Dissertation submitted to

***The Tamil Nadu Dr. M.G.R. Medical University, Chennai***

*In partial fulfilment of the award of degree of*

# MASTER OF PHARMACY

## (PHARMACEUTICAL CHEMISTRY)


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**COLLEGE OF PHARMACY**

Madurai Medical College,
**Madurai – 625 020.**

**Mrs.R.Tharabai, M.Pharm.,**
Assistant Reader,
Department of Pharmaceutical Chemistry,
Madurai Medical College,
Madurai – 625 020.

# CERTIFICATE

This is to certify that dissertation entitled **"Development and validation of RP-HPLC method, UV-Spectrophotometric simultaneous equation and absorbance ratio method of ofloxacin and nitazoxanide in combined tablet dosage form. Development and validation of HPTLC method, UV-Spectrophotometric simultaneous equation and absorbance ratio method of montelucast and theophylline in combined tablet dosage form."** by **Miss.M.Sangeetha** under my guidance and supervision in the Department of Pharmaceutical Chemistry, Madurai Medical College, Madurai – 625 020 in partial fulfillment of the requirement for the award of degree of Master of Pharmacy in Pharmaceutical Chemistry during the year 2007 – 2008.

Station : Madurai

Date : **(R. THARABAI)**

**Mr. M. Chandran, M.Pharm.,**
The Professor and Head,
Department of Pharmaceutical Chemistry,
Madurai Medical College,
Madurai – 625 020.

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This dissertation is forwarded to The Controller of Examination, The Tamil Nadu Dr.M.G.R.Medical University, Chennai.

Station : Madurai

Date :

**(M. CHANDRAN)**

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# CONTENTS

# INTRODUCTION

Analytical chemistry may be defined as the science and the art of determining the composition of materials in terms of the elements of composition contained. In this it is of prime importance to gain the qualitative and quantitative information of the substance and chemical species i.e., to find out what a substance is composed of and exactly how much.

Analytical techniques hold the key to the design development standardization and quality control of medical products. They are equally important in pharmacokinetics and in drug metabolism studies both of which are fundamental to the assessment of bioavailability and the duration of clinical response.

Analytical instrumentation plays an important role in the production and evaluation of new products and in the production of consumers and the environment.

Chemical analysis is generally applied in two areas.

- Quantitative analysis
- Qualitative analysis

**Quantitative analysis:**

The realm of quantitative analysis is to determine how much of each of component or of a specified component is present in the sample.

**Qualitative analysis:**

The realm of qualitative analysis is to determine the component present in the sample or perhaps confirming its presence (or) absence in the sample.

Highly specific sensitive analytical techniques hold the key to the design, development, standardization and quality control of medicinal products. Modern physical methods of analysis are extremely sensitive, providing precise and accurate information about the standards of chemicals (or) drugs up to a nanogram level.

**Spectroscopy**[2,7]

It is the measurement and interpretation of electromagnetic radiations absorbed or emitted when the molecules or atoms or ions of the sample undergo transition from one energy state (ground state) to another (excited state).

## UV- Visible spectroscopy:

It involves the measurement of amount of UV radiation absorbed by a substance in the solution. The wavelength between 190-390 nm (practically 200-400 nm) is considered to be UV radiations / region Wavelength is defined as the distance between any two consecutive parts of the wave whose vibrations are in phase. For example from the crest of one wave to that of the next .Its symbol is **λ**.Coloured compounds absorb in visible range i.e 400 – 800 nm.The wavelength at which maximum absorbance takes place is called as λmax.

The assay of the absorbing substance can be carried out by using :

- Standard absorbtivity value
- Use of calibration graph
- Single point standardization
- Standard absorbtivity value

This procedure is adopted by official compendia for the stable substance that have reasonably broad absorption bands and which are practically un affected by variation of instrumental parameters .The use of standard A (1% 1cm) value avoids the need to prepare a standard solution of the reference substance in order to determine its absorpitivity.[3]

## Use of calibration graph:

In this procedure the absorbance of a number typically (4 – 6) of standard solution of the reference substance at concentrations encompassing sample concentration are measured and the calibration graph is constructed. The concentration of the analide in the sample solution is read from the graph as a concentration corresponding to the absorbance of the solution.[3]

## Single point standardization

This procedure involves the measurement of the absorbance of a sample solution and of a standard solution of the reference substance. The standard and sample solutions are prepared in the reference manner. Ideally of the concentration of the standard solution should be close to that sample solution. The concentration of the substance in the sample is calculated using

$$C_{\text{test}} = \frac{A_{\text{text}} \text{ x } C_{\text{Standard}}}{A_{\text{Standard}}}$$

Where $C_{test}$ and $C_{Standard}$ are the concentrations in the sample and standard solution $A_{text}$ and $A_{Standard}$ are the absorbance of the sample and standard solution respectively. The use of UV and visible spectroscopy for quantitative analysis employs the method of comparing the absorbance of standards and samples at a selected wavelength .The analysis of mixtures of two or more complements is facilitated by activity of absorbance. Other

applications include measurement of absorption of complexes to establish their composition of all chromogenic compounds are not suitable for quantitative measurement i.e., the choice of the system and procedure depends largely on the chemistry of the species to be determined.[3]

Assay of substances in multi component samples they are :

- Simultaneous Equation method
- Absorbance ratio method
- Derivative spectroscopy method
- Chemical derivatisation method
- Multi – component mode of analysis

**Simultaneous equation method:**

If a sample contains two absorbing drugs (X and Y) each of which absorbs at the λmax of the other. It may be possible to determine the quantity of both drugs by the technique of simultaneous equation (or) Vierodt's method.

Criteria for obtaining maximum precision based upon absorbance ratios have been suggested that place limits on the relative concentrations of the component of the mixture.

$$\frac{A2/A1}{ax_2/ax_1} \quad \text{and} \quad \frac{ay_1/ay_2}{A_2/A_1}$$

Where

$ax_1$, $ax_2$ = Absorptivities of X at $\lambda_1$ and $\lambda_2$

$ay_1$, $ay_2$ = Absorptivites of Y at $\lambda_1$ and $\lambda_2$

$A_1$, $A_2$ = Absorbances of the diluted Sample at $\lambda_1$ and $\lambda_2$

The ratio should lie outside the range of 0.1 - 2.0 for the precise determination of (Y and X) two drugs respectively.

These criteria are satisfactory only when the $\lambda_{max}$ of the two components is reasonably dissimilar. The additional criteria includes that the two additional criteria do not interact chemically thereby negating the initial assumption that the total absorbance is the sum of the individual absorbance's.

**Derivative spectroscopic method**:

This method involves the conversion of normal spectrum to its first, second or higher derivative spectrum the transformations that occur in the derivation spectra are understood by reference to an ideal absorption band. For the purpose of spectral analysis to relate chemical structure to electronic transition and for analysis situations in which mixtures contribute interfering absorption. A method of manipulating the spectral data called derivative spectroscopy was developed. In this technique spectra are obtained by plotting the first or higher derivation of absorbance or transmittance with

respect to wavelength versus wavelength. Often these plots reverse spectral details which is lost in an ordinary spectrum In addition concentration measurement of an analyte in the presence of their interference can something be made easily or more accurately.

**Chemical Derivatisation Method**:

Indirect spectrophotometic assays are based on the conversion of the analyte by a chemical reagent to a derivative that has different spectral properties.

This method is employed:

If the analyte absorbs weekly in the uv-region, a more sensitive method of assay is obtained by converting the substance to a derivative with a more intensely absorbing chromophore.

- The interference from irrelevant absorption may be avoided by converting the analyte to its derivative which absorbs in the visible region where irrelevant absorption is negligible.
- Indirect spectrophotometric procedure is also used to improve the selectivity of the assay of an ultra violet absorbing substance in a sample that contains other UV-absorbing components.

## CHROMATOGRAPHY

Modern pharmaceutical formulations are complex mixtures containing one or more therapeutically active ingredients., to a number of inert materials like diluents, disintegrants, colourants and flavours in order to ensure quality and stability of the final product ,the pharmaceutical analyst must be able to separate the mixtures into individual components prior to quantitative analysis .Amongst the most powerful techniques available to the analyst for the separation of these mixtures, a group of highly efficient methods which are collectively called as chromatography. It's a group of technique which works on the principle of separation of components of a mixture into individual components, depending on their affinities for the solutes between two immiscible phases. One of the phases is affixed bed of large surface area, while the other is a fluid, which moves through the surface of the fixed phase. The fixed phase is called stationary phase and the other is termed as the mobile phase. Depending on the type of chromatography employed the mobile phase may be a pure liquid or a mixture of solutions (eg.buffer) or it may be gas (pure or homogenous mixture)

**Classification of chromatography:-**

It can be classified according to the nature of the stationary and mobile phases

The different types of chromatography are

- Adsorption chromatography
- Partition chromatography
- Ion exchange chromatography
- Size exclusion or gel permeation chromatography

The modern instrumental techniques GLC,HPLC and HPTLC provide excellent separation and allow accurate assay of very low concentrations of wide variety of substance in complex mixtures.

**High Performance liquid chromatography**.

HPLC is one among most useful tools available for quantitative analysis. Reverse phase chromatography refers to the use of a polar mobile phase with a non polar stationary phase in contract to normal phase being employed with a non polar mobile phase.

Liquid chromatography is based upon the phenomenon that, under the same conditions, each component in a mixture interacts with its environment differently from other components. Since HPLC is basically a separating technique analysis. It is always used in conjunction with another

analytical tool for quantitative and qualitative analysis. The mode of operation of this system is isocratic. i.e. one particular solvent or mixture is pumped throughout the analysis for some determinations the solvent composition may be altered gradually to give gradient elution.

The rate of distribution of drugs between stationary and mobile phase is controlled by diffusion process, if diffusion is minimized, a faster and effective separation can be achieved. The techniques of high performance liquid chromatography are so called because of its improved performance when compared to classical column chromatography. Advances in column technology high pressure pumping system and sensitive detectors have transformed liquid column chromatography into high speed, efficient, accurate and highly resolved method of separation

**Modes of separation**:

In normal phase mode the stationary phase (e.g. silica gel) is polar in nature and the mobile phase is non-polar. In this technique non-polar compounds travel faster and are eluted first. This is because less affinity between solute and stationary phase. Polar compounds are retained for longer time in the column because more affinity towards stationary phase and takes more time to be eluted from the column. This is not advantageous in pharmaceutical applications since most of the drug molecules are polar in

nature and takes longer time to be eluted and detected. Hence this technique is not widely used in pharmacy.

**Importance of polarity in HPLC:**

Polarity is a term that is used in chromatography as an index of the ability of compounds to interact with one another. It is applied very freely to solutes. Stationary and mobile phase HPLC, the eluting power or solvent strength of the mobile phase I mainly determined by its polarity. If the polarities of stationary phase and the mobile phase are similar, it is likely that the interactions of solute with each phase may also be similar, resulting in poor separation. Retention of solutes is usually altered by changing the polarity of the mobile phase.

**Apparatus:**[2,4,5,6]

**Pumps:**

Pumps are required to deliver a constant flow of mobile phase at pressures ranging from 1 to 550 bar. Mechanical pumps of the reciprocating piston type give a pulsating supply of mobile phase. Dual piston reciprocating pumps produce an almost pulse free flow because so that as one is filling the other is pumping. Modern injectors are based on injection valves which allow the sample at atmospheric pressure to be transferred to the high pressure mobile phase immediately before the column inlet. With the injector is in the LOAD position, the sample is injected from a syringe

through a needle port into the loop. The valve lever is then turned through $60^0$ to the INJECT position and the sample is injected from a syringe through a needle port, into the loop. The valve lever is then turned through $60^0$ to the inject position and the sample is swept into the flowing mobile phase. If an excess of sample is flushed through the loop in the LOAD position, the volume injected is the volume of the loop. Which is typically 10 – 20 q l for analytical separations and 0.1ml for semi-preparative or preparative separations. This complete filling procedure offers the analyst the highest reproducibility and is capable of giving relative standard deviations of less then 0.2%.

**Columns:**

HPLC columns are made of high quality stainless steel polished internally to a mirror finish. Standard analytical columns are 4-5mm internal diameter and 10-30cm in length shorter columns 3-6cm in length containing small particle size packing material (3-5q m) produce similar or better efficiencies in terms of the number of theoretical plates about 7000, that those of 20cm columns containing 10 q m irregular particles and are used when short analysis times and high throughput of samples are required. Microbore columns of 1-2mm internal advantages of lower detection limits and lower consumption of solvent, the latter being important if expensive HPLC grade solvents are used. HPLC may also be carried out on the semi-

preparative or preparative scales by using columns of 7-10mm or 20-40mm internal diameter respectively.

**Packing materials and mobile phases**:[3]

Many of the packing materials used in HPLC are based on materials used in classical column chromatography. The commonly used packing materials and their associated solvent systems in each of the chromatographic modes are

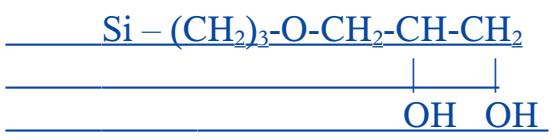
1) Partition HPLC
2) Adsorption HPLC

**Adsorption HPLC:**

Adsorbents used in classical column chromatography, unmodified silica has proved to be the most widely used in HPLC. The functional group responsible for adsorption is the silanol (Si-OH) group which interacts with the sample solutes by hydrogen bonding.There is therefore increasing retention of solutes with increasing solute polarity. Alumina is used as an adsorbent less frequently than silica, although for some separations in particular of aromatic substances and of structural isomers, greater selectivity is obtained with alumina.

**Partition HPLC:**

Packing materials based on silica are also used in partition chromatography. Early applications of partition HPLC involved coating the silica with a polar liquid stationary phase. Eg. Ethane-1,2-diol. Such packing materials have now been replaced by silica to which polar phases are chemically bonded. Example,

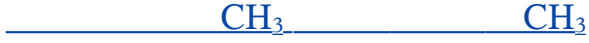


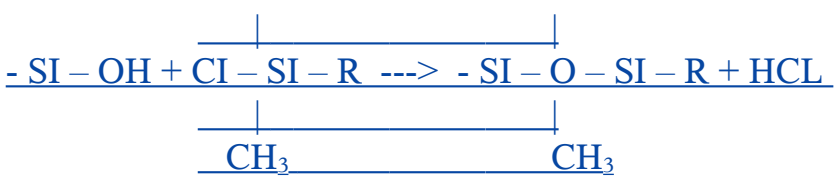
Eg: LiChrosorb Diol

Si – $(CH_2)_3$ –CN e.g. Q Bond pak CN

Si – $(CH_2)_3$ –NH2 e.g. Polygosil $NH_2$.

In reversed –phase partition HPLC the relative polarities of the stationary and mobile phase are the opposite to those in normal phase HPLC, i.e. the stationary phase is less polar than the mobile phase, and consequently the solutes are eluted in the order of their decreasing polarities. The stationary phase is silica, chemically bonded through a siloxane (si-o-si-o) linkage to a low polar functional group. These phases are prepared by treating the surface silanol groups of silica with an organochlorosilane reagent





Where R = $C_6H_{13}$ (Hexyl)

$C_8H_{17}$ (Octyl)

$C_{18}H_{37}$ (Octadecyl).

Untreated silanol groups may be ‘capped’ by treatment with trimethylchlorosilane to eliminate adsorption effects.

The mobile phase in reversed-phase HPLC generally comprises water and a less polar organic solvent modifier. e.g. methanol or acetonitrile. Separations in these systems are considered to be due to different degrees of hydrophobicity of the solutes. The less polar solutes partitioning to a greater extent into the non-polar stationary phase and consequently being retained on the column longer than the more polar solutes. The rate of elution of the components is controlled by the polarity of the organic modifier and its proportion in the mobile phase. The rate of elution is increased by reducing the polarity, e.g. by increasing the proportion of the organic solvent or by using acetonitrile instead of methanol. The simple alteration of the composition of the mobile phase or of the flow rate allows the rate of elution of the solutes to be adjusted to an optimum value and permits the separation of a wide range of chemical types.

## Detectors:

The detection of the separated components in the eluate from the column is based upon the bulk property or the solute property of the individual components. The most commonly used detectors in the HPLC are.

### i. Photometric detectors :

These normally operate in the ultraviolet region of spectrum and are the most extensively used detectors in pharmaceutical analysis. They are of five principal types :

- Single wavelength detectors
- Multi-wavelength detectors
- Variable wavelength detectors
- Programmable detectors
- Diode array detectors.

### ii. Fluorescence detectors:

These are essentially filter fluorimeters or spectrofluorimeters equipped with granting monochromators and micro flow cells. Their sensitivity depends on the fluorescence properties of the components in the eluate.

**iii. Refractive index detectors**:

These are differential refractometers which respond to the change in the bulk property of the refractive index of the solution of the component in the mobile solvent system. The sensitivity of the refractive index detector is much less than that of specific solute property detectors.

**iv. Electrochemical detectors** :

These are based on standard electrochemical principles involving amperometry voltammetry and polarography. These detectors are very sensitive for substances that are electroactive.

**Validation** [8,9,10]

Validation means assessment of validity or action of proving effectiveness. Method validation is the process of proving that an analytical method is acceptable for its intended purpose. For pharamaceutical methods guidelines from the united states pharmacopeia (USP), interenational conference on harmonization (ICH) and the food and drug administration (FDA) provide a frame work for performing such validations

**Parameters used for assay validation**.

The validation of the assay procedure was carried out as per ICH guidelines using the following parameters.

**Specificity**:

Specificity is the ability to asses unequivocally the analyte in the presence of components which may be expected to be present lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures. For the chromatographic methods developing a separation involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The response of the analyte in test mixtures containing the analyte and all potential sample components (placebo formulation, process impurities, etc.) is compared with the response of a solution containing only the analyte. Specificity criteria for an assay method is that the analyte peak will have baseline chromatographic resolution of at least 1.5 from all other sample components. If this cannot be achieved the unresolved components at there maximum expected level will not affect the final assay result by more than 0.5%

**Linearity :**

Linearity of an analytical procedure is its ability (within a given range) to obtained test results, which are directly proportional to the concentration (amount) of analyte in the sample. For assay methods, this study is generally performed by preparing standard solutions at five concentration levels. Five levels are required to allow detection of curvature

in the plotted data acceptability of linearity data is often judged by examining the correlation and y- intercept of the linear regression line for the response versus concentration plot. A correlation coefficient of >0.999 is generally considered as evidence of acceptable fit of the data to the regression line. The y- intercept should be less than a few percent of the response obtained for the analyte at the target level.

**Accuracy:**

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found .The accuracy of a method is the closeness of the measured value to the true value for the sample. Accuracy is usually determined in one of four ways.

1. Accuracy can be assessed by analyzing a sample of known concentration and comparing the measured value to the true value.

2. To compare test results from the new method with results from an existing .alternate method that is known to be accurate.

3. The most widely used recovery study, is performed by spiking analyte in blank matrices. For assay methods, spiked samples are prepared in triplicate at three levels over a range of 50-150% of the target concentration.

4. The technique of standard additions, which can also be used to determine recovery of spiked analyte. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte.

Accuracy criteria for an assay method is that the mean recovery will be 100+2% at each concentration over the range of 80-120% of the target concentration.

**Range:**

Range of analytical procedure is the interval between the upper and lower concentration amounts of analyte in the sample including these concentration for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The range is determined using data from the linearity and accuracy studies. Range criteria for an assay method is that the acceptable range will be defined as the concentration interval over which linearity and accuracy are obtained per previously discussed criteria and that yields a precision of 3% RSD.

**Precision:**

The precision of an analytical procedure express the closeness of agreement between a series of measurements obtained from multiple

sampling of the same homogenous sample under the prescribed conditions. Precision of an analytical procedure is usually expressed the variance, standard deviation or coefficient of variance of a series of measurements. The first type of precision study is instrument precision or injection repeatbility. A minimum of 10 injections of one sample solution is made to test the performance of the chromatographic instrument. The second type is repeatability or intra-assay precision. Intra-assay precision data are obtained on one day. Aliquots of a homogenous sample, each of which has been independently prepared according to the method procedure. from these precision studies, the sample preparation procedure the number of replicate samples to be prepared, and the number of injections required for each sample in the final method procedure will be set. An example of precision criteria for an assay method is that the instrument precision (RSD) will be 1% and the intra assay precision will be 2%

**Detection limit**

The detection limit of an individual analyte procedure is the lowest amount of analyte in a sample which can be detected but not necessarily qualitated as an exact value. Detection limit based on the standard derivation of the response and the slope.

Detection limit (or) limit of detection may be expressed as,

$$DL = \left[\frac{3.3\sigma}{S}\right]$$

Where

σ = standard deviation of the response

S= slope of the calibration curve (of the analyte)

**Quantitation limit**:

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

Quantitation limit based on the standard deviation of the response and the slope. It can be expressed as,

$$QL = \left[\frac{10\sigma}{S}\right]$$

Where

σ = standard deviation of the response

S = slope of the calibration curve (of the analyte)

**Ruggedness:**

The ruggedness of an analytical method is the degree of reproducibility of test result obtained, the analysis of conditions such as different laboratories, different analysis using different instrument, on different days. Different source of reagent, elapsed assay times, assay temperature conditions. Ruggedness is a measure of reproducibility of test result under the variation in conditions normally expected from analyst to

analyst. The criteria of the ruggedness is the RSD should be not more than 2%.

**Robustness:**

The robustness of a method is its ability to remain unaffected by small changes in parameter such as percent organic content and $p^H$ of the mobile phase, buffer concentration, and temperature and injection volume. These method parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment. The criteria for robustness is the RSD should be not more than 2%.

**System suitability testing**:

System suitability testing is an integral part of many analytical procedures the tests are based on the concept that the equipment, electronics, analytical operation and samples to be analyzed constitute an integral system that can be evaluated as such. Typically the process involves making five injections of a standard solution and evaluating several chromatographic parameters such as resolution, area % reproducibility, number of theoretical plates and tailing factor.

**Retention time : ( $R_t$)**

This is the time of emergence of the peak maximum of a compound after injection.

**Retention volume: (RV)**

The volume of mobile phase required to elute one half of the compound from the column as indicated by the peak maximum and is given by $R_V = R_t$ x flow rate

**Asymmetry factor (or ) tailing factor (T )**

$$T = \frac{Y_x}{2A}$$

where ,

$y_x$ = the width of the peak at one twentieth of the peak height.

A = the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one twentieth of the peak height.

The value lies between 0.95 to 1.05.

**Number of theoretical plates (N )**

$$N = 5.54 \text{ x } \left( \frac{R_t}{W_{\frac{h}{2}}} \right)^2$$

The assessment of performance of efficiency of a column is in terms of the number of theoretical plates

$R_t$ = retention time

$W_{h\backslash 2}$ = width of peak at half height

**Resolution (R):**

$$R = \frac{2\left(R_{t2} - R_{t1}\right)}{W_2 + W_1}$$

This gives the resolution between the measured peaks on the chromatogram.

Where

$Rt_1$ and $Rt_2$ = retention time of two component.

$Wt_1$ and $Wt_2$ = the respective peak width. Resolution between measured peaks on the chromatogram must be > 1

## STATISTICAL PARAMETERS:

Statistics consists of a set of methods and rules for organizing and interpreting observations.

**Lineanrity coefficient ( $\gamma$)**

$$\gamma = \frac{\Sigma(X-\bar{X})\,(Y-\bar{Y})}{\Sigma(X-\bar{X})^2\,(Y-\bar{Y})^2}$$

Slope (m)

$$m = \frac{\Sigma(X-\bar{X})\,(Y-\bar{Y})}{\Sigma(X-\bar{X})^2}$$

Standard deviation ($\sigma$)

$$(\sigma) = \sqrt{\frac{\Sigma(X-\bar{X})^2}{n-1}}$$

$$\text{R.S.D (\%)} = \frac{S.D}{\bar{X}} \times 100$$

Where

$\Sigma$ = Sum of observation

X = Mean of arithmetic average ($\Sigma x/n$)

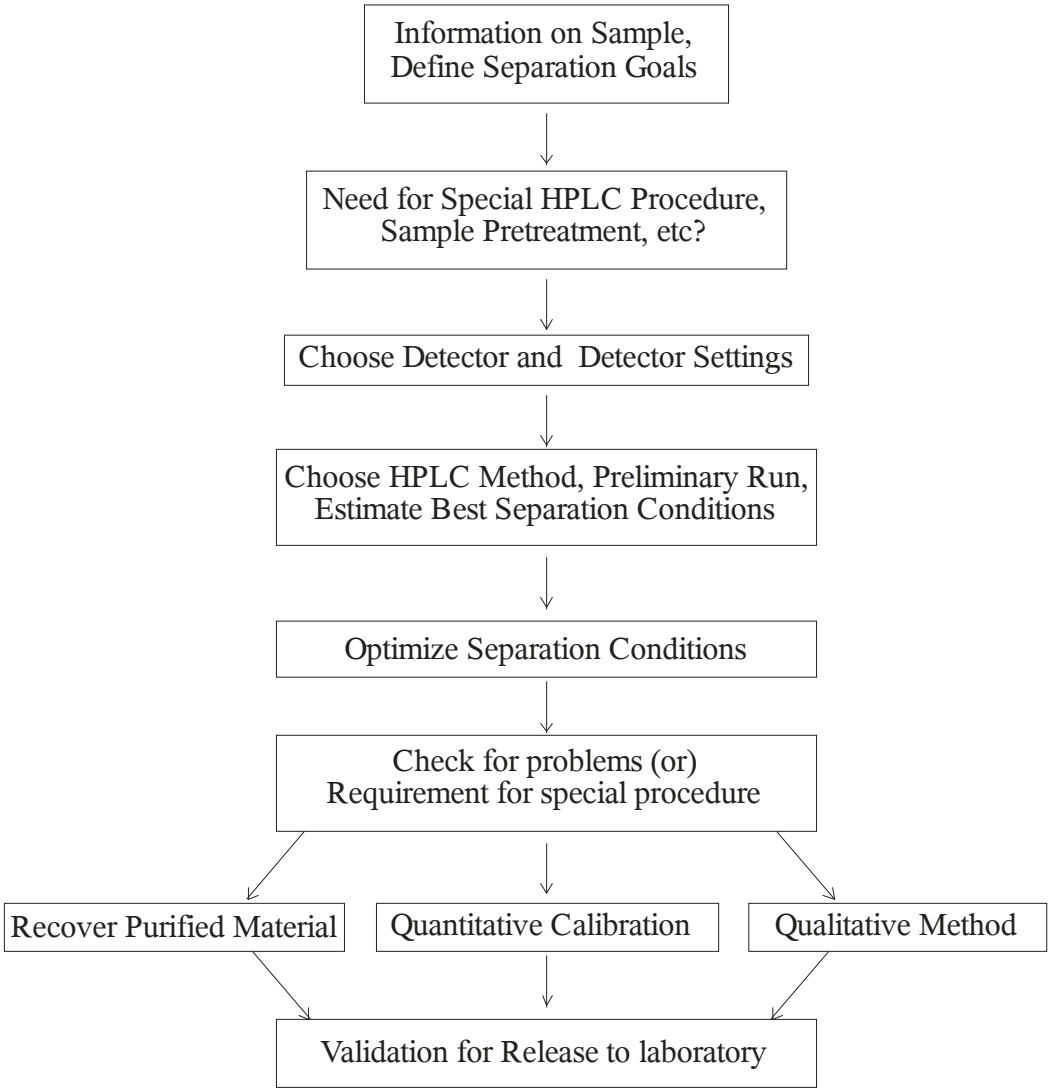
X = Individual observed value.

x-x = Deviation of a value from the mean.

N = Number of observation

R.S.D =Relative standard deviation

**DEVELOPMENT OF REVERSE PHASE HPLC METHOD6**




"Validation does not make a method good or efficient; it merely demonstrates that an analytical method performs in accordance with the claims made for it".

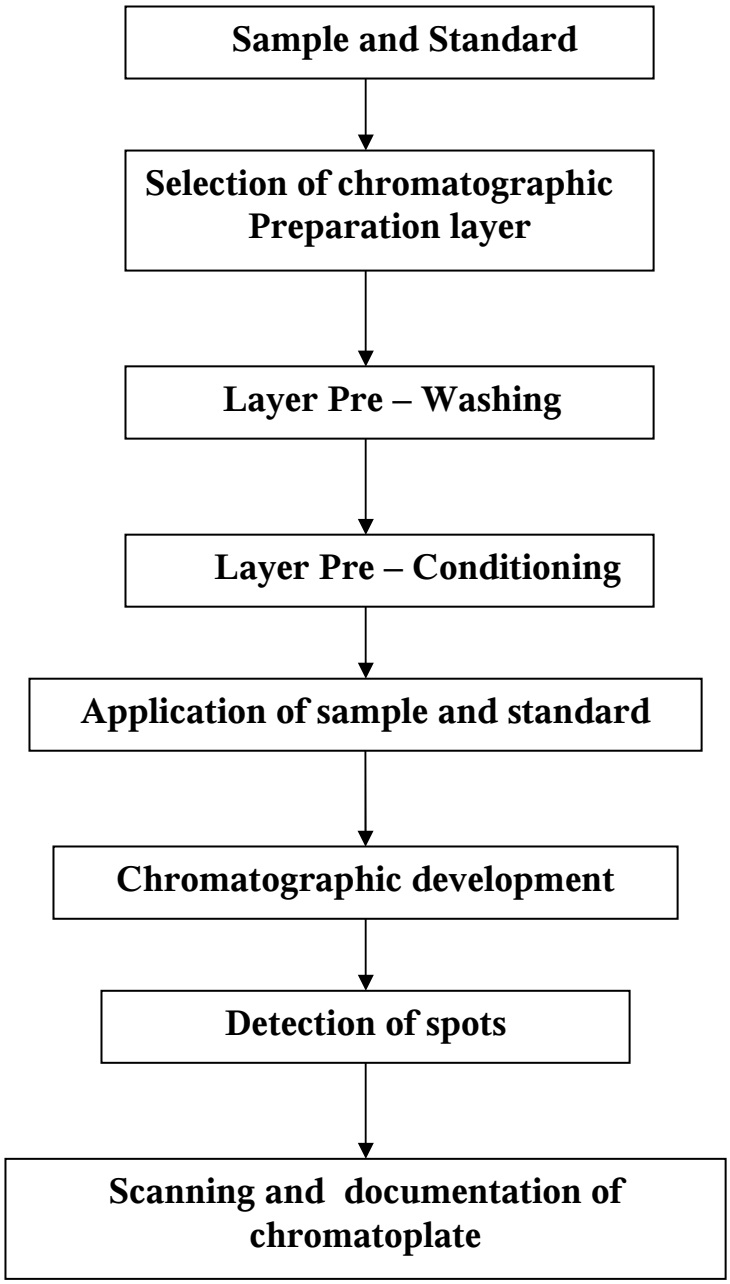
## HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY[17]

Thin layer chromatography (TLC) also know as planar – chromatography or flat bed chromatography a multi stage distribution process. The three chromatography techniques TLC, GLC or HPLC is applicable for an analysis depends on several parameters solubility or volatility of the sample required separation, efficiency, concentration of the analyte, detection limit, cost of analysis, number of sample under analysis sample preparation to be separated. TLC as practiced today is in two forms some are using it a qualitative tool for separation of simple mixture where speed low cost and simplicity are required others use it as a powerful separation tool for quantitative analysis with high sample throughput the latter now referred to as HPTLC. HPTLC can simultaneously handle several samples even of divergent nature and composition supporting several analysis at a given time HPTLC is the most simple separation technique it can be considered a time machine that can speed your work and allows you to do many things at a time usually not possible with other analytical techniques.

## Various steps involved in TLC/HTPC/Planar Chromatography

- Selection of TCL/HPTLC plates and srobebt sample preparation including any clean up and pre- chromatographic derivatization
- Application of samples
- Development (Separation)
- Detection including post-chromatographic
- Derivatization
- Quantitation
- Documentation.

# SCHEMATIC REPRESENTATION OF HPTLC



**Plates:**

**Pre – coated Plates:**

The precoated plates with different support material (glass, aluminium, plates) and with different sorbent layers are available in different format and thickness usually plates with sorbent thickness of 100 – 250µm are using for qualitative and quantitative analysis.

**Glass support:**

It is resistant to heat and chemicals easy to handle and always offers superior flat and smooth surface for chromatographic work. Disadvantage being fragility, relativity high weight additional packing material and higher production cost.

**Polyester(Plastic) Sheet(0.2 mm thick)**

More economical as they are produced even in rool from unbreakable less packing material less shelf space for storage can be cut to any required format spots can cut and eluted charring reactions are possible but temperature should not exceed 120 °.

**Aluminium sheet(0.1mm thick)**

Aluminium Sheets as support offer the same advantage as polyester support but containing high concentration of mineral acids or concentrated ammonia, they chemically attack aluminium.

**Plate Size:**

Pre–coated TLC/HPTLC plates in size of 20 x 20 cm with aluminium or polyester support are usually procured. These plates can be cut to size and shape good cut edges are obtained any layer which has been loosened as a result of cut should be removed by lightly drawing the spatula over the cut edge. This is necessary to obtain constant RF values.

**Pre- washing of pre- coated plates:**

Pre- washing of plates must be done to at least 1-2 cm longer than the subsequent actual chromatographic development .as a result of pre-washing signal to noise ratio is substantially low and base lines are straighter reduction in signal to noise ratio as a result of pre-washing improves the limit of detection (LOD) of the procedure.

Methanol is the most commonly employed solvent for pre-washing but it cleaning power is not good. It is preferable to carry out pre-washing process with mobile phase containing acid or alkali should be avoided as they may not be completely removed during subsequent activation mixtures of Chloroform in Methanol (1:1); Ethyl acetate – Methanol (1:1Chloroform:Methanol:Ammonia(90:10:1) have been used as solvents for pre-washing. Methylene-chloride-methanol(1:1) is best suited for removing any impurity one may also try pre-washing with 1% ammonia or 1% acetic acid in methanol.

**Activation of pre-coated plates:**

Freshly opened box of TLC/HPTLC plates usually does not require activation However plates exposed to high humidity or kept on hand for long time may have to be activated by placing in oven at 110-120 0c for 30 minutes prior to sample spotting after the plates are removed form pre-wash chambers .They should always be dried in vertical position as in horizontal position drops of solvent may fall on the plate as a result of condensation. Activation at higher temperature and for longer time should be avoided as it. It may lead to very active layers and there will be risk of samples being decomposed or artifact being formed.

**Sample preparation:**

Sample preparation is an important pre-requisite for success of thin layer chromatographic separation. The sample preparation procedure is to dissolve the dosage form with complete recovery of intact compounds of interest and minimum of matrix with a suitable concentration of analytes for direct application on the HPTLC plates. Solvent for dissolving the sample should be non- polar and volative polar solvents are likely to induce circular chromatography at origin for reversed-phase chromatography usually polar solvents are used for dissolving the sample however such polar solvents must wet the sorbent so that sample penetrates the layer uniformly.

**Application of sample :**

Sample application is the most critical step for obtaining good resolution for quantification by HPTLC. Use of automatic application devices, is recommended for quantitative analysis while using graduated capillaries one must ensure that they fill and empty completely. Usually application of 1-10 µl volume for TLC and 0.5 - 5µl for HPTLC is recommended keeping the size of starting zone down to minimum ; 2-4 mm (TLC) and 0.5 1mm (HPTLC) in the concentration range of 0.1 – 1 µg /ml for TLC /HPTLC .

**Mobile phase (MP)**

Mobile phase commonly called solvent system is traditionally selected by controlled process of trial and error and also based on one's own experiment. Mobile phase should be chosen taking into consideration chemical properties of analytes and the sorbent layer. Use of mobile phase containing more than three or four components should normally be avoided as it is often difficult to get reproducible ratios of different components. Various components of mobile phase should be measured separately and then placed in the mixing vessel laboratories equipped with complete HPTLC system usually use smaller development chamber such as twin trough chambers (10x 10 cm) where comparatively smaller volumes of mobile phase usually 10-15 ml required. Mobile phase should be measured

with volumetric pipettes. Different components of mobile phase should be first mixed in mixing vessel and then introduced into the developing chamber. Chambers usually containing multi-component mobile phase once used is not recommended for re-use and future development work due to differential evaporation and adsorption by the layer and also once the chamber I opened each solvent component will evaporate disproportionately depending on their volatilities.

**Pre- conditioning (chamber saturation):**

Chamber saturation has pronounced influence on the separation profile. When the plate is introduced into an unsaturated chamber, the solvent evaporates form the plate mainly at the solvent front therefore larger quantity of the solvent shall be required for a given distance hence resulting is increase in Rf values. If the tank is saturated (by lying with filter paper) prior to development throughout the chamber. As soon as the plate is placed in such saturated chamber it soon gets pre-loaded with solvent vapours hence less solvent shall be required to travel a particular distance resulting in lower Rf values

**Development and drying :**

Ascending ,descending, two dimensional horizontal, multiple overrun (continuous), gradient radial (circular), anti-radial (anti–circular), multi modal (multi – dimensional) forced flow planar chromatography are the most common modes of development After development the plate is removed form the chamber and mobile phase is removed as completely and as quickly as possible. This step should preferably be preformed in fume cup board to avoid contamination of laboratory atmosphere. The plates should always be laid horizontally so that while mobile phase migrate evenly to the separated substances will migrate evenly to the surface where they can be easily detected. Usually analyst may employ hand dryer to faster removal of the mobile phase.

**Detection and visualization:**

As soon as the development process is complete the plate is removed form the chamber and dried to remove the mobile phase completely .the zones can be located by various physical, chemical, biological–physiological methods there is apparently no difficultly in detecting coloured substances or colorless substances absorbing in shortwave ultra violet (UV) region (254 nm) or with intrinsic fluorescence such as Riboflavin, quinine sulphate. The substances which do not have above properties have to be transferred into detectable substances by means of

chromogenic or fluorogenic reagents which are more expensive time consuming and complicated. Detection sensitivity depends on the specificity for the reagent employed .Iodine is the universal detection reagent. Detection under UV light is the first choice and is non – destructive in most of the cases and is commonly employed for densitometric scanning.

**Validation of HPTLC procedure for pharmaceuticals Analysis:**

**Selectivity:**

Ability of the developed analytical method to detect analysis analyte quantitatively in the presence of other components which are expected to be present in the sample matrix or other related substances. Results are expressed as 'Resolution' if the expected impurities or related substances are available, they should be chromatographed along with the analyte / sample to check the system suitability retention factor, tailing factor and resolution the main parameters to be observed

**Linearity (sensitivity):**

Ability of the method with in a given range to obtain test result in direct proportion to the concentration of analyte in the sample – calibration curve for the analyte

**Precision:**

It provides an indication of random error, results should be expressed as relative standard deviation (RSD) or coefficient of variation (10V)

Repeatability (precision on replication) Precision under same conditions (same analyst, same apparatus short interval of time and identical reagents)

Using the same sample

- Repeatability of measurement of peak area – RSD > 1% based on seven times measurement of same spot
- Repeatability of position – RSD > 2% based on seven times repositioning the instrument after each measurement.
- Repeatability of sample application – equal volume applied as seven spots > 3%

Considering that impurity limit of drug substance is usually rather low(sum of impurities usually > 1% acceptable if RSD > 8 .Solutions are prepared form seven separately weighed amounts of same sample determine on the same chromatoplate using three calibration points within the curve.

**Reproducibility (precision on comparison)**

Precision under different conditions (different analyte different laboratory ,different days and reagents from different sources) using the same sample RSD > 10% within laboratory reproducibility.

**Accuracy (trueness)**

Accuracy of an analysis is determined by systematic error involved. It is defined as closeness of agreement between the actual (true) value and mean analytical value obtained by applying the test method a number of times. The accuracy is acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method. If the obtained for repeatability of the method. If the recovery rate is found to be 100% it implies that the proposed analytical method is free from constant and proportional systematic error. A blank matrix and known impurities must be matrix and known impurities must be available to test the accuracy of the method if the matrix or impurities are not available it must be recorded.

**Ruggedness:**

This is one of the mot important parameters for validation of any analytical method following experiments are usually recommended to evaluate ruggedness of a TLC / HPTLC method.

a) Sample preparation: composition, quantity of solvent, pH, shaking time temperature and number of extractions to be checked.

b) Sample application: volume applied, spot (shape and size)/ band spot, stability.

c) Separation to be performed at least on three different plates from the same or different manufacturers

d) Chromatographic conditions chamber saturation, eluent composition, eluent volume, temperature, humidity and development distance (migration distance)

e) Spot visualization : post –chromatographic derivatization, spraying dipping reaction temperature and time

f) Quantitative evaluation: Drying of plates, detection wavelength used.

**Limit of Detection (LOD)**

Lowest amount of analyte that can be detected - > 10% of the individual impurity limit if this is not possible then amount of analyte to be applied has to be increased LOD is determined on the basis of signal and noise ration mean of 15 noise peak areas and their absolute SD values are determined. LOD is the amount of applied sample producing a peak area which is equal to the sum of mean blank area and three times standard deviation. The ideal detection limit is usually 5-10 folds higher than with matrix.

**Limit of quantitation (LOQ)**

Lowest amount of analyte which can be quantitatively determined in a sample with defined precision and accuracy under stated conditions. LOQ > 20% of the individual impurity. LOQ–amount of applied sample producing a peak area which is equal to sum of mean blank and ten times its SD. Alternatively decreasing amount of substance (volume – concentration) are applied in triplicate on the same plate and RSD calculated from peak area of triplicate samples. LOQ is determined as smallest quantity of the substance for which RSD of the peak area of triplicate sample is either > 5% or when RSD for repeatability of the method does not exceed for

repeatability of the method does not exceed 5% not greater than this repeatability value.

**Stability:**

Analyte should not decompose during development of the chromatogram and should be stable in solution and on the sorbent for atleast 30 and 15 mts respectively. The intensity of the spot on the chromatogram should be constant for at least 60 min chemical reaction of the solvents and their impurities can form artifact which may produce extra spot/peak thus leading to false assay values. Evaporation of organic solvent to dryness also may lead to formation of artifact, complete removal of organic solvent should be avoided.

# REVIEW OF LITERATURE

**Ready GKS et al**; carried out the derivative spectrophotometric and graphical absorbance ratio method for simultaneous estimation of norfloxacin and tinidazole in two component tablet formulations. Norfloxacin has an absorbance maxima at 275 nm and Tinidazole has an absorbance maxima at 317.8nm in 10% v/v N,N- dimethylformamide. The Beer's law is obeyed in the concentration range of 0 -24 meug /ml for Nortloxacin and 0 – 36 meug /m1 for Tinidazole.[18]

**Incilay suslu et al**., carried out the extractive – spectrophotometric determination of ofloxacin by the Application of bromophenol blue and bromocresol purple. A simple rapid and extractive spectrophotometric methods were developed .These methods were based on the formation of yellow ion-pair complexes between the basic nitrogen of the drug and bromophenol blue and bromocresol purple as sulphonphthalein dyes in phthalate buffer pH 3.0 and pH 3.1 respectively .The formed complexes were extracted with chlorofrom and measured at 414 nm for ofloxacin – bromophenol blue and 408 nm for ofloxacin – bromocresol purple. Beer's law was obeyed in the ranges 0.87 – 17.35 and 0.58 – 14.46 mcg/ml[19].

**Panzade P.D.et al.,** carried out a simple spectrophotometric method for simultaneous quantitative estimation of ofloxacin and tinidazole by

using simultaneous equation method. In 0.1N NaoH ofloxacin and tinidazole shows λmax at 294 nm and 317nm respectively[20].

**Shervington ,L.A.et al**., developed a rapid and reliable HPLC method for the simultaneous separation and quantitation of five quinolones antibiotics. Naildixic acid norfloxacin ,ofloxacin,ciprofloxacin and lomefloxacin..The calibration curves were linear over the concentration range of 1.20-4.8mg/100 ml .The separation was performed on a phenomenx ODS C18column using an isocratic ion pairing mobile phase consisting of 35% (v/v) aqueous acetonitrile together with tetrabutylammonium acetate sodium dodecyl sulphate and citric acid (pH3.4)[21]

**Liao et al**., carried out an HPLC method for the quantitative determination of Chlorhexidine acetate in combination with ofloxacin in lotion is reported .The average recovery was 100.64% for Chlorhexidine acetate and 99.88% for ofloxacin[22].

**Hopkala – H et al**., carried out the derivative UV spectrophotometry for the determination of ciprofloxacin, norfloxacin and ofloxacin in tablets[23]

**Wang - L et al.,** developed a spectrophotometric method for the determination of ofloxacin in tablets was described [24].

**Argekar – AP et al**, developed a Quantitative determination of lomefloxacin, ofloxacin pefloxacin and enrofloxacin in pharmaceutical dosages, bulk drugs and process monitoring of enrofloxacin by HPLC–RP[25].

**Zhang – X2 et al**., carried out the studies on preparation and determination of ofloxacin injection and its stability under light and heat for upto 1 year was evaluated using spectrophotometry .ofloxacin injection was stable under light and heat for 1 year[26].

**Zhang – H et al**., developed a UV spectrophotometric method for the determination of ofloxacin granules by UV spectrometry. Recovery and accuracy of this method were 98.71 and 0.99% respectively. The results were not different from those obtained using HPLC[27].

**Mathur – SC et al**., carried out a simple accurate spectrophotometric method for the determination of ofloxacin in tablets was described[28].

**Srividya – B et al**., carried out a simple, rapid ,selective and precise high performance thin layer chromatographic determination of ofloxacin. The samples were spotted on precoated silica gel 60 F254 plates and developed with a mobile phase consisting of n- butanol, methanol, strong ammonia and water (4:1:0.9:1.3).Densitometric analysis of ofloxacin was carried out at 292nm, the Rf value for ofloxacin was 0.53 .The average recovery of ofloxacin was 99.35% at the levels tested[30].

**EV-LD et al.,** determined the microbiological assay for determination of ofloxacin injection. A simple, sensitive and specific agar diffusion bioassay for the antibacterial ofloxacin was developed using a strain of micrococcus luteus ATCC 9341 as the test organinism. Ofloxacin at concentration ranging 12-27 mug/ ml could be measured in injection. The results obtained by three methods were statistically analysis variance (ANOVA) and the results obtained indicate that there is no significant difference among these methods[31].

**Zhang – SY et al**., determined the simultaneous estimation of ofloxacin ,ketoconazole and dexamethasone in Bio-Tai ointment by HPLC[32].

**Mashru – RC et al**., carried out the spectrophotometric method for the determination of pefloxacin and ofloxacin in pharmaceutical formulations. It was determined that the method is simple ,accurate and reproducible for the routine analysis of the drugs in bulk form and in formulations[34].

**Zhang- X2 et al.,** carried out a first order derivative spectrophotometric method used for the determination of ofloxacin in gel form . Interference of other ingredients could be eliminated. Recovery was 99.9% with an RSD of 0.7% [35]

**Jiang – JY et al.,** developed a ultraviolet spectrophotometry of ofloxacin injection. The wavelength of detection was 293nm. Absorption coefficient was 927,and average recovery was 99.9%[36].

**Ertan – R et al.,** studied on the quantitative determination of ofloxacin ,one of the fluoroquinolone derivatives. Quantitative analysis methods,including acidimetry and non-aqueous titration of ofloxacin tablet formulations were investigated..UV spectrometry and high pressure liquid chromatograpy were also utilized for the determination of the active compound in tablet formulations and the sensitivities of the methods were compared[37].

**Yin –C et al.,** carried out the determination of four quinolones by high performance capillary electrophoresis method. Lomefloxacin hydrochloride, enoxacin, ciprofloxacin hydrochloride and ofloxacin is described[38].

**Kitade –T et al**., carried out an easy and sensitive method for the quantitative determination of ofloxacin by using solid substrate room-temperature phosphorimetry on a poly (vinyl alcohol) substrate. The method did not require a dry gas flush during the measurement of phosphorescence. The phosphorescence intensity of ofloxacin was enhanced using NaoH and KI as enhancers. The influence of different conditions such as solution pH and concentration of heavy atoms used as the enhancer were studied.[39]

**Narayana et al;** developed and validated the RP – HPLC method for the estimation of nitazoxanide in bulk Drug and tablets[40].

**I.Sullayman adagu et al;** carried out the invitro activity of nitazoxanide and related compounds against isolates of Giardia intertinalis, entamoeba histolytica and trichomonas vaginalis. The activates of the N – (nitrothiazolyl) salicylamide nitazoxanide and its metabolite tizoxanide were compared with metronidazole invitro in microplates against six axenic isolates of Giardia intestinalis. Tizoxanide was eight times more active than metronizole against metronizole susceptible isolates and twice as active against a resistant isolate[41].

**Marcelo donadel malesuik et al**., developed a validated stability - indicating LC method for Nitazoxanide in pharmaceutical formulations. An isocratic LC separation was performed on a phenomenax synergi fusion C18 column using a mobile phase of 0.1 % O – Phosphoric acid solution .pH 6.0 Acetonitrile (45:55 v/v ) at a flow rate of 1.0 ml/ min. Detection was achieved with a photodiode array detector at 240 nm. The detector response for Nitazoxanide was linear over the concentration range from 2 to 100 mg /ml (r=0.9999)[42].

**Salvador namur et al .,** carried out the development and validation of a High Performance Thin-layer Chromatographic method with densitometry, for quantitative analysis of Tizoxanide (a metabolite of

Nitazoxanide ) in human plasma. The analyte was extracted from human plasma by Cation – exchange solid – phase extraction. In HPTLC the stationary phase was silica gel 60 f254 and the mobile phase was Toluene – Ethyl acetate: acetic acid 6.2 : 13.4:0.4(v/v). The working range was 400 – 16000ng /ml. The average recovery was 85.55[43].

**Kapse GK et al ;** carried out a Spectrophotometric method for the Estimation of Nitazoxanide in pharmaceutical formulation. Three simple and sensitive spectrophotometric methods in the visible region have been developed. These method are based on the reaction of reduced Nitazoxanide with P – dimethylamino benzaldehyde, P-demethylamino – cinnamaldehyde and vanillin in acidic conditions to form pink, orange red and orange yellow coloured chormogens with absorption maxima at 559 nm ,534.5 nm and 475 nm respectively. The reduction of Nitazoxanide was carried out with zinc granules and 5N hydrochloric acid at room tempature in methanol. Beers law is obeyed in the concentration range of 5 – 25 mg/ml and 10 – 50 mg/ml respectively[44].

**Ashoks, Jadhav et al.,** carried out a validated stability indicating RP-LC method for nitazoxanide, a new antiparasitic compound. The drug substance was subjected to stress conditions of hydrolysis, photolysis and thermal degradation. The considerable degradation of nitazoxanide was observed under base and peroxide hydrolysis[45].

**Lakshminarayana KV et al,** developed and validated a spectrophotometric method for the estimation of nitazoxanide in tablet dosage form. Two simple and sensitive visible spectrophotometric method (A and B) have been developed for the quantitiative estimation of nitazoxanide. Methods were based on the formation of reddish yellow coloured and green coloured chromogens, which were measured at 544nm and 715nm repectively[46].

**Alsarra et al.,** developed a stability-indicating HPLC method for the determination of montelucast in tablets and human plasma and its application to pharmacokinetic and stability studies .The intra day and interday precisions showed coefficients of variations ranged from 5.87% to 9.60% and from 2.13% to 6.18% at three different levels of concentrations[47].

**Radhakrishna et al.,** compared HPLC and derivative spectrophotometric methods for the simultaneous determination of montelucast and loratadine .HPLC separation was achieved with a symmetry C18 column and sodium phosphate buffer (pH 3.7): acetonitrile (20:80 v/v) as eluent ,at a flow rate of 1.0ml/min.UV detection was performed at 225nm.In the UV second –order derivative spectrophotometry.for the determination of loratidine the zero –crossing technique was applied at 276.1nm but for montelucast peak amplitude at 359.7nm(tangent method) was used[48].

**Liu-L et al;** developed a stereoselectoive high performance liquid chromatography with column switching for the determination of montelucast (MK-0476) and its s –enantiomer in human plasma[49].

**Alsarra et al;** developed a spectrofluorometric determination of montelucast in dosage forms and spiked human plasma .The highest fluorescence intensity was obtained in methanol at 390nm using 340nm for excitation[50]

**Amin RD et al;** carried the determination of montelucast – 0476 in human plasma by liquid chromatography.the method involves precipitation of protein and reversed – phase liquid chromatogaphy with fluorescence detection. The assay is linear in the range of 30 – 3000 ng /m1-1 of MK – 0476 and the limit of detection is 5ng m1-1. The interday accuracy values at these concentrations are 94 and 104% respectively the absolute recovery of MK – 0476 is 99%[52]

**Alsarra et al**; developed a voltammetric determination of montelucast sodium in dosage forms and human plasma.It was studied using cyclic voltammetry ,direct current (DCT) differential pulse polarography (DPP) and alternating current (ACT) polarography. The mean % recovery (n=5) was 101.38+/- 3.85 . The number of electrons transferred in the reduction process could be accomplished and a proposal of the electrode reaction was proposed[54].

**Sentuerk-Z et al.,**carried out the determination of theophylline and ephedrine HCL in tablets by ratio spectra derivative spectrophotometry and LC. The first derivative amplitudes at 231.8 and 250.3nm were selected for the assay of theophylline and ephedrine . For High performance liquid chromatography mobile phase of Methanol-Water (40:60) with detection at 217nm.Linearity was obtained in the concentration range of 5-150mug[55].

**Shishoo-CJ el al.,** developed a sensitive HPLC method for bioavailability and bioequivalence studies of theophylline SR formulations.The HPLC method was applied in a 2 way cross over study with an 8 day washout period of the bioavailability and bioequivalence in 6 healthy male volunteers[56].

**Budvari-Barany Z et al.,** carried out two HPLC methods and used for the determination of purity of caffeine ,theophylline and theobromine.The 2 systems enabled the detection of 0.1-0.2% of the compounds in each other[57].

**Pokrajac-m et al.,** compared the spectrodensitometric determination of theophylline to HPLC, RIA and EMIT procedures .The sample estimated were obtained from pediatric patients with drug concentrations mainly within the therapeutic range. The method was statistically comparable and gave good results when compared to HPLC and especially to EMIT but not to RIA[58].

**Chen-TM el at.,** carried the simultaneous determination of ephedrine Hcl, phenobarbital and theophylline in regular and sustained action tablets using Butabarbital as the internal standard .Recoveries utilizing the method ranged from 97.5-100.1% for I,96.3-100% for II and 98.1-100.3% for III in conventional tablets[59].

**Culzoni MJ el al.,** developed a chemometrics assited UV spectroscopic strategies for the determination of theophylline in syrups[60].

**Elsayed-MAH et al.,** carried out the spectrophotometric determination of theophylline formulations.Ampuls were analysed without interference from excipients or preservatives .A mixture of minophylline and Phenobarbital in syrup was analysed for both components with good accuracy and high reproducibility[61].

**De-Fabrizio –F et al.,** developed a column chromatography and UV spectroscopy.They were used for the separation and quantitative determination of theophylline and etophylline in syrup formulations[62].

**Banner AS et al.,** carried out the interference of drugs with the determination of theophylline by the UV spectrophotometric procedure.It was found that the presence of barbiturates ,phenytoin and acetaminophen may yield values falsely low by as much as 10 mcg/ml[63].

**Mirfazaelin et al**, developed a quantitative Thin layer chromatography for the determination of theophylline in plasma. The method involves extraction of the drug and internal standard (acetaminophen) by Chloroform-Isopropanol (75:25) followed by the separation on TLC silica plates using Acetic acid:Isopropanol:Toluene, (1:12:6) as the eluting solvent[65].

**Mehdi ansari et al.,** determined the simultaneous quantitation of theophylline and guaifenesin in syrup by HPLC ,Derivative ratio spectrophotometry .For quality control purposes .The first derivative method can be applied only for THP and depends on the use of the absorption spectrum of the binary mixtures.In HPLC method methanol:water (40:60v/v) is used as mobile phase. Caffeine was used as the internal standard and the substances were detected at 280nm[66].

**EI-Gindy et al.,** developed the simultaneous estimation of diprophylline,phenobarbitone and papaverine hydrochloride by HPLC and chemometric assited spectrophotometric methods.Mobile phase consisting of 0.02 Potassium di hydrogen phosphate pH 3.5 –acetonitrile (55:45v/v). Quantitation was achieved with UV detection at 210nm based on the peak area[67].

**Brouwers -J et al.,** determined the intra aluminal theopylline concentrations after oral intake of an immediate and a slow release dosage form. Human intestinal fluids were aspirated from two sampling sites (duodenum and jejunum) at different time points after oral intake of theophylline.osmolarity and pH of the aspirates were mesured and theophylline concentrations were determined by HPLC-UV[68].

**Ku-YR et al.,** determined xanthine bronchodilators in adulterated Chinese herbal preparations by HPLC.A method employing solid –phase extraction and HPLC for the extraction and quantitative determination of 5 bronchodilating xanthines[70].

**Mazzei et al.,** carried out the HPLC analysis of theophylline bioequivaiance study of two sustained release formulations at steady state.An accurate and reproducable isocratic reversed phase HPLC method with UV detection is described for the determination.of theophylline anhydrous in 2 sustained – release formulations[71].

**De – fabrizio – f et al.,** carried out column chromatography and UV spectrometry for the separation and quantitative determination of theophylline(I) and elophylline(II) in syrup formulation. An aliquot of diluted syrup was pipetted into a chromatography column containing a strong anion exchange resin which retains theophylline and allows etophylline to pass through it. It was subsequently eluted with in Hcl and

determined by difference spectrometry at 284nm;etophylline was determined at 272.[72]

**Scott –NR et al;** developed an HPLC method with ion pair complexation is described for the determination of methylxanthine metabolites of caffeine and theophylline in urine. Comparison of this procedure with direct injection of diluted urine specimens showed the latter analysis for the quantitation of the major urinary metabolites[76].

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# AIM OF THE PRESENT WORK

Antibacterial drugs namely ofloxacin and nitazoxanide are selected for analysis by UV spectrophotometric method and reverse phase high performance liquid chromatography. It is estimated by simultaneous equation method as the $\lambda_{max}$ of the drug is dissimilar and their absorbance ratio lies outside the range 0.1 to 2. It is also estimated by Absorbance ratio methods the drugs absorbs at the $\lambda_{max}$ of the other.

The next objective is to develop and validate a reverse phase high performance liquid chromatography which would be simple, rapid, efficient and reliable for the analysis of both the drugs in combined dosage form.

Antiasthmatic drugs namely Montelucast and Theophylline existing in combined dosage from are selected for analysis by UV spectrophotometric method and High performance thin layer chromatography. In UV both simultaneous equation and absorbance ratio methods are carried out as the drugs absorbs at the $\lambda_{max}$ of the other and the ratio of absorbance at any two wavelengths is a constant value independent of concentration or path length.

# UV-SPECTROPHOTOMETRIC SIMULTANEOUS EQUATION METHOD OF OFLOXACIN AND NITAZOXANIDE IN COMBINED TABLET DOSAGE FORM

## PRINCIPLE:

If a sample contains two absorbing drugs (X and Y) each of which absorbs at the $\lambda_{max}$ of the other. It may be possible to determine the quantity of both drugs by the technique of simultaneous equation (or) Vierodt's method.

Criteria for obtaining maximum precision, based upon absorbance ratios have been suggested that place limits on the relative concentrations of the component of the mixture.

$\frac{A_2 / A_1}{ax_2 / ax_1}$ and $\frac{ay_2 / ay_1}{A_2 / A_1}$

Where $ax_1$, $ax_2$ = Absorptivities of X at $\lambda_1$ and $\lambda_2$

$ay_1$, $ay_2$ = Absorptivities of X at $\lambda_1$ and $\lambda_2$

$A_1$, $A_2$ = Absorbances of the diluted sample at $\lambda_1$ and $\lambda_2$.

The ratio should lie outside the range of 0.1 – 2.0 for the precise determination of (Y and X) two drugs respectively.

These criteria are satisfactory only when the $\lambda_{max}$ of the two components is reasonably dissimilar. The additional criteria includes that two components do not interact chemically, there by negating the initial

assumption that the total absorbance is the sum of the individual absorbance's.

## MATERIALS

I. **Market Sample**:

1. ZENFLOX (Discovery mankind pharmaceuticals)
2. LABEL CLAIM:

Ofloxacin - 200mg

Nitazoxanide - 500mg

II. **Equipments Used**:

- ATCO Balance
- SHIMADZU UV - spectrophotometer double beam digital UV-1700

**III. Solvent Used:**

- Methanol AR

## FIXATION OF VARIOUS PARAMETERS

**$\lambda_{max}$**

The wavelength at which maximum absorption takes place is called $\lambda_{max}$

**Determination of Absorption Maximum ($\lambda_{max}$) for Ofloxacin**

**Procedure:**

50mg of authentic ofloxacin sample was accurately weighed and transferred to 50ml volumetric flask, methanol was added, dissolved and the volume was made upto 50ml with methanol.

1ml of this stock solution was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol.

The absorbance of solution was measured against solvent blank in UV-region of 200-400nm. The $\lambda_{max}$ was found to be 296nm. This spectrum of maximum absorbance is shown in graph-1

## BEER'S LAW PLOT FOR OFLOXACIN

The intensity of a beam of parallel monochromatic radiation decreases exponentially with the number of absorbing molecules. More simply it is stated that the absorbance is proportional to the concentration.

$$\text{Log } I_0 / I_t = KC$$

Beer's law plot was constructed by measuring the absorbance of various concentration of drug against solvent blank.

## PROCEDURE

50mg of authentic ofloxacin sample was accurately weighed and transferred to 50ml volumetric flask , methanol was added, dissolved and the volume was made up to 50ml with methanol.

From this aliquots of 0.1ml, 0.2ml, 0.3ml,.0.4ml, 0.5ml, 0.6ml, 0.7ml, 0.8ml, 0.9ml, 1.0ml, was pipetted out in to separate 100ml volumetric flask. Then the volume was made upto 100ml with methanol. The absorbance of each solution was found out at 296nm against a reagent blank. The readings are presented in Table- 1 and graph-2

## Table – 1

## DATA FOR BEER'S LAW PLOT FOR OFLOXACIN

## (Linearity)

| S.No. | Concentration µg/ml | Absorption |
|---|---|---|
| 1 | 5 | 0.7670 |
| 2 | 10 | 0.9535 |
| 3 | 15 | 1.1145 |
| 4 | 20 | 1.3694 |
| 5 | 25 | 1.5447 |
| 6 | 30 | 1.7698 |
| 7 | 35 | 1.9423 |
| 8 | 40 | 2.1425 |
| 9 | 45 | 2.3944 |
| 10 | 50 | 2.5645 |

Linearity Co-efficient ($\gamma$ ) = 0.9988

Slope (m) = 0.04039

Intercept (c) =0.5454

## DEVIATIONS FROM BEER'S LAW

A system is said to obey Beer's law, where a plot of concentration vs absorbance gives a straight line by using a line of best fit. When a straight line is not obtained ; the system is said to be deviated from Beer's law.

For the drug ofloxacin maximum deviation was found in the Concentration range above 50µg/ml. The readings are presented in Table -2 and graph-3

**Table 2**

**DATA FOR DEVIATIONS FORM BEER'S LAW PLOT FOR OFLOXACIN**

| S.No. | Concentration µg/ml | Absorbance |
|---|---|---|
| 1. | 5 | 0.7670 |
| 2. | 10 | 0.9535 |
| 3. | 15 | 1.1145 |
| 4. | 20 | 1.3694 |
| 5. | 25 | 1.5447 |
| 6. | 30 | 1.7698 |
| 7. | 35 | 1.9423 |
| 8. | 40 | 2.1425 |
| 9. | 45 | 2.3944 |
| 10. | 50 | 2.5645 |
| 11. | 55 | 0.2614 |
| 12. | 60 | 0.2648 |
| 13. | 65 | 0.2674 |

**Determination of Absorption Maximum($\lambda_{max}$) for Nitazoxanide**

**Procedure:**

50mg of authentic nitazoxanide sample was accurately weighed and transferred to 50ml volumetric flask and methanol was added, dissolved and the volume was made upto 50ml with methanol

1ml of this stock was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol

The absorbance of solution was measured against solvent bank in UV-region of 200-400 nm. The $\lambda_{max}$ was found to be 410nm. This spectrum of maximum absorbance is shown in graph-4

## BEER'S LAW PLOT FOR NITAZOXANIDE

The intensity of a beam of parallel monochromatic radiation decreases exponentially with the number of absorbing molecules. More simply it is stated that the absorbance is proportional to the concentration.

$$\text{Log } I_0 / I_t = KC$$

Beer's law plot was constructed by measuring the absorbance of various concentration of drug against solvent blank.

**PROCEDURE**

50mg of authentic Nitazoxanide sample was accurately weighed and transferred to 50ml volumetric flask and methanol was added dissolved and the volume was made up to 50ml with methanol.

From this aliquots of 0.1ml, 0.2ml, 0.3ml, 0.4ml, 0.5ml, 0.6ml, 0.7ml, 0.8ml, 0.9ml, 1ml, was pipetted out in to separate 100ml volumetric flask. Then the volume was made upto 100ml with methanol. The absorbance of each solution was found out at 410nm against a reagent blank. The readings are presented in Table-3 and graph-5

## TABLE-3

## DATA FOR BEER'S LAW PLOT FOR NITAZOXANIDE

| S.No. | Concentration µg/ml | Absorbance |
|---|---|---|
| 1. | 5 | 0.2498 |
| 2. | 10 | 0.4836 |
| 3. | 15 | 0.7299 |
| 4. | 20 | 0.9672 |
| 5. | 25 | 1.2094 |
| 6. | 30 | 1.4564 |
| 7. | 35 | 1.6984 |
| 8. | 40 | 1.9394 |
| 9. | 45 | 2.1774 |
| 10. | 50 | 2.4186 |

Linearity coefficient $\gamma = 0.9999$

Slope (m) = 0.04831

Intercept (c) =0.00447

## DEVIATIONS FROM BEER'S LAW

A system is said to obey Beer's law, where a plot of concentration vs absorbance gives a straight line by using a line of best fit. When a straight line is not obtained; the system is said to be deviated from Beer's law.

For the drug, Nitazoxanide maximum deviation was found in the concentration range above 50µg/ml. The readings are presented in Table – 4 and graph-6

**Table – 4**
**Data for Deviations from Beer's law plot for Nitazoxanide**

| S.No. | Concentration µg/ml | Absorption |
|---|---|---|
| 1 | 5 | 0.2498 |
| 2 | 10 | 0.4836 |
| 3 | 15 | 0.7299 |
| 4 | 20 | 0.9672 |
| 5 | 25 | 1.2094 |
| 6 | 30 | 1.4564 |
| 7 | 35 | 1.6984 |
| 8 | 40 | 1.9394 |
| 9 | 45 | 2.1774 |
| 10 | 50 | 2.4186 |
| 11 | 55 | 2.5540 |
| 12 | 60 | 2.5548 |
| 13 | 65 | 2.5594 |

**Preparation of ofloxacin standard solution**

50mg of authentic ofloxacin sample is accurately weighed and transferred to 50ml volumetric flask and 5ml of methanol was added and shaken until it dissolves and the volume was made upto 50ml with methanol.

From this 1ml was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol to give a concentration of 10µg/ml.

**Preparation of Nitazoxanide standard solution**

50mg of authentic nitazoxanide sample is accurately weighed and transferred to 50ml volumetric flask and 5ml of methanol was added and shaken until it dissolves and the volume was made upto 50ml with methanol.

From this 1ml was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol to give a concentration of 10µg/ml.

The absorbance & absorptivity values are shown in Table- 5a &5b respectively.

**Preparation of sample solution**

Twenty tablets are weighed and average weight was calculated. The tablets are ground to a fine powder. A powder equivalent to 200mg of ofloxacin and 500mg of nitazoxanide was accurately weighed and transferred to 50ml volumetric flask and 15ml of methanol was added and shaken until it dissolves and the volume was made upto 50ml with methanol. This solution was filtered through whatmann filter paper.

From this 1ml was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol.

The absorbance of each solution was found out at 296nm ($\lambda_{max}$ of ofloxacin) and 410nm ($\lambda_{max}$ of Nitazoxanide) against a reagent blank.

The analysis data are given in table-6

**Calculation:**

$\lambda_1 = 296nm$

$\lambda_2 = 410nm$

$ax_1$ and $ax_2$ – Absorptivities of ofloxacin at $\lambda_1$ and $\lambda_2$

$ay_1$ and $ay_2$ – Absorptivities of Nitazoxanide at $\lambda_1$ and $\lambda_2$

Cx and Cy – Concentration of ofloxacin and Nitazoxanide of sample respectively in grams / 100 ml.

$A_1$ and $A_2$ – Absorbance of sample at $\lambda_1$ and $\lambda_2$

$$\text{Absorptivity (a)} = A/bc = \frac{\text{Absorbance}}{\text{b x concentration of substance}}$$

Determination of ofloxacin

$$Cx = \frac{A_2\, ay_1 - A_1\, ay_2}{ax_2\, ay_1 - ax_1\, ay_2}$$

Determination of Nitazoxanide

$$Cy = \frac{A_1\, ax_2 - A_2\, ax_1}{ax_2\, ay_1 - ax_1\, ay_2}$$

## Table-5a

## Absorbance values for standard and sample

| Wavelength | Ofloxacin | Nitazoxanide | Sample |
|---|---|---|---|
| 296<br>$\lambda_1$<br>Ofloxacin | 0.1843 (X1) | 0.0232 (y1) | 1.9746 (A1) |
| 410<br>$\lambda_2$<br>Nitazoxanide | 0.0632 (X 2) | 0.2541 (y2) | 1.6864 A2) |

## Table-5b

## Absorptivity values for ofloxacin and nitazoxanide.

| Parameter | Absorptivity at 296nm | | Absorptivity at 410nm | |
|---|---|---|---|---|
| | Ofloxacin | Nitazoxanide | Ofloxacin | Nitazoxanide |
| *Mean | $ax_1$=956.4 | $ay_1$=0 | $ax_2$= 24.9 | $ay_2$=483.6 |

Absorptivity values are the mean of six determinations. . $ax_1$ and $ax_2$ are absorptivities of ofloxacin at 296 nm, and 410nm, respectively; $ay_1$ and $ay_2$ are abxorptivities of nitazoxanide at 296nm and 410nm respectively.

-

## CRITERIA FOR OBTAINING MAXIMUM PRECISION

$(A_2 / A_1)/ (ax_2 / ax_1)$ and $(ay_2 / ay_1)/ (A_2 / A_1)$ was found to be 2.49 & 12.82 respectively. This ratio should lie outside the range 0.1-2.0 for the precise determination.

**Table-6**

**Analysis data of tablet formulations**

| Parameters | UV-spectrophotometry | |
|---|---|---|
| | Ofloxacin | Nitazoxanide |
| Label Claim | 200mg | 500mg |
| Amount found | 199.54 mg | 496.25mg |
| *% Drug content | 99.77 | 99.25 |

*value for % Drug content are mean of five estimations.

## RECOVERY STUDIES

To check the accuracy of the developed method and to study the interference of formulation additives, analytical recovery experiments were carried out by standard addition method at 80, 100 and 120% level. From the total amount of drug found the percentage recovery was calculated. The results are reported in Table-7.

**TABLE-7**

**Recovery studies**
**Ofloxacin**

| Range | Amount found | Recovery |
|---|---|---|
| 80% | 198.2 mg | 99.46% |
| 100% | 199.76 mg | 99.88% |
| 120% | 199.08 mg | 99.54% |

**Nitazoxanide**

| Range | Amount found | Recovery |
|---|---|---|
| 80% | 198.2 mg | 99.46% |
| 100% | 199.76 mg | 99.88% |
| 120% | 199.08 mg | 99.54% |

*Recovery is the mean of three estimations

# SIMULTANEOUS UV SPECTROPHOTOMETRIC ESTIMATION OF OFLOXACIN AND NITAZOXANIDE IN COMBINED TABLET DOSAGE FORM BY ABSORBANCE RATIO METHOD

## PRINCIPLE

This method depends on the property of a substance which obeys Beer's law at all wavelengths, the ratio of absorbance at any two wavelengths is a constant value independent of concentration or path length. Two different dilutions of the same sample give the same absorbance ratio; this ratio is referred to as a 'Q' value.

## Preparation of Ofloxacin standard solution

50mg of authentic ofloxacin sample is accurately weighed and transferred to 50ml volumetric flask and methanol was added and shaken until it dissolves and the volume was made upto 50ml with methanol.

From this 1ml was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol.

## Preparation of Nitazoxanide standard solution

50mg of authentic nitazoxanide sample is accurately weighed and transferred to 50ml volumetric flask and methanol was added and shaken until it dissolves and the volume was made upto 50ml with methanol.

From this 1ml was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol. The absorbance & absorptivity values are shown in table-8a & 8b respectively.

**Determination of Iso Absorptive point:**

The standard solutions of Ofloxacin and Nitazoxanide are scanned over 200-400nm. When the graph was plotted with wavelength against absorbance, the wavelength of equal absorptivity was found at 350nm. The overlain spectra is shown in figure-A

**Preparation of sample solution**

Twenty tablets are weighed and average weight was calculated. The tablets are ground to a fine powder. A powder equivalent to 200 mg Ofloxacin and 500mg of Nitazoxanide was accurately weighed and transferred to 50ml volumetric flask and methanol was added and shaken until it dissolves and the volume was made upto 50ml with methanol. This solution was filtered through whatmann filter paper.

From this 1ml was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol.

The absorbance of each solution was found out at 350nm (Iso Absorptive point) and 410nm ($\lambda_{max}$ of Nitazoxanide) against a reagent blank. The analysis value are given in table-9

**Calculation:**

$\lambda_1$ = 350nm (Iso absorpritive point)

$\lambda_2$ = 410nm ($\lambda_{max}$ of Nitazoxanide)

$ax_1$ and $ax_2$ – Absorptivities of Ofloxacin at $\lambda_1$ and $\lambda_2$

$ay_1$ and $ay_2$ – Absorptivities of Nitazoxanide at $\lambda_1$ and $\lambda_2$

Cx and Cy – Concentration of Ofloxacin and Nitazoxanide (Sample) in grams per 100ml

A1 and A2 – Absorbance of sample at $\lambda_1$ and $\lambda_2$

$$\text{Absorptivity (a)} = A/bc = \frac{\text{Absorbance}}{\text{B x concentration of substance}}$$

Determination of Cx and Cy

$$Cx = \frac{Q_m - Q_y}{Q_x - Q_y} \times \frac{A_1}{Ax_1}$$

$$Cy = \frac{Q_m - Q_x}{Q_y - Q_x} \times \frac{A_1}{Ax_1}$$

Where

$$Q_m = \frac{A_2}{A_1}$$

$$Q_x = \frac{ax_2}{ax_1}$$

$$Q_y = \frac{ay_2}{ay_1}$$

## Table-8a

## Absorbance values for standard and sample

| Wavelength | Ofloxacin | Nitazoxanide | Sample |
|---|---|---|---|
| 350 $\lambda_1$ Iso absorptive point | 0.0130($X_1$) | 0.0130 ($Y_1$) | 0.1854 ($A_1$) |
| 410 $\lambda_2$ Nitazoxanide | 0.0049 ($X_2$) | 0.0967$Y_2$) | 0.9984 ($A_2$) |

## Table-8b

## Absorptivity values for Ofloxacin and Nitazoxanide

| Parameter | Absorptivity at 350nm | | Absorptivity at 410nm | |
|---|---|---|---|---|
| | Ofloxacin | Nitazoxanide | Ofloxacin | Nitazoxanide |
| *Mean | ax1=65.4 | ay1=65.4 | ax2= 24.9 | ay2=483.6 |

*Absorptivity values are the mean of six determinations. ax1 and ax2 are absorptivities of Ofloxacin at 350nm, and 410nm, respectively; ay1 and ay2 are absorptivities of nitazoxanide at 350nm and 410nm respectively.

## Table-9

## Analysis data of tablet formulations

| Parameters | UV-spectrophotometry | |
|---|---|---|
| | Ofloxacin | Nitazoxanide |
| Label Claim | 200mg | 500mg |
| Amount found | 201.3mg | 501.4mg |
| *% Drug content | 100.67 | 100.29 |

*value for % Drug content are mean of five estimations

## RECOVERY STUDIES

To check the accuracy of the developed method and to study the interference of formulation additives, analytical recovery experiments were carried out by standard addition method at 80, 100 and 120% level. From the total amount of drug found the percentage recovery was calculated. The results are reported in Table-10.

**Table-10**

**Recovery studies**

**Ofloxacin**

| Range | Amount found | Recovery |
|---|---|---|
| 80% | 201.2mg | 100.62% |
| 100% | 199.5mg | 100.27% |
| 120% | 201.1mg | 100.57% |

**Nitazoxanide**

| Range | Amount found | Recovery |
|---|---|---|
| 80% | 501.4mg | 100.28% |
| 100% | 501.0mg | 100.27% |
| 120% | 501.2mg | 100.25% |

*Recovery is the mean of three estimations

# DEVELOPMENT AND VALIDATION OF REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF OFLOXACIN AND NITAZOXANIDE IN COMBINED TABLET DOSAGE FORM

## Instruments:

- Shimadzu liquid chromatograph LC – 10 AT VP
- Mettler Toledo AG 285 Balance CP-225D
- DIGISUN-DI-707 pH meter
- Millipore filter (10.45/ µ m)
- Whatman filter paper
- Sonicator

## Reagents and Chemicals

- Acetonitrile
- HPLC grade water
- Buffer
- Methanol

## REFERENCE STANDARDS:

## Ofloxacin and Nitazoxanide:

These two reference standards were obtained as gift samples from Discovery Mankind Pharmaceuticals. The authenticity and purity of the sample was certified by the same.

**Sample Tablet brand used** : ZENFLOX

Discovery Mankind Pharmaceuticals

**Label claim** : Ofloxacin - 200mg

Nitazoxanide - 500mg

## METHOD DEVELOPMENT AND OPTIMIZATION:

## SELECTION OF WAVELENGTH:

The known concentration of Ofloxacin and Nitazoxanide were taken and dissolved in methanol. The wavelength were tried at 250nm, 260nm, 270nm and the Peaks of the drugs were showing fronting and tailing. The peak areas were also found to be minimum.

Finally 280nm were selected for the analysis.

## OPTIMIZATION OF CHROMATOGRAPHIC PARAMETAERS

### (a) SELECTION OF MODE OF OPERATION:

As both the drugs were are polar in nature, a RP-HPLC method was Proposed.

**(b) SELECTION AND STANDARDISATION OF MOBILE PHASE:**

ZENFLOX is combination of Ofloxacin 200mg and Nitazoxanide 500mg. The method development of Ofloxacin and Nitazoxanide required adequate resolution of two drug peaks in the chromatogram.

**DIFFERENT COMBINATIONS OF BUFFER AND SOLVENTS:**

Buffer (Tetra butyl ammonium hydrogen sulphate) and acetonitrile (60:40)

Buffer (Tetra butyl ammonium hydrogen sulphate) and acetonitrile (50:50)

Buffer (Tetra butyl ammonium hydrogen sulphate) : methanol: acetonitrile (10:30:60)

Buffer (Tetra butyl ammonium hydrogen sulphate) : methanol:acetonitrile (20:20:60)

Peaks of ofloxacin and Nitazoxanide were well resolved with solvent system Buffer (Tetra butyl ammonium hydrogen sulphate): methanol: acetonitrile .(20:20:60) and it is shown in figure-4

**SELETION OF FLOW RATE:**

The Flow rate for Ofloxacin and Nitazoxanide were tried with 0.4ml, 0.5ml, and 0.7ml and the peaks of the drugs were showing fronting and

tailing with 0.4ml and 0.7ml respectively and finally 1ml per minute was selected for the analysis.

**PREPARATION OF BUFFER SOLUTION:**

Buffer solution was prepared by using 0.6788g of tetra butyl ammonium hydrogen sulphate in 200ml of HPLC grade water,filtered through 0.45µ nylon membrane and degassed.

**PREPARATION OF MOBILE PHASE:**

Mix the Buffer, methanol: and acetonitrile in the ratio of 20:20:60 and degass it. Filtered through 0.45µ membrane.

**DILUENT**

Mobile phase is used as diluent.

**DETERMINATION OF RETENTION TIME:**

**(A) STANDARD SOLUTION OF OFLOXACIN**:

Accurately 0.200g of ofloxacin was taken in a 100ml volumetric flask and the volume was adjusted to 100ml with mobile phase. 5 ml was taken in a separate 50ml volumetric flask and the volume was adjusted to 50 ml with mobile phase to get concentration of 200 µg/ml of ofloxacin. 20µl of this solution was injected and chromatogram was obtained and it is in shown Figure-3.

**STANDARD SOLUTION OF NITAZOXANIDE**

Accurately 0.500g Nitazoxanide was taken in a 100ml volumetric flask and the volume was adjusted to 100ml with mobile phase. 5 ml was taken in a separate volumetric flask of 50ml and the volume was adjusted to 50ml with mobile phase to get a concentration of 500 µg/ml. 20µl of this solution was injected and the chromatogram was recorded and shown in Figure-2

**(C) PREPARATION OF MIXED STANDARD SOLUTION:**

0.200g of ofloxacin and 0.500g of Nitazoxanide was transferred into a 100ml dried volumetric flask. The compounds were first dissolved in 20ml of mobile phase and it was sonicated. Then the volume was adjusted to 100ml with mobile phase. From the stock solution 5ml was transferred to a 50ml volumetric flask and the volume was adjusted to 50ml with mobile phase to get a concentration of 200µg/ml of Ofloxacin and 500µg/ml of Nitazoxanide. 20 µl of the resulting solution was injected and chromatogram was recorded shown in Figure-4.

**FIXED CHROMATOGRAPHIC CONDITIONS:**

INSTRUMENT : Shimadzu liquid chromatograph

LC-10 AT VP

COLUMN : C18

WAVELENGTH : 280nm

TEMPERATURE : Ambient temperature.

FLOW RATE : 0.5ml/min

INJECTION VOLUME : 20µl.

MOBILE PHASE : Buffer (Tetra butyl ammonium hydrogen sulphate ): methanol and acetonitrile.(20:20:60)

RETENTION TIME : 4.67 min for ofloxacin 7.77 min for nitazoxanide.

**QUANTITATIVE DETERMINATION OF THE DRUGS BY USING THE DEVELOPED METHOD**

Sample : Ofloxacin and Nitazoxanide

Label Claim : Ofloxacin – 200mg

Nitazoxanide -500mg

**METHOD:**

Twenty tablets were weighed and powdered. Average weight (1.3595) of sample tablet ZENFLOX (equivalent to 200mg of Ofloxacin and 500mg of Nitazoxanide ) was taken into 100ml dried volumetric flask. The powder was first dissolved in 20ml of mobile phase and sonicated and finally the volume was adjusted to 100ml with mobile phase. From this solution 5ml was transferred to 50ml volumetric flask and volume was adjusted to 50ml with mobile phase to get a concentration of 200µg/ml of Ofloxacin and 500µg/ml of Nitazoxanide. 20µl of the solution was injected and the chromatogram obtained is shown in Figure 5

The amount of Ofloxacin & Nitazoxanide present in the tablet formulation was calculated by comparing the peak area of the standard and reports are given in Table-8

**Amount of drug present in the tablet:**

$$\frac{\text{Sample area}}{\text{Standard Area}} \text{ x } \frac{\text{Standard dilution}}{\text{Sample dilution}} x \frac{\text{Potency}}{100} \text{ x Average weight}$$

$$\text{Percentage purity} = \frac{\text{Amount present}}{\text{Label claim}} \text{ x } 100$$

## Table -11

## Quantitative Estimation

| S. No. | Brand Name | Content | Label Claim (mg) | Peak area | Amount present (mg) | Percent Purity% w/v |
|---|---|---|---|---|---|---|
| 1. | ZENFLOX | ofloxacin | 200mg | 9563.4884 | 201.04 | 100.20% |
| | | Nitazoxanide | 500mg | 5917.5990 | 12.66 | 101.281% |

Acceptance critearia: 98-102%w/v

Assay for ofloxacin:

Amount Present =

$$\frac{9563.4884}{10093.2956} \times \frac{201.0}{1286.2} \times \frac{99.87}{100} \times 1359.5 = 201.04\text{mg}$$

% Label Claim

$$\frac{201.04}{200} \times 100 = 100.20\ \%$$

Assay for nitazoxanide:

Amount Present =

$$\frac{5917.5990}{6251.4783} \times \frac{500.10}{1286.2} \times \frac{99.87}{100} \times 1359.5 = 499.71\ \text{mg}$$

% Label Claim =

$$\frac{499.71}{} \times 100 = 99.94\%$$

500

## VALIDATION

Validation of an analytical method is a process to establish by laboratory studies that the performance characteristics of the method meet the requirements for the intended analytical application. Performance characteristics are expressed in terms of analytical parameters.

**Design of experiment**:

Typical analytical parameters used in assay validation are,

- Specificity
- Linearity and range
- Limit of quantification
- Limit of detection
- Accuracy
- Precision
  - System precision
  - Method precision
- Robustness
- Ruggedness
- System suitability studies

- Resolution
- Number of theoretical plates
- The tailing factor.

## SPECIFICITY

The specificity of an analytical method is its ability to measure accurately and specifically the analytes in the presence of compounds that may be expected to be present in the sample matrix.

**Determination:**

The specificity of the analytical method was determined by injecting the placebo solution under the same experimental conditions as the assay.

**Preparation of placebo:**

Placebo is prepared by mixing all the excipients without active ingredients.

**Procedure**:

- 225mg placebo was accurately weighed and transferred into a 25ml volumetric flask and the volume was made to 25ml with the mobile phase. The solution was filtered through Millipore filter paper and degassed. 20µl of this solution was injected and chromatogram was recorded and shown in Figure-6

- 200mg Ofloxacin and 500mg Nitazoxanide were weighed and transferred into a 100ml volumetric flask and volume was adjusted to 100ml with mobile phase. Further 5ml of the solution was taken and the volume was made upto 50ml to get a concentration of 200µg/ml Ofloxacin & 500µg/ml Nitazoxanide. To this solution 90mg of placebo was added and it was sonicated, filtered through a Millipore filter paper .20µl of this solution was injected, chromatogram was recorded and shown in Figure.-7. The mixed standard solution was also injected without placebo and it was recorded and shown in Figure -8 and the reports are shown in Table-12 &13

## Table-12

## Specificity for Ofloxacin

| S.No. | Sample | Area obtained |
|---|---|---|
| 1. | Standarad | 10093.2956 |
| 2. | Standard+Placebo | 10039.6397 |
| 3. | Placebo | 0 |

## Table-13

## Specificity for Nitazoxanide

| S.No | Sample | Area obtained |
|---|---|---|
| 1. | Standarad | 6251.4783 |
| 2. | Standard+Placebo | 6200.3386 |
| 3. | Placebo | 0 |

## LINEARITY AND RANGE:

Linearity of an analytical method is its ability to elicit test result that are directly proportional to the concentration of analyte in samples within a given range.

**Determination:**

The linearity of the analytical method was determined by mathematical treatment of test result obtained by analysis of samples with analyte concentrations across the claimed range. Area was plotted graphically as a function of analyte concentration. Percentage curve fitting was calculated.

**Method**:

**Preparation of mixed standard stock solution:**

Accurately weighed 200mg Ofloxacin and 500mg of Nitazoxanide were transferred in to a 100ml of standard flask and it was dissolved with mobile phase and the volume was made upto 100 ml with mobile phase. From the resulting solution,4, 4.5, 5, 5.5, 6ml were transferred into 5 different 50ml volumetric flask. The volume was made with mobile phase to get a final concentration of 160.8, 180.9, 201.0, 221.1, 242.5µg/ml of

ofloxacin 400,450, 500,550, 600µg/ml of Nitazoxanide. 20µl of the resulting solution was injected and chromatogram was recorded.

The chromatograms of ofloxacin and Nitazoxanide are shown in Figure 9 - 13

**Acceptance Criteria**

- Correlation coefficient should not be less than 0.99

The linearity datas and analytical performance parameters of ofloxacin and nitazoxanide are shown in Table-14- 16 and calibration curve are shown in graph – A and B

**Table-14**

**LINEARITY DATA**

**OFLOXACIN**

| S.No. | Concentration(µg/ml) | Peak Area |
|---|---|---|
| 1. | 160.8 | 8248.9069 |
| 2. | 180.9 | 9103.6437 |
| 3. | 201.0 | 10119.4923 |
| 4. | 221.1 | 11246.5845 |
| 5. | 242.5 | 12475.5709 |

**Table-15**

**NITAZOXANIDE**

| S.No. | Concentration(µg/ml) | Peak Area |
|---|---|---|
| 1. | 400 | 5279.7606 |
| 2. | 450 | 5539.6733 |
| 3. | 500 | 5843.0991 |
| 4. | 550 | 6154.6587 |
| 5. | 600 | 6485.6500 |

**Table-16**

**ANALYTICAL PERFORMANCE PARAMETERS**

| S. no. | Drug name | Linear dynamic range(µ/ml) | Correlation coefficient | Slope | Intercept |
|---|---|---|---|---|---|
| 1. | Ofloxacin | (160.8-242.5) | 0.9981 | 52.068 | -237.242 |
| 2. | Nitazoxanide | (400-600) | 0.9990 | 6053.5285 | 2762.8040 |

## ACCURACY

The accuracy of an analytical method is the closeness of the results obtained by that method to the true value. Accuracy may often be expressed as percent recovery by the assay of known added amount of analyte.

**Determination:**

The accuracy of the analytical method was determined by applying the method to the analysed samples to which known amounts of analyte had been added. The accuracy was calculated from the test results as the percentage of analyte recovered by the assay.

**Acceptance criteria:**

Percentage recovery should be within 98-102%

**PROCEDURE:**

Mixed standard stock solution 2ml and sample stock solution 2ml were mixed together in 50 ml volumetric flask and the volume was made upto 50ml with mobile phase to get 80% range. Similarly 100% and 120% range was prepared. 20µl of this solution was injected three times and chromatograms were shown in Fig. 14 to 22 and values in table 17 and 18

## Table-17

## Recovery Study of ofloxacin.

| S.No. | RANGE | Area obtained | Amount Recovered(mg) | % Recovery |
|---|---|---|---|---|
| 1. | 80% | 7620.3121 | 200.2 | 100.11 |
| | | 7590.2365 | 199.4 | 99.72 |
| | | 7602.4132 | 199.7 | 99.88 |
| 2 | 100% | 9546.9764 | 200.6 | 100.33 |
| | | 9558.0072 | 200.8 | 101.42 |
| | | 9566.8988 | 201.1 | 100.55 |
| 3 | 120% | 11272.7721 | 197.4 | 98.73 |
| | | 11524.9356 | 201.8 | 100.94 |
| | | 11289.2563 | 197.7 | 98.88 |

## Table-18

## Recovery Study of Nitazoxanide

| S.No. | RANGE | Area obtained | Amount Recovered(mg) | % Recovery |
|---|---|---|---|---|
| 1. | 80% | 4755.4487 | 501.8 | 100.37 |
| | | 4725.5896 | 498.1 | 99.74 |
| | | 4698.5682 | 495.1 | 99.17 |
| 2 | 100% | 5947.2235 | 502.1 | 100.42 |
| | | 5926.2385 | 500.3 | 100.06 |
| | | 5888.5844 | 497.1 | 99.43 |
| 3 | 120% | 7151.5948 | 503.15 | 100.63 |
| | | 7086.4323 | 503.15 | 99.71 |
| | | 7114.9244 | 500.55 | 100.11 |

## PRECISION

Precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of a homogenous sample. Precision of analytical method is usually expressed as the standard deviation and relative standard deviation.

**Determination:**

The precision of the analytical method was determined by assaying sufficient number of sample and relative standard deviation was calculated.

The precision of the instrument was determined by assaying the samples consecutively, number of time and relative standard deviation was calculated.

**Acceptance Criteria:**

The relative standard deviation should be with in 2%

**SYSTEM PRECISION :**

**Procedure:**

Accurately weighed 200mg of ofloxacin and 500mg of Nitazoxanide were transferred into a 100ml standard flask and it was dissolved with mobile phase. From the resulting solution 5ml was transferred into 50ml volumetric flask. The volume was made up with mobile phase to 50ml.

The system precision was evaluated by measuring 6 successive injection of 20µl of standard solution. The peak response were measured from the chromatogram shown in Figure 23-28 and system precision data area shown in Table-19& 20.

**Method Precision**:

**Procedure**

Twenty tablets were weighed and powdered. Average weight (1.3595) of sample tablet ZENFLOX (equivalent to 200mg of Ofloxacin and 500mg Nitazoxanide hydrochloride) was taken into 100ml dried volumetric flask. The powder was first dissolved in 20ml of mobile phase and sonicated and finally the volume was adjusted to 100ml with mobile phase. From this solution 5ml was transferred to 50ml volumetric flask and volume was adjusted to 50ml with mobile phase to get a concentration of 200µg/ml of Ofloxacin and 500µg/ml of Nitazoxanide. 20µl of the solution was injected and the chromatogram obtained is shown in Figure 29-34 and method precision data are shown in table 21-23

**Table-19**

**System Precision data**

| S.No. | Area of Ofloxacin | Area of nitazoxanide |
|---|---|---|
| 1. | 10039.6397 | 6200.3386 |
| 2. | 10066.1488 | 6344.9496 |
| 3. | 10111.2275 | 6272.8360 |
| 4. | 10093.2956 | 6251.4783 |
| 5. | 10119.4923 | 6256.6252 |
| 6. | 10124.3483 | 6263.4962 |
| MEAN | 10092.3587 | 6264.9539 |
| S.D | 33.4147 | 46.668 |
| %RSD | 0.3310 | 0.7449 |

**Table-20**

| Relative Standard Deviation | ofloxacin | Nitazoxanide | Acceptance Criteria |
|---|---|---|---|
| | 0.3310 | 0.7449 | 2% |

**Table -21 Method Precision of Ofloxacin**

| S.No. | Area Obtained | Assay value in(mg) | % Label claim w/v |
|---|---|---|---|
| 1. | 9536.4994 | 200.4 | 100.23 |
| 2. | 9546.9764 | 200.6 | 100.34 |
| 3. | 9558.0072 | 200.9 | 100.46 |
| 4. | 9566.8988 | 201.0 | 100.50 |
| 5. | 9524.2345 | 200.2 | 101.10 |
| 6. | 9521.9143 | 200.1 | 100.08 |
| | MEAN | | 100.28 |
| | STANDARD DEVIATION | | 0.1784 |
| | RELATIVE STANDARD DEVIAION | | 0.1779 |

**Table-22 Method Precision of Nitazoxanide**

| S.No. | Area Obtained | Assay value in(mg) | % Label claim w/v |
|---|---|---|---|
| 1. | 5910.4990 | 499.0 | 99.80 |
| 2. | 5947.2235 | 502.1 | 100.42 |
| 3. | 5926.2383 | 500.3 | 100.06 |
| 4. | 5888.5844 | 497.1 | 99.43 |
| 5. | 5898.9423 | 498.0 | 99.60 |
| 6. | 5954.9132 | 502.7 | 100.55 |
| | MEAN | | 99.97 |
| | STANDARD DEVIATION | | 0.04075 |
| | RELATIVE STANDARD DEVIAION | | 0.04076 |

**Table-23 Method Precision report for Ofloxacin and Nitazoxanide**

| Relative Standard Deviation | Ofloxacin | Nitazoxanide | Acceptance Criteria |
|---|---|---|---|
| | 0.1779 | 0.04076 | 2% |

**Limit of detection (LOD)**

It is the lowest amount of analyte in a sample that can be detected but not necessarily quantities as an exact value under the stated, experimental conditions. The detection limit is usually expressed as the concentration of analyte.

It is given by

$$L.O.D = \frac{3.3 \times \sigma}{m}$$

σ = standard deviation of the response

m= slope of the calibration curve

**TABLE-24**

**LIMIT OF DETECTION**

| DRUG | STANDARD DEVIATION | SLOPE | L.O.Dμg/ml |
|---|---|---|---|
| OFLOXACIN | 32.1946 | 52.068 | 2.040 |
| NITAZOXANIDE | 0.0791 | 6053.5285 | 0.0000431 |

**Limit of Quantitation:**

The Quantitation limit of an analytical procedure is the lowest amount of analyte which can be Quantitatively determined with suitable Precision and Accuracy.

It is given by

$$L.O.Q = \frac{10x\sigma}{m}$$

σ = standard deviation of the response

m= slope of the calibration curve

**TABLE-25**

**LIMIT OF QUANTITATION**

| DRUG | STANDARD DEVIATION | SLOPE | L.O.Q µg/ml |
|---|---|---|---|
| OFLOXACIN | 32.1946 | 52.068 | 6.183 |
| NITAZOXANIDE | 0.0791 | 6053.5285 | 0.0001306 |

## RUGGEDNESS

The Ruggedness of an analytical method is degree of reproducibility of test result obtained by the analysis of the same sample under a variety of normal test condition, such as different laboratories, different analyst, different instruments, different lots of reagents, different elapsed assay times, different assay temperature, different days, etc.

Ruggedness is normally expressed as the lack of influence on test result of operational and environmental variables of the analytical method.

**Determination:**

The ruggedness of an analytical method was determined by analysis of aliquots from homogeneous lots by different analysts using operational and environmental conditions that may differ but were still with in the specified parameters of the assay. The degree of reproducibility of test result was then determined as a function of the assay variables. This reproducibility was assayed under normal conditions to obtain a measure of the ruggedness of analytical method.

The assay of ofloxacin & Nitazoxanide were performed in different conditions like different analyst on different days.

**Method:**

The standard and sample solutions were prepared by different analysts on different days and the resulting solution were injected and chromatograms are recorded and shown in Figure 35-42 and ruggedness of the method and report of Ofloxacin & Nitazoxanide are shown in Table 26.

**Table-26**

**RUGGEDNESS**

| Analyst | Date | Amount Found | | %purity | |
|---|---|---|---|---|---|
| | | Ofloxacin | Nitazoxanide | Ofloxacin | Nitazoxanide |
| I | 26Nov 2007 | 200.28 | 500.55 | 100.14 | 100.11 |
| II | 26nov 2007 | 198.46 | 497.40 | 99.23 | 99.48 |
| I | 27Nov 2007 | 198.12 | 502.00 | 99.06 | 100.40 |
| II | 27Nov 2007 | 197.50 | 494.60 | 98.50 | 98.92 |

## ROBUSTNESS

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

**Determination:**

The robustness of an analytical method was determined by analysis of aliquots from homogenous lots by differing physical parameters that may differ but were still within the specified parameter of the assay for example change in physical parameters like flow rate and lambda max.

**Method:**

**Standard solution preparation:**

200mg Ofloxacin and 500mg Nitazoxanide were transferred into a 100ml volumetric flask, volume was adjusted to 100 ml with mobile phase. Further 5ml of the solution was taken and the volume was made upto 50ml to get a concentration of 200µg/ml of ofloxacin and 500µg/ml of nitazoxanide.

**Sample preparation:**

Twenty tablets were weighed and powdered. Average weight 1.3595g of sample tablet ZENFLOX (equivalent to 200mg ofloxacin and 500mg ) was taken into 100ml dried volumetric flask. The powder was first dissolved in 20ml of mobile phase, sonicated and finally the volume was adjusted to 100ml with mobile phase. From this solution 5ml was transferred in to 50ml volumetric flask and volume was adjusted to 100ml with mobile phase. 20µl of this solution was injected and individual chromatograms were recorded and they are shown in Figure 43-58 and datas are shown in Table-27-34.

## Table 27

## Chromatographic condition:- change in flow rate (0.4ml/min)

| Change in flow rate | 0.4 ml/min |
|---|---|
| Column | $C_{18}$ |
| Wave length | 280nm |
| Temperature | Ambient25°c |
| Injection Volume | 20µl |

## Table 28

## Change in flow rate (0.4ml/min)

| S.No. | Drug | Average Standard Area | Average Sample Area | % Purity w/v |
|---|---|---|---|---|
| 1. | Ofloxacin | 10079.2339 | 9646.3056 | 101.53% |
| 2. | Nitazoxanide | 6238.1923 | 5953.5489 | 100.74% |

## Table 29

## Chromatographic condition:- change in flow rate (0.6ml/min)

| Change in flow rate | 0.6ml/min |
|---|---|
| Column | $C_{18}$ |
| Wave length | 280nm |
| Temperature | Ambient25°c |
| Injection Volume | 20µl |

## Table 30

## Change in flow rate (0.6ml/Min)

| S.No. | Drug | Average Standard Area | Average Sample Area | % Purity w/v |
|---|---|---|---|---|
| 1. | Ofloxacin | 10059.4621 | 9836.3404 | 101.62% |
| 2. | Nitazoxanide | 6372.9144 | 5980.4439 | 99.05% |

## Table 31

## Chromatographic condition:- change in Lambda Max -282nm

| Column | $C_{18}$ |
|---|---|
| Wave length | 282nm |
| Temperature | Ambient25°c |
| Injection Volume | 20µl |
| Flow rate | 0.5ml/min |

## Table -32

## Change in Lambda Max-282nm

| S.No. | Drug | Average Standard Area | Average Sample Area | % Purity w/v |
|---|---|---|---|---|
| 1. | Ofloxacin | 10083.0568 | 9664.7177 | 101.68% |
| 2. | Nitazoxanide | 6522.9706 | 6120.1818 | 99.04% |

## Table - 33

## Chromatographic condition:- change in Lambda Max -278nm

| Column | $C_{18}$ |
|---|---|
| Wave length | 278nm |
| Temperature | Ambient25°c |
| Injection Volume | 20µl |
| Flow rate | 0.5ml/min |

## Table - 34

## Change in Lambda Max-278nm

| S.No. | Drug | Average Standard Area | Average Sample Area | % Purity w/v |
|---|---|---|---|---|
| 1. | Ofloxacin | 10103.7055 | 9694.6796 | 101.79% |
| 2. | Nitazoxanide | 6604.9728 | 6029.0529 | 101.15% |

## SYSTEM SUITABILITY PARAMETERS

System suitability testing is an integral part of many analytical procedures. The test is based on the concept that the equipment, electronics, analytical operation and sample to be analysed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

**Method:**

A solution of 200µg/ml of ofloxacin and 500µg/ml of Nitazoxanide were prepared by diluting with mobile phase and same was injected and a chromatogram was recorded and they are shown in Figure 59 and system suitability report are shown in the following

**Table - 35**

**System suitability parameters**

| S.No. | Parameters | ofloxacin | nitazoxanide |
|---|---|---|---|
| 1. | Theoretical plates | 12,082.1306 | 8,361.6466 |
| 2. | Tailing factor | 1.00 | 1.00 |
| 3. | Resolution | 6.2 | |

# UV-SPECTROPHOTOMETRIC SIMULTANEOUS EQUATION METHOD OF MONTELUCAST AND THEOPHYLLINE IN COMBINED TABLET DOSAGE FORM

## PRINCIPLE

If a sample contains two absorbing drugs (X and Y) each of which absorbs at the $\lambda_{max}$ of the other. It may be possible to determine the quantity of both drugs by the technique of simultaneous equation (or) Vierodt's method.

Criteria for obtaining maximum precision, based upon absorbance ratios have been suggested that place limits on the relative concentrations of the component of the mixture.

$$\frac{A2/A1}{ax_2/ax_1} \quad \text{and} \quad \frac{ay_1/ay_2}{A_2/A_1}$$

Where

$ax_1$, $ax_2$ = Absorptivities of X at $\lambda_1$ and $\lambda_2$

$ay_1$, $ay_2$ = Absorptivites of Y at $\lambda_1$ and $\lambda_2$

$A_1$, $A_2$ = Absorbances of the diluted Sample at $\lambda_1$ and $\lambda_2$

The ratio should lie outside the range of 0.1–2.0 for the precise determination of (Y and X) two drugs respectively.

These criteria are satisfactory only when the $\lambda_{max}$ of the two components is reasonably dissimilar. The additional criteria includes that two components do not interact chemically, there by negating the initial assumption that the total absorbance is the sum of the individual absorbance's.

## MATERIALS

### I. Market Sample

1. ZOMONT-THEO (Panacea biotech)
2. LABEL CLAIM:

Montelucast - 10mg

Theophylline - 200mg

### II. Equipments Used

- ATCO Balance
- SHIMADZU UV - spectrophotometer double beam digital UV-1700

### III. Solvent Used

- Methanol AR

## FIXATION OF VARIOUS PARAMETERS

**$\lambda_{max}$**

The wavelength at which maximum absorption takes place is called $\lambda_{max}$

**Determination of Absorption Maximum ($\lambda_{max}$) for Montelucast**

**Procedure:**

100mg of authentic montelucast sample was accurately weighed and transferred to 50ml volumetric flask, methanol was added, dissolved and the volume was made upto 50ml with methanol.

5ml of this stock solution was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol.

The absorbance of solution was measured against solvent blank in UV-region of 200-400nm. The $\lambda_{max}$ was found to be 344nm. This spectrum of maximum absorbance is shown in graph-7

## BEER'S LAW PLOT FOR MONTELUCAST

The intensity of a beam of parallel monochromatic radiation decreases exponentially with the number of absorbing molecules. More simply it is stated that the absorbance is proportional to the concentration.

$$\text{Log } \frac{I_o}{I_t} = KC$$

Beer's law plot was constructed by measuring the absorbance of various concentration of drug against solvent blank.

## PROCEDURE

100mg of authentic montelucast sample was accurately weighed and transferred to 50ml volumetric flask , methanol was added, dissolved and the volume was made up to 50ml with methanol.

From this aliquots of 2.5ml, 3ml, 3.5ml,.4ml, 4.5ml, 5ml, 5.5ml, 6ml, 6.5ml, 7.0ml,7.5ml was pipetted out in to separate 100ml volumetric flask. Then the volume was made upto 100ml with methanol. The absorbance of each solution was found out at 344nm against a reagent blank. The readings are presented in Table- 35and graph-8

## Table – 35

## DATA FOR BEER'S LAW PLOT FOR MONTELUCAST (Linearity)

| S.No. | Concentration µg/ml | Absorption |
|---|---|---|
| 1 | 50 | 0.0379 |
| 2 | 60 | 0.0448 |
| 3 | 70 | 0.0519 |
| 4 | 80 | 0.0598 |
| 5 | 90 | 0.0667 |
| 6 | 100 | 0.0742 |
| 7 | 110 | 0.0819 |
| 8 | 120 | 0.0896 |
| 9 | 130 | 0.0968 |
| 10 | 140 | 0.1049 |
| 11 | 150 | 0.1144 |

Linearity Co-efficient ($\gamma$) = 0.9996

Slope (m) = 0.0007566

Intercept (c) = -0.0008

## DEVIATIONS FROM BEER'S LAW

A system is said to obey Beer's law, where a plot of concentration vs absorbance gives a straight line by using a line of best fit. When a straight line is not obtained; the system is said to be deviated from Beer's law.

For the drug montelucast maximum deviation was found in the concentration range above 150µg/ml. The readings are presented in Table -36 and graph-9.

### Table-36

### DATA FOR DEVIATIONS FORM BEER'S LAW PLOT FOR MONTELUCAST

| S.No. | Concentration µg/ml | Absorbance |
|---|---|---|
| 1. | 50 | 0.0379 |
| 2. | 60 | 0.0448 |
| 3. | 70 | 0.0519 |
| 4. | 80 | 0.0598 |
| 5. | 90 | 0.0667 |
| 6. | 100 | 0.0742 |
| 7. | 110 | 0.0819 |
| 8. | 120 | 0.0896 |
| 9. | 130 | 0.0968 |
| 10. | 140 | 0.1049 |
| 11. | 150 | 0.1144 |
| 12. | 160 | 0.1264 |
| 13. | 170 | 0.1278 |

**Determination of Absorption Maximum ($\lambda_{max}$) for Theophylline**

**Procedure:**

100mg of authentic theophylline sample was accurately weighed and transferred to 50ml volumetric flask and methanol was added, dissolved and the volume was made upto 50ml with methanol.

5ml of this stock was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol

The absorbance of solution was measured against solvent bank in UV-region of 200-400 nm. The $\lambda_{max}$ was found to be 289nm. This spectrum of maximum absorbance is shown in graph-10

## BEER'S LAW PLOT FOR THEOPHYLLINE

The intensity of a beam of parallel monochromatic radiation decreases exponentially with the number of absorbing molecules. More simply it is stated that the absorbance is proportional to the concentration.

$$\text{Log } I_0 / I_t = KC$$

Beer's law plot was constructed by measuring the absorbance of various concentration of drug against solvent blank.

## PROCEDURE

100mg of authentic Theophylline sample was accurately weighed and transferred to 50ml volumetric flask and methanol was added dissolved and the volume was made up to 50ml with methanol.

From this aliquots of 5ml, 5.5ml, 6ml, 6.5ml, 7ml, 7.5ml, 8ml, 8.5ml, 9ml, 9.5ml,10ml was pipetted out in to separate 100ml volumetric flask. Then the volume was made upto 100ml with methanol. The absorbance of each solution was found out at 289nm against a reagent blank. The readings are presented in Table-37 and graph-11

## TABLE-37

## DATA FOR BEER'S LAW PLOT FOR THEOPHYLLINE

| S.No. | Concentration µg/ml | Absorbance |
|---|---|---|
| 1. | 100 | **0.1702** |
| 2. | 110 | **0.1872** |
| 3. | 120 | **0.2064** |
| 4. | 130 | **0.2263** |
| 5. | 140 | **0.2382** |
| 6. | 150 | **0.2598** |
| 7. | 160 | **0.2768** |
| 8. | 170 | **0.2949** |
| 9. | 180 | **0.3146** |
| 10. | 190 | **0.3278** |
| 11. | 200 | **0.3488** |

Linearity coefficient $\gamma = 0.9995$

Slope (m) = 0.001778

Intercept (c) =0.0813

## DEVIATIONS FROM BEER'S LAW

A system is said to obey Beer's law, where a plot of concentration vs absorbance gives a straight line by using a line of best fit. When a straight line is not obtained; the system is said to be deviated from Beer's law.

For the drug, Theophylline maximum deviation was found in the concentration range above 200µg/ml. The readings are presented in Table – 38 and graph-12

**Table – 38**

**Data for Deviations from Beer's law plot for Theophylline**

| S.No. | Concentration µg/ml | Absorption |
|---|---|---|
| 1 | 100 | 0.1702 |
| 2 | 110 | 0.1872 |
| 3 | 120 | 0.2064 |
| 4 | 130 | 0.2263 |
| 5 | 140 | 0.2382 |
| 6 | 150 | 0.2598 |
| 7 | 160 | 0.2768 |
| 8 | 170 | 0.2949 |
| 9 | 180 | 0.3146 |
| 10 | 190 | 0.3278 |
| 11 | 200 | 0.3488 |
| 12 | 210 | 0.3524 |
| 13 | 220 | 0.3568 |

**Preparation of montelucast standard solution**

100mg of authentic montelucast sample is accurately weighed and transferred to 50ml volumetric flask and 5ml of methanol was added and shaken until it dissolves and the volume was made upto 50ml with methanol.

From this 5ml was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol to give a concentration of 100µg/ml.

**Preparation of Theophylline standard solution**

100mg of authentic theophylline sample is accurately weighed and transferred to 50ml volumetric flask and 5ml of methanol was added and shaken until it dissolves and the volume was made upto 50ml with methanol.

From this 5ml was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol to give a concentration of 100µg/ml.

The absorbance & absorptivity values are shown in Table- 39a &39b respectively.

**Preparation of sample solution**

Twenty tablets are weighed and average weight was calculated. The tablets are ground to a fine powder. A powder equivalent to 10mg of montelucast and 200mg of theophylline was accurately weighed and transferred to 50ml volumetric flask and 15ml of methanol was added and shaken until it dissolves and the volume was made upto 50ml with methanol. This solution was filtered through whatmann filter paper.

From this 5ml was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol.

The absorbance of each solution was found out at 344nm ($\lambda_{max}$ of montelucast) and 289nm ($\lambda_{max}$ of Theophylline) against a reagent blank. The analysis data are given in table-40

**Calculation:**

$\lambda_1$ = 289nm $\lambda_2$ = 344nm

$ax_1$ and $ax_2$ – Absorptivities of Theophylline at $\lambda_1$ and $\lambda_2$

$ay_1$ and $ay_2$ – Absorptivities of montelucast at $\lambda_1$ and $\lambda_2$

Cx and Cy – Concentration of Theophylline and montelucast of sample respectively in grams / 100 ml.

$A_1$ and $A_2$ – Absorbance of sample at $\lambda_1$ and $\lambda_2$

$$\text{Absorptivity (a)} = A/bc = \frac{\text{Absorbance}}{\text{b x concentration of substance}}$$

Determination of Theophylline

$$Cx = \frac{A_2\, ay_1 - A_1\, ay_2}{ax_2\, ay_1 - ax_1\, ay_2}$$

Determination of montelucast

$$Cy = \frac{A_1\, ax_2 - A_2\, ax_1}{ax_2\, ay_1 - ax_1\, ay_2}$$

**Table-39a**

**Absorbance values for standard and sample**

| Wavelength | Theophylline | montelucast | Sample |
|---|---|---|---|
| 289 $\lambda_1$ Theophylline | 0.0742 (X1) | 0.0246 (y1) | 1.5204 (A1) |
| 344 $\lambda_2$ montelucast | 0.0081 (X 2) | 0.1702 (y2) | 0.3354 (A2) |

**Table-39b**

**Absorptivity values for montelucast and theophylline**

| Parameter | Absorptivity at 289nm | | Absorptivity at 344nm | |
|---|---|---|---|---|
| | Theophylline | Montelucast | Theophylline | Montelucast |
| *Mean | $ax_1$=74.26 | $ay_1$=24.66 | $ax_2$= 8.152 | $ay_2$=170.26 |

Absorptivity values are the mean of six determinations. $ax_1$ and $ax_2$ are absorptivities of Theophylline at 289 nm, and 344nm, respectively; $ay_1$ and $ay_2$ are abxorptivities of Montelucast at 289nm and 344nm respectively.

**CRITERIA FOR OBTAINING MAXIMUM PRECISION**

$(A_2 / A_1)/ (ax_2 / ax_1)$ and $(ay_2 / ay_1)/ (A_2 / A_1)$ was found to be 2.00 & 31.29 respectively. This ratio should lie outside the range 0.1-2.0 for the precise determination.

**Table-40**

**Analysis data of tablet formulations**

| **Parameters** | **UV-spectrophotometry** | |
|---|---|---|
| | **Montelucast** | **Theophylline** |
| Label Claim | 10mg | 200mg |
| Amount found | 9.986 mg | 199.98mg |
| *% Drug content | 99.86 | 199.98 |

*value for % Drug content are mean of five estimations.

## RECOVERY STUDIES

To check the accuracy of the developed method and to study the interference of formulation additives, analytical recovery experiments were carried out by standard addition method at 80, 100 and 120% level. From the total amount of drug found the percentage recovery was calculated. The results are reported in Table-41.

### TABLE-41

### Recovery studies

**Montelucast**

| Range | Amount found | Recovery |
|---|---|---|
| 80% | 9.985 mg | 99.85% |
| 100% | 10.18 mg | 100.09% |
| 120% | 9.982 mg | 99.82% |

**Theophylline**

| Range | Amount found | Recovery |
|---|---|---|
| 80% | 199.98 mg | 99.99% |
| 100% | 200.12mg | 100.06% |
| 120% | 199.98mg | 99.99% |

*Recovery is the mean of three estimations

# SIMULTANEOUS UV SPECTROPHOTOMETRIC ESTIMATION OF MONTELUCAST AND THEOPHYLLINE IN COMBINED TABLET DOSAGE FORM BY ABSORBANCE RATIO METHOD

## PRINCIPLE:

This method depends on the property of a substance which obeys Beer's law at all wavelengths, the ratio of absorbance at any two wavelengths is a constant value independent of concentration or path length. Two different dilutions of the same sample give the same absorbance ratio; this ratio is referred to as a 'Q' value.

## Preparation of Montelucast standard solution

100mg of authentic montelucast sample is accurately weighed and transferred to 50ml volumetric flask and methanol was added and shaken until it dissolves and the volume was made upto 50ml with methanol.

From this 5ml was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol.

**Preparation of Theophylline standard solution**

100mg of authentic theophylline sample is accurately weighed and transferred to 50ml volumetric flask and methanol was added and shaken until it dissolves and the volume was made upto 50ml with methanol.

From this 5ml was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol.

The absorbance & absorptivity values are shown in table-42a & 42b respectively.

**Determination of Iso Absorptive point:**

The standard solutions of Montelucast and Theophylline are scanned over 200-400nm. When the graph was plotted with wavelength against absorbance, the wavelength of equal absorptivity was found at 302nm. The overlain spectra is shown in figure-B

**Preparation of sample solution**

Twenty tablets are weighed and average weight was calculated. The tablets are ground to a fine powder. A powder equivalent to 10 mg Montelucast and 200mg of Theophylline was accurately weighed and transferred to 50ml volumetric flask and methanol was added and shaken until it dissolves and the volume was made upto 50ml with methanol. This solution was filtered through whatmann filter paper.

From this 5ml was pipetted out in to separate 100ml volumetric flask and the volume was made up to 100ml with methanol.

The absorbance of each solution was found out at 302 (Iso Absorptive point) and 344nm ($\lambda_{max}$ of montelucast) against a reagent blank.

The analysis value are given in table-43

**Calculation:**

$\lambda_1$ = 302nm (Iso absorpritive point)

$\lambda_2$ = 344nm ($\lambda_{max}$ of montelucast)

$ax_1$ and $ax_2$ – Absorptivities of theophylline at $\lambda_1$ and $\lambda_2$

$ay_1$ and $ay_2$ – Absorptivities of montelucast at $\lambda_1$ and $\lambda_2$

Cx and Cy – Concentration of theophylline and montelucast (Sample) in grams per 100ml

A1 and A2 – Absorbance of sample at $\lambda_1$ and $\lambda_2$

$$\text{Absorptivity (a)} = A/bc = \frac{\text{Absorbance}}{\text{B x concentration of substance}}$$

Determination of Cx and Cy

$$Cx = \frac{Q_m - Q_y}{Q_x - Q_y} \times \frac{A_1}{Ax_1}$$

$$Cy = \frac{Q_m - Q_x}{Q_y - Q_x} \times \frac{A_1}{Ax_1}$$

Where

$$Q_m = \frac{A_2}{A_1}$$

$$Q_x = \frac{Ax_2}{Ax_1}$$

$$Q_y = \frac{Ay_2}{Ay_1}$$

## Table-42a

## Absorbance values for standard and sample

| Wavelength | Theophylline | Montelucast | Sample |
|---|---|---|---|
| 302<br>$\lambda_1$<br>Iso absorptive point | 0.08014 | 0.08014 | 0.16946 (A1) |
| 344<br>$\lambda_2$<br>Montelucast | 0.17026 | 0.00815 | 0.3354(A2) |

## Table-42b

## Absorptivity values for Montelucast and Theophylline

| Para-meter | Absorptivity at 302nm | | Absorptivity at 344nm | |
|---|---|---|---|---|
| | Theophylline | Montelucast | Theophylline | Montelucast |
| *Mean | ax1=80.14 | ay1=80.14 | ax2= 8.152 | ay2=170.26 |

* Absorptivity values are the mean of six determinations. ax1 and ax2 are absorptivities of theophylline at 302nm, and 344nm, respectively; ay1 and ay2 are absorptivities of montelucast at 302nm and 344nm repectively.

## Table-43

## Analysis data of tablet formulations

| Parameters | UV-spectrophotometry | |
|---|---|---|
| | Montelucast | Theophylline |
| Label Claim | 10mg | 200mg |
| Amount found | 99.80% | 99.94% |
| *% Drug content | 9.982mg | 199.88mg |

*value for % Drug content are mean of five estimations

## RECOVERY STUDIES

To check the accuracy of the developed method and to study the interference of formulation additives, analytical recovery experiments were carried out by standard addition method at 80, 100 and 120% level. From the total amount of drug found the percentage recovery was calculated. The results are reported in Table-10.

**Table-44**

**Recovery studies**

**Montelucast**

| Range | Amount found | Recovery |
|---|---|---|
| 80% | 9.985mg | 99.85% |
| 100% | 10.10mg | 101.00% |
| 120% | 9.86mg | 99.86% |

**Theophylline**

| Range | Amount found | Recovery |
|---|---|---|
| 80% | 199.98mg | 99.99% |
| 100% | 200.20mg | 100.10% |
| 120% | 199.98mg | 99.99% |

*Recovery is the mean of three estimations

# SIMLULTANIOUS ESTIMATION OF MONTELUCAST AND THEOPHYLLINE IN TABLETS BY HPTLC

**1. Selection and pre washing of plate**:

A precoated silica gel 60 $f_{254}$ on aluminium sheet with 100 to 250 micro m thickness was selected for study . Pre washing of plate was done with methanol and then it was activated by keeping in an oven at 115° for 10 mts.

**2. Selection of Solvent:**

Ideal property of a solvents employed fo HPTLC are,

- Drugs should be soluble in the solvent used
- Drug should show stability in the solvent used.
- Drug should be stable.
- Solvent should be volatile.

Accordingly methanol is selected as the solvent of sample for further studies.

**3. Selection of wave length**

UV spectra of montelucast and theophylline was found the λmax of montelucast and theophylline was found to be 289nm and 344 nm. Wave length of 254nm was selected for the study.

**4. Development of Optimum Mobile Phase**:

A solvent system that would give dense compact spots, good separation of both drugs from each other and separation from solvent front and application position was to be selected. Initially different solvent system were tried as below.

| Mobile phase | Observation |
|---|---|
| n-hexane: Methanol:Glacial acetic acid | No good separation |
| Toluene: Acetone:Methanol | No movement of theophylline |
| Chloroform:Ethyl acetate :Methanol | No good separation |
| Ethyl acetate: Methanol | Seperation was poor |
| Toluene:Ethyl acetate :acetone:Formic acid | Both the drugs moves along with solvent front |
| Ethyl acetate:Chloroform:Ethanol:Ammonia | Good separation |

In the above system (except Ethyl acetate: Chloroform: Ethanol: Ammonia) either the analytes ran along with the solvent front or did not run or tailing was absorbed. That is the resolution was very poor.

**Optimization of mobile phase (Ethyl acetate: Chloroform:Ethanol:Ammonia)**

Different ratios of Ethyl acetate:chloroform:ethanol:ammonia were tried in order to achive an optimum resolution with good peak and sharper of both drugs. A ratio of Ethylacetate: Chloroform: Ethanol: Ammonia (6:4:3:1) was selected because it gave compact spots and good resolution between analytes and good separation from solvent front and sample application position. The trail chromatograms for this mobile phase system are shown in the chromatograms.

**Instrument**:

- CAMAG TLC scanner-3
- Millipore filter (10-45/um)
- Whatmann filter paper
- Sonicator.

**Reagents and chemicals**:

Ethyl acetate

Chloroform

Ethanol

Ammonia

**Reference standard:**

Motelucast

Theophylline.

**Sample brand used:**

ZOMONT-THEO (Panacea biotech)

**Label claim:**

Montelucast-10mg

Theophylline -200mg.

**Fixed chromatographic conditions:**

**Instrument** : Camag TLC scanner 3.

**Test plate** : Precoated plates silica gel 60 f254 aluminium.

**Format** : 8x7cm

**Thickness** : 200µm

**Spotting volume** : 2-10µl

**Amount / spot**

Montelucast –0.02-0.1µl

Theophylline – 0.4 -2µl.

**Mobile phase:** Ethylacetate:Chloroform:Ethanol:Ammonia (6:4:3:1)

**Speration technique** : Ascending

**Detection** : UV

**Wavelength**: 254nm.

**Quantitative determination of drugs by using the developed method:**

**Sample**: ZOMONT-THEO (Panacea biotech)

**Label claim**:

Montelucast - 10mg

Theophylline` - 200mg

**Preparation of standard solution:**

Weigh accurately about 10mg of montelucast and 200mg of theophylline into a 100ml volumetric flask. The powder was first dissolved in 25ml of methanol and sonicated for few minutes and filtered through what man filter paper. Finally the volume was adjusted to 100ml with methanol. Form this solution 5ml was transferred to 50ml volumetric flask and the volume was adjusted to 50ml with methanol.

**Preparation of sample solution:**

20 tablets were weighed and powdered .Average weight of (1.142g) sample tablet Zomont-theo (equivalent to 10mg of montelucast and 200mg of theophylline ) was taken into a 100ml volumetric flask, the power was first dissolved in 25ml of methanol and sonicated for few minutes and filtered through whatman filter paper. Finally the volume was adjusted to 100ml with methanol. From this solution 5ml was transferred to 50ml volumetric flask and the volume was adjusted to 50ml with methanol to get concentration of 10mg of montelucast and 200mg of theophylline.

**Method:**

4µl of this standard and sample solution was spotted over the TLC plate and it was placed on the saturated solvent chamber containing a mobile phases(Ethyacetate:Chloroform: Ethanol: Ammonia)(6:4:3:1) system and allowed to develop the solvent.

After the solvent has traveled to a distance of 7cm, the plates were removed form the chamber and dried. The components were detected by examination under UV detection.

After the detection of spots, the TLC plates were placed on the TLC scanner and set the x axis and y axis value by adjusting light of the deuterium lamp fallen just below the spot after the scanning lamp was completed the chromatogram was recorded and it is shown in figure

Amount of drug present in the tablet

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{standard dilution}}{\text{sample dilution}} \times \frac{\text{potency}}{100} \times \text{avg.weight}$$

$$\text{Percentage purity} = \frac{\text{amount present}}{\text{Label claim}} \times 100$$

## Table:45

## Quantitative estimation

| Brand name | content | Label claim | Peak area | Amount present(mg) | Percentage purity%w/w |
|---|---|---|---|---|---|
| ZOMONT-THEO | Montelucast | 10mg | 622.6 | 10.13mg | 101.33 |
| | Theophylline | 200mg | 2375.0 | 199.82mg | 99.91 |

Acceptance criteria:98-102%

**Assay of montelucast:**

$$\frac{622.6}{762.1} \times \frac{11.8}{1.0842} \times \frac{99.8}{100} \times 1.142 \quad 10.13\text{mg}$$

$$\%\text{label claim} = \frac{10.13}{10} \times 100 = 101.33\%$$

**Assay of theophylline:**

$$\frac{2375}{2570.0} \times \frac{205.7}{1.0842} \times \frac{99.87}{100} \times 1.142$$

$$\text{Label claim} = \frac{199.82}{200} \times 100 = 99.91\%$$

# Validation

The word validation means assessment of validity action of proving effectiveness. Validation of an analytical method is a process to establish by laboratory studies that the performance characteristics of the method meet the requirement for the intended analytical application performance. Characteristics are expressed in terms of analytical parameters.

**Design of experiment:**

Typical analytical parameters used in assay validation are:

- linearity and range
- limit of quantitation
- limit of detection
- accuracy
- stability

**Linearity and Range:**

Linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample

**Preparation of montelucast standard solution:**

10mg of standard montelucast was weighed accurately and transferred to 100ml volumetric flask. Add 25ml of methanol to dissolve the drug and the volume is made upto 100ml with menthol.

From the resulting solution 3ml, 4ml, 5ml, 6ml 7ml were transferred into five different 50ml volumetric flask. The volume was made with methanol to get a final concentration of 6, 8 ,10 12, 14µg/ ml. 4µl of the resulting solution was injected and chromatogram was recorded.The chromatogram was shown in figure

**Preparation of theophylline standard solution:**

200mg of standard montelucast was weighed accurately and transferred to 100ml volumetric flask add 25ml of methanol to dissolve the drug. And the volume is made upto 100ml with menthol.

From the resulting solution 4ml, 4.5ml, 5ml, 5.5ml 6ml were transferred into five different 50ml volumetric flask. The volume was made with methanol to get a final concentration of 100mg ,150mg, 200mg,250mg, 300mg. 4µl of the resulting solution was injected and chromatogram was recorded. The chromatogram was shown in figure

**Acceptance criteria:**

Correlation coefficient should not be less than 0.99.The linearity data and analytical performance parameters of monlelucast, and theophylline are shown in table and calibration curve are shown in graph.

**Table-46**

**Linearity data**

**Montelucast**

| S. No. | concentration (mglml) | Area obtained |
|---|---|---|
| 1. | 6 | 624.9 |
| 2. | 8 | 756.3 |
| 3. | 10 | 1078.1 |
| 4. | 12 | 1187.1 |
| 5. | 14 | 1256.3 |

**Table-47**

**Theophylline**

| S.No | concentration mg/ml | area obtained |
|---|---|---|
| 1. | 100 | 769.2 |
| 2. | 150 | 961.9 |
| 3. | 200 | 1154.6 |
| 4. | 250 | 1384.3 |
| 5. | 300 | 1648.9 |

## Table-48

**Analytical Performance Parameters:**

| S. No | Drug name | Linearity range (µg/ml) | Correlation Coefficient(γ) | Slope | Intercept |
|---|---|---|---|---|---|
| 1. | Montelucast | 6-14 | 0.9962 | 79.23 | 310.858 |
| 2. | Theophylline | 100-300 | 0.9974 | 4.3636 | 128.36 |

**Limit of detection(L.O.D)**

It is lowest amount of analyte in a sample that can be detected but not necessarily quantities as an exact value under the stated experimental conditions. The detection limit is usually expressed as the concentration of analyte .

It is given by

$$DL = \left[\frac{3.3\sigma}{S}\right]$$

σ - standard deviation of the response

M - slope of the calibration curve.

**Table-49**

**Limit of detection.**

| Drug | Standard Deviation | Slope | L.O.D mglml |
|---|---|---|---|
| Monlelucast | 3.162 | 79.23 | 0.131 |
| Theophylline. | 79.05 | 4.3636 | 59.782 |

**Limit of quantitation:**

The quantitation limit of an analytical procedure is the lowest amount of analyte which can be quantitatively determined with suitable precision and accuracy.

It is given by.

$$QL = \left[\frac{10\sigma}{S}\right]$$

σ= standard deviation of the response.

M = slope of the calibration curve

**Table-50**

**Limit of quantitation**

| Drug | Standard deviation | Slope | L.O.Q mg/ml |
|---|---|---|---|
| Montelucast | 3.162 | 79.23 | 0.399 |
| Theophylline | 79.05 | 4.3636 | 181.157 |

**ACCURACY:**

The accuracy of an analytical procedure express the closeness of the measured value to the true value for the sample.

Accuracy may often be expressed as percent recovery by the assay of know added amount of analyte.

**Determination:**

The accuracy of the analytical method was determined by applying the method to the analysed samples to which known amounts of acceptance criteria.Percentage recovery should be within 98- 102% w/w.

## TABLE-41

## Recovery studies

### Montelucast

| Range | Amount found | Recovery |
|---|---|---|
| 80% | 10.15 | 101.53% |
| 100% | 10.23 mg | 102.36% |
| 120% | 10.13 mg | 101.33% |

### Theophylline

| Range | Amount found | Recovery |
|---|---|---|
| 80% | 200.22 mg | 100.11% |
| 100% | 197.63mg | 98.81% |
| 120% | 199.82mg | 99.91% |

**Stability studies :**

When the developed chromatographic plate is exposed to atmosphere the analytes are likely to be decomposed hence it is necessary to conduct stability studies. Stability of the analyte on plate was studied at different time intervals and peak areas are were compared with the peak area of freshly scanned plate the developed plate was found to be 2 hours and 3 hours for montelucast and theophylline.

# RESULTS AND DISCUSSION

## Ofloxacin and nitazoxanide:

UV spectrophotometry and reverse phase high performance liquid chromatography were developed for analysing ofloxacin and nitazoxanide in combined tablet dosage form.

For UV spectrophotometry simultaneous equation and absorbance ratio method was carried out. Linearity was obtained in the concentration range of 5 to 50 µg/ml for both drugs. In simultaneous equation method the % Drug content was found to be 99.97% and 100.31% . In absorbance ratio method the % drug content was found to be 100.67% and 100.27%. Recovery experiments were performed and it was within 98 – 102% .

In HPLC method, HPLC conditions were optimized to obtain an adequate separation of eluted compounds. Initially various mobile phase were tried, to separate drugs. Mobile phase and flow rate selection was based on peak parameters (height, tailing, theoretical plates, etc). The system with buffer (Tetra butyl ammonium hydrogen sulphate): methanol Acetonitrile (20:20:60:v/v) with 0.5 ml/min flow rate is quite robust. The optimum wavelength for detection was 280nm at which better detector response for drugs was obtained. The average retention times for ofloxacin and nitazoxanide is 4.67min and 7.77 min respectively.

According to USP system suitability test are an integral part of chromatographic method. They are used to verify the reproducibility of the chromatographic system. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared stock solution. The parameters are shown in table 41.

The calibration curve was found to be linear for both ofloxacin & Nitazoxanide.The percentage purity was 100.20 %and 99.94% for ofloxacin and nitazoxanide respectively.

The mean recoveries were found to be in the range of 98% to 102%.

Limit of detection for ofloxacin and nitazoxanide was found to be 2.040 µg/ml & 0.0000431µg/ml respectively.

Limit of quantitation for ofloxacin & nitazoxanide was found to be 6.183 µg/ml & 0.0001306 µg/ml respectively.

Robustness of the proposed method was determined by changing the wavelength and flow rate.

Ruggedness of proposed method was determined by analysis of aliquots from homogenous slot by different analyst in different days using similar operational environmental condition. The results were within 98-102%.

**Montelucast and Theophylline**:

Montelucast and theophylline in combined dosage form was estimated by UV spectrophotometry and high performance thin layer chromatography.

In UV both simultaneous and absorbance ratio method was carried out. Linearity was obtained in the concentration range of 50 µg/ml to 150 µg/ml for montelucast and 100 µg/ml to 200 µg/ml for theophylline.

The percentage drug content was found to 99.86% and 99.99% for montelucast and theophylline respectively using simultaneous equation method.

The percentage drug content using absorbance ratio method was found to be 99.94 and 99.80 for montelucast and theophylline respectively.

The recovery experiments were also performed for both method which was within the range of 98-102%.

In HPTLC method, HPTLC conditions were optimized to obtain an adequate separation of eluted compounds. Initially various mobile phase were tried, to separate drugs. Mobile phase was selected depending upon the separation of the combined drugs. The Mobile phase of Ethyl acetate:chloroform:ethanol:ammonia(6:4:3:1) was selected. The optimum wavelength for detection was 254nm at which better detector response for

drugs was obtained. The average Rf values for montelucast and theophylline and  min respectively.

The calibration curve was found to be linear for both montelucast & theophylline.The percentage purity was 101.33% and 99.91% for montelucast and theophylline respectively.

The mean recoveries were found to be in the range of 98% to 102%.

Accuracy was found at 80%, 100%, 120% level.They were found to be in the range of 98-102% level.

Limit of detection for montelucast and theophylline was found to be 0.131 µg/ml &59.78 µg/ml respectively.

Limit of quantitation for montelucast & theophylline was found to be 0.399 µg/ml & 181.75 µg/ml respectively.

Stability studies was also carried out to check the stability of the plates and decomposition of the drugs after a specified time.

# SUMMARY AND CONCLUSION

Ofloxacin and Nitazoxanide available in combined tablet dosage form were analysed by UV–spectrophotometric simultaneous equation and absorbance ratio method and reverse phase high performance liquid chromatography.

On comparing these methods reverse phase high performance liquid chromatography was found to be more accurate, simple, and rapid than UV spectrophotometric\methods.

Montelucast and theophylline in combined tablet dosage form was estimated using High performance thin layer chromatography and UV-spectophotometry employing simultaneous equation method and absorbance ratio method.

On comparing these methods High performance thin layer chromatography was found to be more accurate ,simple and rapid than UV spectrophotometry methods.

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