 30µg/ml and CHLO 10µg/ml, changes in the optimized chromatographic condition were done and effect on the peak area was measured using this approach. % RSD of small change in the HPLC parameter were found to be less than 2 % indicating the robustness of the developed method.

Robustness data of EFO and CHLO (n=3)

EFFECT OF CHANGE IN VOLUME OF ORGANIC AND AQEOUS PHASE (ACN:METHANOL:WATER)						
(45:35:20 v/v/v)			**(40:45:25 v/v/v)**		**(40:35:25 v/v/v)**	
	Peak Area	**%RSD**	**Peak Area**	**%RSD**	**Peak Area**	**%RSD**
EFO (30 µg/ml)	486664.67	1.31	484013.67	0.27	486390.33	1.23
CTD (10 µg/ml)	123694.33	0.92	124725.00	0.76	124524.33	0.46
EFFECT OF CHANGE IN FLOWRATE						
0.9 ml/min			**1 ml/min**		**1.1 ml/min**	
	Peak area	**%RSD**	**Peak area**	**%RSD**	**Peak area**	**%RSD**
EFO (30 µg/ml)	486206.00	**1.07**	484013.67	0.27	486540.33	**0.89**
CTD (10 µg/ml)	124017.33	0.51	124725.00	0.76	123687.33	1.4

EFFECT OF CHANGE IN DETECTION						
239 nm			**241 nm**		**243 nm**	
	Peak Area	**%RSD**	**Peak Area**	**%RSD**	**Peak Area**	**%RSD**
EFO (30 µg/ml)	486931	**1.2**	484013.67	0.27	486981.67	**1.16**
CTD (10 µg/ml)	124759.667	0.81	124725.00	0.76	124390.67	0.48

(7) Limit of Detection and Quantitation:

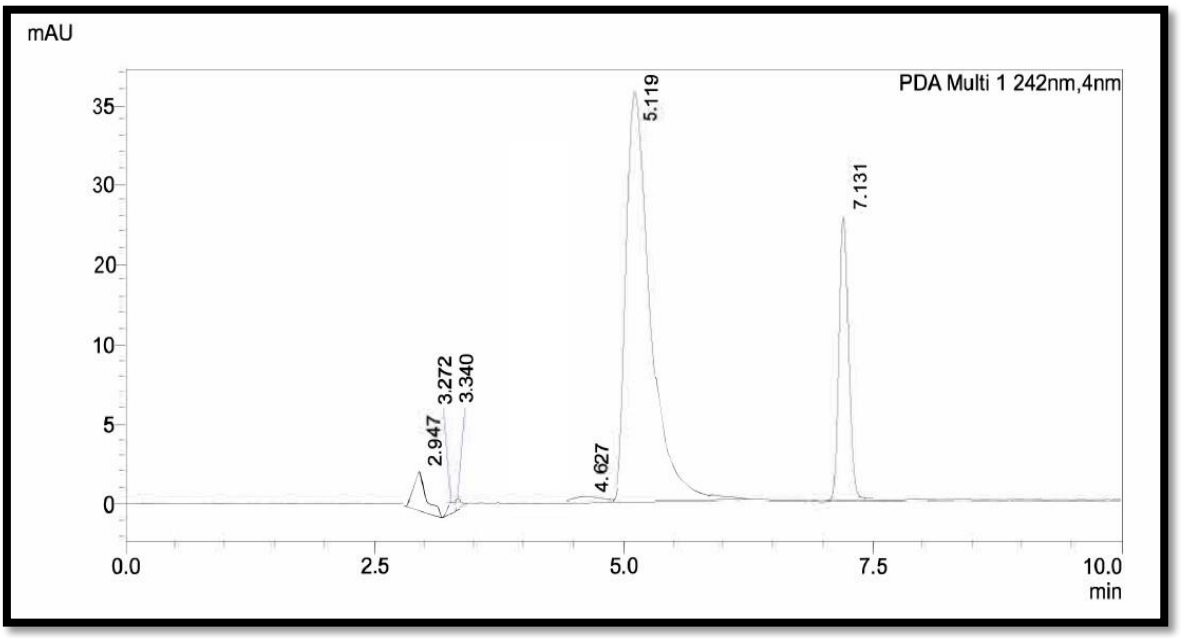
The EFO and CHLO both had LODs and LOQs were shown in Table 7.15 indicate the sensitivity of method.

LOD and LOQ data of EFO and CHLO

	EFO (µg/ml)	**CHLO (µg/ml)**
LOD	0.925	0.483
LOQ	2.80	1.46

Application of the proposed method for analysis of CHLO and FIMA in synthetic mixture.

The HPLC method was used to determine the amounts of EFO and CHLO in the synthesised combination.



Chromatogram of EFO and CHLO in synthetic mixture Analysis data of formulation (n=3)

Sr. No	Drug	Concentration (µg/ml)	Peak area ± SD	% Assay ± SD	%RSD
1	EFO	40	673774.7 ± 10000.3	100.79 ± 0.63	0.63
2	CHLO	12.5	161899.3 ± 1077.62	100.93 ± 1.40	1.39

Summary of validation parameters

PARAMETERS	HPLC method	
	EFO	CHLO
Concentration range(µg/ml)	15-75 µg/ml	100-500 µg/ml
Regression equation	y = 17737x - 42350	y = 13757x - 11435
Correlation Coefficient(r^2)	0.9994	0.9949
Accuracy(%Recovery) (n=3)	98.39 – 101.86 %	98.22 – 101.78 %,
Repeatability (%RSD) (n=7)	1.37	0.82
Intra-day Precision (%RSD) (n=3)	0.59- 1.29 %	0.30 – 0.91 %
Inter-day precision (%RSD) (n=3)	0.67- 1.73 %	0.69 – 1.33 %
Assay (%Recovery) (n=3)	100.79 ± 0.63	100.93 ± 1.40
Specificity	specific	specific
Robustness	Robust	Robust
LOQ(µg/ml)	0.925	0.483
LOD(µg/ml)	2.80	1.46

For two compounds, all of the parameters satisfied the ICH method validation criteria and were deemed to be suitable for routine quantitative analysis in pharmaceutical dosage forms. The linearity, accuracy, and precision of the results revealed to be within limits, with lower detection and quantification limits.

Conclusion:

overall results for the HPLC method development and validation revealed that this method can be used for the QC analysis of estimation EFO and CHLO in the synthetic mixture and cab be used for routine analysis. The develop method were accurate, sensitive and robust.Based on the results of the HPLC method used to analyse EFO and CHLO in their synthetic mixture, it can be concluded that the method has linearity in the

range of EFO and CHLO against concentration over the ranges of 15-75 µg/ml for EFO and 5-25 µg/ml for CHLO. At 242 nm, the regression coefficients (R2) for EFO and CHLO were more than 0.99. The EFO and CHLO both had LODs and LOQs indicating develop method were sensitive. For EFO and CHLO, the % assay was found to be more than 100 %w/w. For precision, repeatability, intra-day, and inter-day studies, the %R.S.D. was determined to be less than 2%. As a result, the

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