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A NEW VALIDATED RP-HPLC METHOD FOR DETERMINATION OF OFLOXACIN AND FLAVOXATE HYDROCHLORIDE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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Abstract:

In the present research work gastro retentive floating matrix formulation of ketoconazole by using various polymers were developed. Then the formulation was developed by using different concentrations of polymers of guar gum, carbopol 934 and HPMC K100 M as polymeric substances. The formulation blend was subjected to various preformualation studies, flow properties and all the formulations were found to be good indicating that the powder blend has good flow properties. Among all the formulations the formulations HPMC K100 M as polymer were retarded the drug release up to desired time period i.e., 12 hours in the concentration of 80 mg. (F8 Formulation, 99.78% Drug release). The optimized formulation dissolution data was subjected to release kinetics; from the release kinetics data it was evident that the formulation followed peppas release kinetics.

Keywords: *Ketoconazole, guar gum, carbopol 934, HPMC K100 M, and Floating tablets.*

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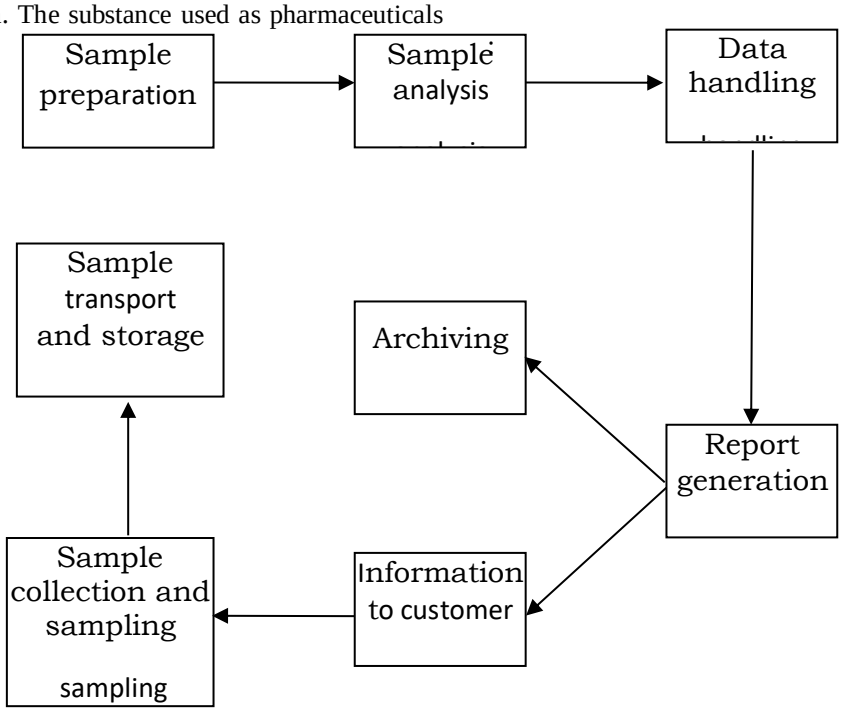
INTRODUCTION:

Pharmaceutical analysis is a branch of practical chemistry that involves a series of process for identification, determination, quantification and purification of a substance, separation of the components of a solution or mixture, or determination of structure of chemical compounds. The substance may be a single compound or a mixture of compounds and it may be in any of the dosage form. The substance used as pharmaceuticals are animals, plants, microorganisms, minerals and various synthetic products.

Analytical chemistry is the science that seeks ever improved means of measuring the chemical composition of natural and artificial materials.

The major stages of an analytical process are described as follows:[1]



Steps in analytical cycle

To be effective and efficient, analyzing samples requires expertise in:

1. The chemistry that can occur in a sample
2. The total amount of sample available
3. Concentration range of analyte
4. Analysis and sample handling methods for a wide variety of problems (the tools-of-the-trade)
5. Accuracy and precision of the method
6. Proper data analysis and record keeping

Analytical chemistry is the science of obtaining, processing, and communicating information about the composition and structure of matter. In other words, it is the art and science of determining what matter is and how much it exists. Analytical chemistry also is concerned with developing the tools used to examine chemical compositions. It is concerned with the chemical characterization of matter both qualitatively and quantitatively. Qualitative analysis gives an indication of the identity of the chemical species in the sample and quantitative analysis determines the amount of one or more of these components.

Analytical methods can be separated into classical and instrumental. Classical methods use separation techniques such as precipitation, extraction, and distillation and qualitative analysis by color, odor, or melting point.

The quantitative analysis is achieved by measurement of weight or volume. Instrumental methods use an apparatus to measure physical qualities of the analyte such as light absorption, fluorescence, or conductivity. The separation of materials is accomplished by using chromatography or electrophoresis methods. [2]

Common Techniques for analysis

For analysis it is useful to consider chemical and physical characteristics that are useful for qualitative or quantitative analysis and thus analysis can be divided into:-

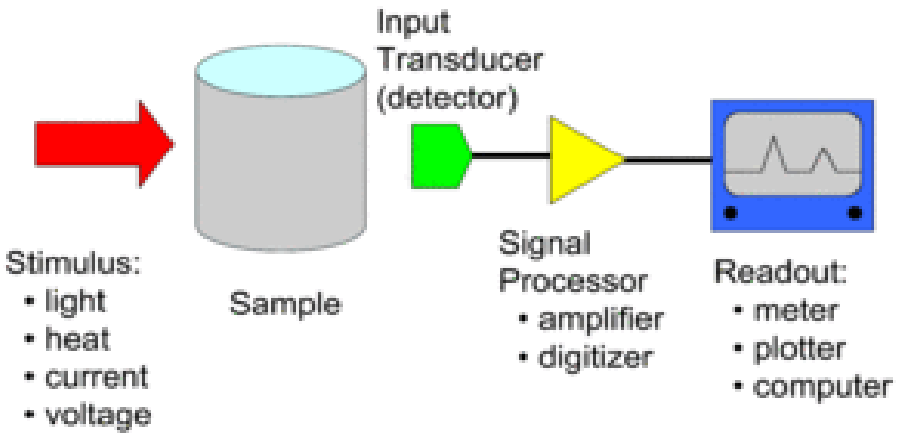
Qualitative analysis: It is performed to establish composition of natural/synthetic substances. These tests are performed to indicate whether the substance or compound is present in the sample or not. Various qualitative tests are detection of evolved gas, formation of precipitates, limit tests, colour change reactions, melting point and boiling point test etc.[3]

Quantitative analysis: These techniques are mainly used to quantify any compound or substance in the sample. These techniques are based in (a) the quantitative performance of suitable chemical reaction and either measuring the amount of reagent added to complete the reaction or measuring the amount of reaction product obtained.

(b) The characteristic movement of a substance through a defined medium under controlled conditions, (c) electrical measurement, (d) measurement of some spectroscopic properties of the compound.

S.no	Physical property measured	Instrumental method based on the measurement of property
1	Absorption of Radiation	Spectrophotometry (X-ray, UV, Visible, IR): Colorimetry, Atomic absorption, NMR.
2	Emission of Radiation	Emission spectroscopy(X-ray, UV, Visible, IR): Flame photometry, Fluorescence (X-ray, UV, Visible)
3	Scattering of Radiation	Turbidimetry, Nephelometry
4	Refraction of Radiation	Refractometry
5	Diffraction of Radiation	X-ray, electron Diffraction methods.
6	Rotation of Radiation	Polarimetry
7	Electrical Potential	Potentiometry
8	Electrical Conductance	Conductometry
9	Electrical Current	Polarography, Amperometric titrations
10	Mass-to-charge ratio	Mass spectrometry

Instrumental Analysis



Block diagram of an analytical instrument showing the stimulus and measurement of response

UV-Spectrophotometry

Spectroscopy is the measurement and interpretation of electromagnetic radiation absorbed or emitted when the molecules or atoms or ions of a sample move from one energy state to another energy state.[4]

Spectroscopy is a general methodology that can be adapted in many ways to extract the information you need (energies of electronic, vibrational, rotational states, structure and symmetry of molecules, dynamic information).

Ultraviolet-Visible Spectrophotometry is one of the most frequently employed techniques in Pharmaceutical analysis.

It involves the measurement of the amount of Ultraviolet (190-380nm) radiation by a substance in a solution.

A compound or drug which possess conjugated double bond absorbs UV radiation at a specific wavelength and this character of the drug is specific for a fixed solvent system. The wavelength at which maximum absorption occurs is called λmax. It is independent of concentration. For a drug to be measured by the ultraviolet analytical method, it should follow the Beer's-Lambert's law. The concentration of an analyte in solution can be determined by measuring the absorbance at some wavelength and applying the Beer-Lambert Law

The Beer-Lambert Law states the amount of intensity of light when passing through any sample decreases exponentially with increase in thickness of the sample and the concentration of the medium. [5]

Lambert Beer's law is a mathematical means of expressing how light is absorbed by matter.

This relationship may be expressed as:

$$A = \varepsilon dc$$

A= absorbance

ε = molar extinction coefficient

d = Path length in cm

c = molar concentration

Spectrophotometric assay

Assay of single component sample

a) Single standard (or) Double-point standardization

It involves the measurement of the absorbance of a sample solution and of a standard solution of the reference substance. The concentration of the substance in the sample is calculated from the proportional relationship that exists between absorbance and concentration.

$$C_{test} = \frac{A_{test} \times C_{std}}{A_{std}}$$

C_{std} = Concentration of the standard solution

C_{test} = Concentration of the sample

A_{test} and A_{std} = Absorbances of the sample and standard solutions.

b) Calibration graph method[6]

In this procedure the absorbances of a number of standard solutions of the reference substance at concentrations encompassing the sample concentrations are measured and a calibration graph is constructed. The concentration of the analyte in the sample solution is read from the graph as the concentration corresponding to the absorbance of the solution.

Assay of multi-component samples:

The assay of components of mixture sample can be done by following methods:

1) Simultaneous Equation method
2) Absorbance Ratio method
3) Geometric Correction method
4) Difference spectrophotometry
5) Derivative spectrophotometry

The basis of all the spectrophotometric techniques for multicomponent samples is the property that at all wavelengths,

a) The absorbance of a solution is the sum of absorbances f the individual components or

b) The measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference (blank) cell.

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 both drugs by the technique of simultaneous equation.

The information required is:

a) The absorptivities of X at λ_1 and λ_2, ax_1 and ax_2 respectively.

b) The absorptivities of Y at λ_1 and λ_2, ay_1 and ay_2 respectively.
c) The absorbances of the diluted sample at λ_1 and λ_2, A_1 and A_2 respectively.
d) Let C_x and C_y be the concentrations of X and Y respectively in the diluted sample.

Two equation are constructed based upon the fact that the sum of individual absorbance of X and Y.

$$\text{At } \lambda_1,\ A_1 = ax_1bc_x + ay_1bc_y$$
$$\text{At } \lambda_2,\ A_2 = ax_2bc_x + ay_2bc_y$$

$$C_x = \frac{A_2ay_1 - A_1ay_2}{ax_2ay_1 - ax_1ay_2}$$

$$C_y = \frac{A_1ax_2 - A_2ax_1}{ax_2ay_1 - ax_1ay_2}$$

Using the above equations, concentrations of individual components in a mixture can be determined. [7]

Difference spectrophotometry

The selectivity and accuracy of spectrophotometric analysis of samples containing absorbing interferents may be markedly improved by the technique of difference spectrophotometry.

The essential feature of a difference spectrophotometric assay is that the measured value is the difference absorbance (ΔA) between two equimolar solutions of the analyte in different spectral characteristics.

The criteria for applying spectrophotometry to the assay of a substance in the presence of other absorbing substances are that:-

a) Reproducible changes may be induced in the spectrum of the analyte by the addition of one or more reagents.
b) The absorbance of the interfering substances is not altered by the reagents.

Derivative Spectrophotometry

Derivative Spectrophotometry involves the conversion of a normal spectrum to its first, second or higher derivative spectrum. The transformations that occur in the derivative spectra are understood by the reference to a Gaussian band which represents an ideal absorption band.

The first derivative (D^1) spectrum is a plot of the rate of change of absorbance with wavelength against wavelength i.e. a plot of the slope of fundamental spectrum against wavelength or a plot of $\delta A\ \delta\lambda\ _{Vs}\ \lambda$.

The second derivative (D^2) spectrum is a plot of the curvature of the D^0 (Zero order spectrum) against wavelength or a plot of $\delta^2 A\ \delta^2\lambda\ _{Vs}\ \lambda$.

The spectral transformations confer two principle advantages on derivative spectrophotometry.

1) An even order spectrum is of narrow spectral bandwidth than its fundamental spectrum.
2) Derivative spectrophotometry discriminates in favour of substance of narrow spectral bandwidth against broad bandwidth substances.

Hence substance of narrow spectral bandwidth displays larger derivatives, amplitudes than those of broad bandwidth substances.

Absorbance Ratio Method

The absorbance ratio method is a modification of the simultaneous equations procedure. It depends on the property that, for a substance which obeys Beers law at all wavelength, the ratio of absorbances at any two wavelengths is a constant value independent of concentration or path length.

Here absorbances are measured at two wavelengths one being the λ of one of the components (λ_2) and the other being a wavelength of equal absorptivity of the two components (λ_1), i.e. an iso-absorptive point.

High-performance Liquid Chromatography [8]

Chromatography is the separation of a mixture into individual components using a stationary and mobile phase. Early liquid chromatography was carried out in glass columns with diameters of 1 to 5 cm and lengths of 50 to 500 cm. The average diameter of the solid stationary phase particles was usually in the 100 to 200 micron range. Recent technology has allowed for the development of packing material with relatively small particle size diameter (3-10 micron). This technology resulted in the development of columns with very high efficiencies, and consequently has involved the use of more sophisticated instrumentation to perform at increased pressure and flows; Hence the term High Performance Liquid chromatography (HPLC).

HPLC is a type of liquid chromatography that employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rate liquid must be pressurized to a few thousands of pounds per square inch.

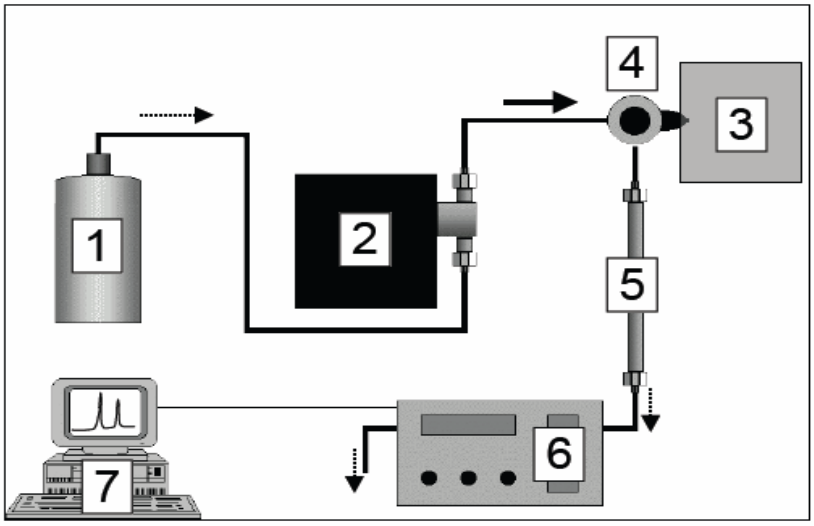
The rate of distribution of drugs between Stationary and mobile phase is controlled by diffusion process. If diffusion is minimized faster and effective separation can be achieved.

The techniques of high performance liquid chromatography are so called because of its improved performance when compared to classical column chromatography advances in column chromatography into high speed, efficient, accurate and highly resolved method of separation.

For the recent study Flavoxate and ofloxacin was selected for estimation of amount of analyte present in formulation and bulk drug. The HPLC method is selected in the field of analytical chemistry, since this method is specific, robust, linear, precise and accurate and the limit of detection is low and also it offers the following advantages

- Speed many analysis can be accomplished in 20min (or) less.
- Greater sensitivity (various detectors can be employed).
- Improved resolution (wide variety of stationary phases).
- Re usable columns (expensive columns but can be used for many analysis).
- Ideal for the substances of low viscosity.
- Easy sample recovery, handling and maintenance.
- Instrumentation leads itself to automation and quantification (less time and less labour).
- Precise and reproducible.
- Integrator itself does calculations.
- Suitable for preparative liquid chromatography on a much larger scale

HPLC Basic Instrumentation [9]



1 – Reservoir
2 – Pump
3 – Auto Sampler
4 – Injection Valve
5 – Column
6 – Detector
7 – Data System

Schematic diagram of a basic HPLC system

HPLC components

The essential components of a complete HPLC system are solvent delivery system (Pump), detector, fixed volume injector loop or autosampler, solvent reservoirs, packed column, data system and recorder. A schematic of a simplified HPLC system is shown in Figure 1.

Column

The column is probably the heart of HPLC system. The development of this column technology leads to the evolution of the HPLC instrumentation systems used today. The conventionally used HPLC columns are particle packed columns. The key of column selection when previous separation is not available resides in knowing the chemistry of the sample.

Columns should never be dry. A dry column will eventually have voids because the packing will shrink away from the wall, which would result in band broadening. Before running a sample in HPLC the column should be equilibrated. Usually column equilibrium is achieved after passage of 10 – 20 column volumes of the new mobile phase through the column. Insufficient column equilibrium usually leads to retention difference.

Pump

The solvent delivery system or as it is commonly called the pump includes two major types, constant volume or flow and constant pressure. Constant volume pumps are mechanically driven systems, most commonly using screw driven syringes or reciprocating pistons. On the other hand, constant pressure pumps are driven or controlled by gas pressure.

Injector or Auto sampler

Samples are usually introduced by syringe injection via a manual injector into the mobile phase stream or by the use of an auto sampler. The important aspects in sample introduction are precise and reproducible injections. This is especially important with quantitative analysis where the reproducibility of the peak response is dependant on the precision of the sample introduction.

Direct syringe injection through a manual injector was the first popular method of sample introduction. As HPLC instrumentation evolved, many auto sampler techniques were applied so that sample introduction has become more precise and rapid.

Detector

HPLC detectors include ultraviolet-visible, fluorescence, electrochemical, refractometer, mass spectrometer and others. The UV visible absorption detector is the most widely used detector in liquid chromatography, since most organic compounds show some useful absorption in the UV region. This detector is fairly universal in application, although sensitivity depends on how strongly the sample absorbs light at a particular wavelength.

Solvent reservoir

Different containers are used as a solvent delivery system reservoir. The best material from which the containers are made is glass. Plastic containers are not recommended as it leads to plasticizer leaching. The container should be covered to prevent solvent evaporation. The tubing from the reservoir can be made of stainless steel or Teflon, and both are satisfactory.

Data handling and analysis

Data handling in HPLC is as important to the success of any experiment or analysis as any other components in the system. It is part of good HPLC techniques to properly label and document the analytical results. The advanced computer softwares used now in data handling and analysis allow easy recording and storage of all chromatographic data.

Types of HPLC techniques

Based on modes of chromatography

- Normal phase chromatography
- Reverse phase chromatography

Based on principle of separation:

- Adsorption chromatography
- Ion exchange chromatography
- Size exclusion chromatography
- Affinity chromatography
- Chiral phase chromatography

Based on elution technique

- Isocratic separation
- Gradient separation

Based on the scale of operation

- Analytical HPLC
- Preparative HPLC

Normal phase chromatography[10]

In normal phase mode the stationary base (eg; silica gel) is polar in nature and the mobile phase is non polar. In this technique, non polar compound travel faster and are eluted first. This is because less affinity between solute and stationary phase and take more time to elute.

Reverse phase chromatography

The popularity of reversed phase liquid chromatography is easily explained by its unmatched simplicity, versatility and scope. Neutral and ionic analytes can be separated simultaneously. Retention in RPLC is believed to occur through nonspecific hydrophobic interaction of the solute with the stationary phase. The near universal application of RPLC stems from the fact that almost all organic compounds have hydrophobic regions in their structure and are capable of interacting with the stationary phase.

A decrease in the polarity of the mobile phase leads to a decrease in retention.

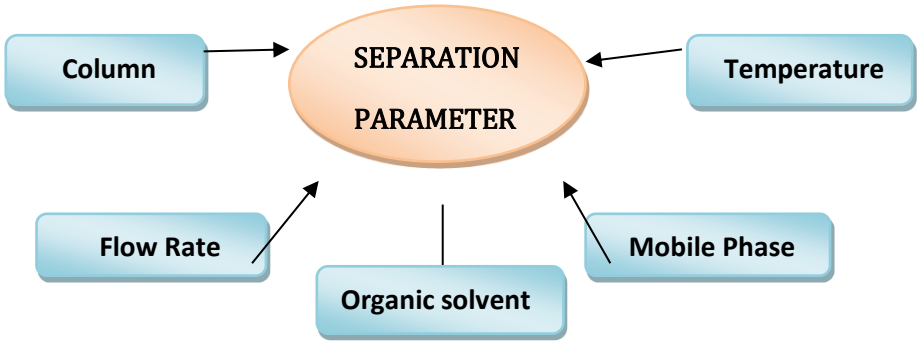
It is also generally observed in RPLC that branched chain compounds are retained to a lesser extent than their straight chain analogues and that unsaturated compounds are eluted before their fully saturated analogs.A wide variety of RP-HPLC columns are available. Most columns are silica based. Silica offers good mechanical stability.

A typical stationary phase is formed by chemically bonding a long-chain hydrocarbon group to porous silica. Typical ligands are n-octadecyl (C18), n-octayl (C8), n-butyl (C4), diphenyl (C2) and cyano propyl.

Parameters affecting separation [11]

Separation in reversed phase chromatography is affected by stationary phase type and column length. It is also affected by organic solvent type and percentage in the mobile phase and by mobile phase pH. Flow rate could also affect separation in reversed phase chromatography; however it is usually limited by the developed backpressure. Moreover temperature of the column also has an effect on separation.

Parameters affecting separation in reversed phase chromatography are shown



Quantitative Methods in chromatography

Internal standard method

In this technique a known quantity internal standard is chromatographed and area is ascertained then a quantity of the internal standard is added to the raw sample prior to any sample pretreatment or separation operation. The peak area of the standard in sample run is compared with the peak are when the standard is run separately This ratio serves as correction factor for variation in sample size for losses in any preliminary operations, or for incomplete resolved adjacent sample component, must not interfere with the sample component and must never be present in sample.

$$\text{Area ratio} = \frac{\text{Area of sample}}{\text{Area of internal standard}}$$

$$\text{Sample concentration} = \frac{\text{Area of sample}}{\text{Area of internal standard}} \times \text{concentration of standard}$$

This technique is often used for the samples having components. It is used to evaluate the absolute purity of sample .the procedure is to total up the areas under all peaks and then calculate the percentage of total area that is contributed by compound of interest. For this method the entire sample must be eluted all components must be separated and peak must be completely resolved.

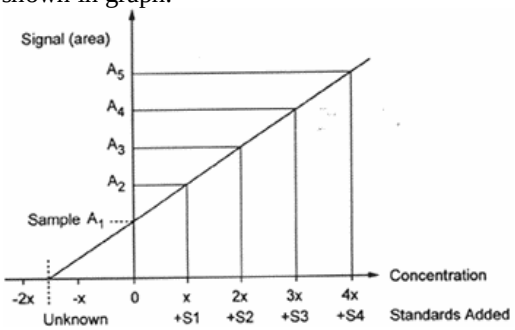
Standard addition method [12]

Standard addition method is used in many techniques in analytical chemistry. It is of limited use in chromatography because of the difficulty of injecting accurately known amounts of sample. A sample mixture is analysed for the analyte of interest by adding a specified amount of this analyte to the sample, thus increasing its concentration. The analysis is then repeated and the resulting increase in peak area due to addition of the standard amount is noted. Hence, the concentration of the analyte in the original sample may be calculated. If the peak area for the first analysis is A1 and with the standard addition of x mg is A2, then the peak area corresponding to x mg (or x mg/litre) is (A2 - A1). Thus, the original amount of the analyte x in the sample corresponding to A1, is given by

Amount x = (x A1) / (A2 - A1) mg/litre.

An allowance for dilution due to addition of the standard amount has to be made. The main difficulty with this method concerns the reproducibility of the sample injection. A precision of better than 1 % should be achieved if valid quantitative results are to be obtained.An alternative approach is first to analyse the sample, noting the area, A1, for the analyte. Successive standard amounts of the analyte are thenadded, each sample standard mixture being analysed and the areas recorded.

A graph of peak area versus concentration is drawn and the amount of analyte in the sample obtained by extending the calibration lineto intersects the abscissa as shown in graph.



External standard method [13]

Automated sample injection systems and multiport injection valves (HPLC) have good reproducibility so that a series of injections can be made with a variation in sample volume of < 1 %. A set of standard mixtures containing known concentrations

of the analytes is analysed and their peak areas recorded. A calibration graph of area versus concentration can be drawn for each analyte to confirm a linear detector response and from which the amount of the analyte in a mixture can be determined. Alternatively for an established method a replicate series of one standard mixture is injected and the area/unit amount of analyte calculated.ASTANDARD = xmg/litre.

The mixture is then analysed and the amount of the components in the sample calculated using the peak area data for the standard mixture.Therefore, if the recorded peak area for the component in a sample mixture is AMIX then the amount of component x is
Amount x = (x AMIX) / (ASTANDARD) mg/litre.

Parameters used in HPLC

Retention time (Rt)

Retention time is the difference in time between the point of injection and appearance of peak maxima .Retention time is the time required for 50% of a component to be eluted from a column Retention time is measured in minutes or seconds retention time is also proportional to the distance moved on a chart paper, which can be measured in cm.

Retention volume (Vr):

Retention volume is the volume of mobile phase required to elute 50% of the component from the column .It is the product of retention time and flow rate.

Retention volume = Retention time * flow rate

Tailing factor (T)

The accuracy of quantification decreases with increase in peak tailing as the integrator encounters difficulty in determining where and when the peak ends. Hence, the calculation of the area under the peak differs with tailing. If the integrator is unable to determine exactly when an up slope or down slope occurs, accuracy drops.

$$T = W_x / 2f$$

Recommendations: T of < 1.5 is preferred.
Resolution (Rs):

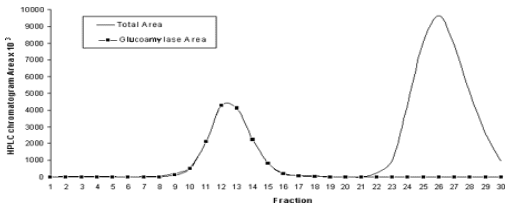
Resolutionn is the measure of extent of separation of two component and the baseline separation achieved. Resolution is generally defined as the distance between the centers of two eluting peaks as measured by retention time or volume divided by average width of respective peaks. for example an Rs value of 1.0 indicates 98% purity has been achieved base line resolution between two well-formed peaks indicates 100% purity and requires an Rs value greater than 1.5 resolution can be determined by using the following formula

$$Rs = \frac{2(Rt1 - Rt2)}{W1+W2}$$

Calculating Rs is the simplified method for quantitating the actual separation achieved between two solute molecules the parameters that contribute to peak resolution column selectivity,column efficiency and the column retention time

$$Rs = 1/4 * (\alpha-1) * (\sqrt{N}) * \frac{k'}{1 + k'}$$

α=Separation Factor, k'=capacity factor, N=Column Plate Number.
Resolution Rs is a function of selectivity, efficiency (number of theoretical plates N and average retention factor K for peaks 1and2.



Chromatogram
Capacity factor [14]
The capacity factor is related to the retention time and is a reflection of the proportion of time a particular solute resides in the stationary phase as opposed to the mobile phase. long retention times result in large values of K^1.
The Capacity Factor is not same as the available binding capacity ,which refers to the mass of the solute that a specified amount of medium is capable of binding under defined conditions the capacity factor K^1can be calculated.

Capacity Factor = Moles of solute in stationary phase
Moles of solute in mobile phase

$$K^1 = \frac{Tr - To}{To} * \frac{Vr - Vo}{Vo}$$

Tr & To are retention times Vr & Vo are retention volumes respectively
Recommendations:

The peak should be well resolved from other peaks and the void volume. Generally the value of

k' is >2.

Efficiency (N): The efficiency of packed column is expressed by number of theoretical plates N. It is a measure of the band spreading of a peak smaller the band spread higher the theoretical plates indicates good column and system performance. Theoretical plates is an imaginary or hypothetical unit of a column where equilibrium has been established between stationary based on mobile phase.

The greater the number of theoretical plates a column has greater its efficiency and corresponding the higher the resolution which can be achieved.

The column plate's number increases with several factors;

1. Well packed columns
2. Longer columns
3. Lower flow rates
4. Smaller column packing particles
5. Lower mobile phase viscosity and higher temperature
6. Smaller samples molecules

The column plates number N is defined by

$$N = 16(Rt/W)^2$$

N = Number of theoretical plates
Rt =Retention time, W=Peak width at base

Manual measurement of baseline bandwidth may be subject to error .Therefore a more practical equation for N is

$$N = 5.54(Rt/W_{1/2})^2 = L/H$$

$W_{1/2}$=Band width at half height,

Selectivity

Selectivity is equivalent to relative retention time of the solute peaks and unlike efficiency depends strongly on the chemical properties of the chromatography medium.

Analytical Method Development

Method development is done

a. For new products
b. For existing products

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are developed to reduce the cost and time for better precision and ruggedness. Trial runs are conducted, method is optimized and validated. When alternate method proposed is intended to replace the existing procedure comparative laboratory data including merit/demerits are made available.

Steps of method development

Documentation starts at the very beginning of the development process, a system for full documentation of the development studies must be established. All data relating to these studies must be recorded in laboratory notebook or an electronic database. Analyte standard characterization.

1. All known information about the analyte and its structure is collected i.e., physical and chemical properties, toxicity, purity, hygroscopic nature, solubility and stability.
2. The standard analyte (100% purity) is obtained. Necessary arrangement is made for the proper storage (refrigerator, desiccators, and freezer).
3. When multiple components are to be analyzed in the sample matrix, the number of components is noted, data is assembled and the availability of standards for each one is determined.
4. Only those methods (MS, GC, HPLC etc.,) that are compatible with sample stability are considered.

Method Requirements [15]

The goals or requirements of the analytical method that need to be developed are considered and the analytical figures of merit are defined. The required detection limits, selectivity, linearity, range, accuracy and precision are defined. Literature search and prior methodology The literature for all types of information related to the analyte is surveyed. For synthesis, physical and chemical properties, solubility and relevant analytical methods. Books, periodicals, chemical manufacturers and regulatory agency compendia such as USP / NF, Association of Official Analytical Chemists (AOAC) and American Society for Testing and Materials (ASTM) publications are reviewed. Chemical Abstracts Service (CAS) automated computerized literature searches are convenient. Choosing a method

a) Using the information in the literatures and prints, methodology is adapted. The methods are modified wherever necessary. Sometimes it is necessary to acquire additional instrumentation to reproduce, modify, improve or validate existing methods for in-house analytes and samples.
b) If there is no prior method for the analyte in the literature, from analogy, the compounds that are similar in structure and chemical properties are investigated and are worked out. There is usually one compound for which analytical method already exist that is similar to the analyte of interest.

Instrumental setup and initial studies

a) The required instrumentation is setup. Installation, operational and performance

qualification of instrumentation using laboratory standard operating procedures (SOP's) are verified.

b) Always new consumables (e.g. solvents, filters and gases) are used, for example, method development is never started, on a HPLC column that has been used earlier.

c) The analyte standard in a suitable injection / introduction solution and in known concentrations and solvents are prepared. It is important to start with an authentic, known standard rather than with a complex sample matrix.

d) If the sample is extremely close to the standard (e.g., bulk drug), then it is possible to start work with the actual sample.

e) Analysis is done using analytical conditions described in the existing literature.

Optimization

During optimization one parameter is changed at a time, and set of conditions are isolated, rather than using a trial and error approach. Work has been done from an organized methodical plan and every step is documented (in a lab notebook) in case of dead ends.

Documentation of analytical figures of merit

The originally determined analytical figures of merit Limit of quantitation (LOQ), Limit of detection (LOD), linearity, time per analysis, cost, sample preparation etc., are documented.

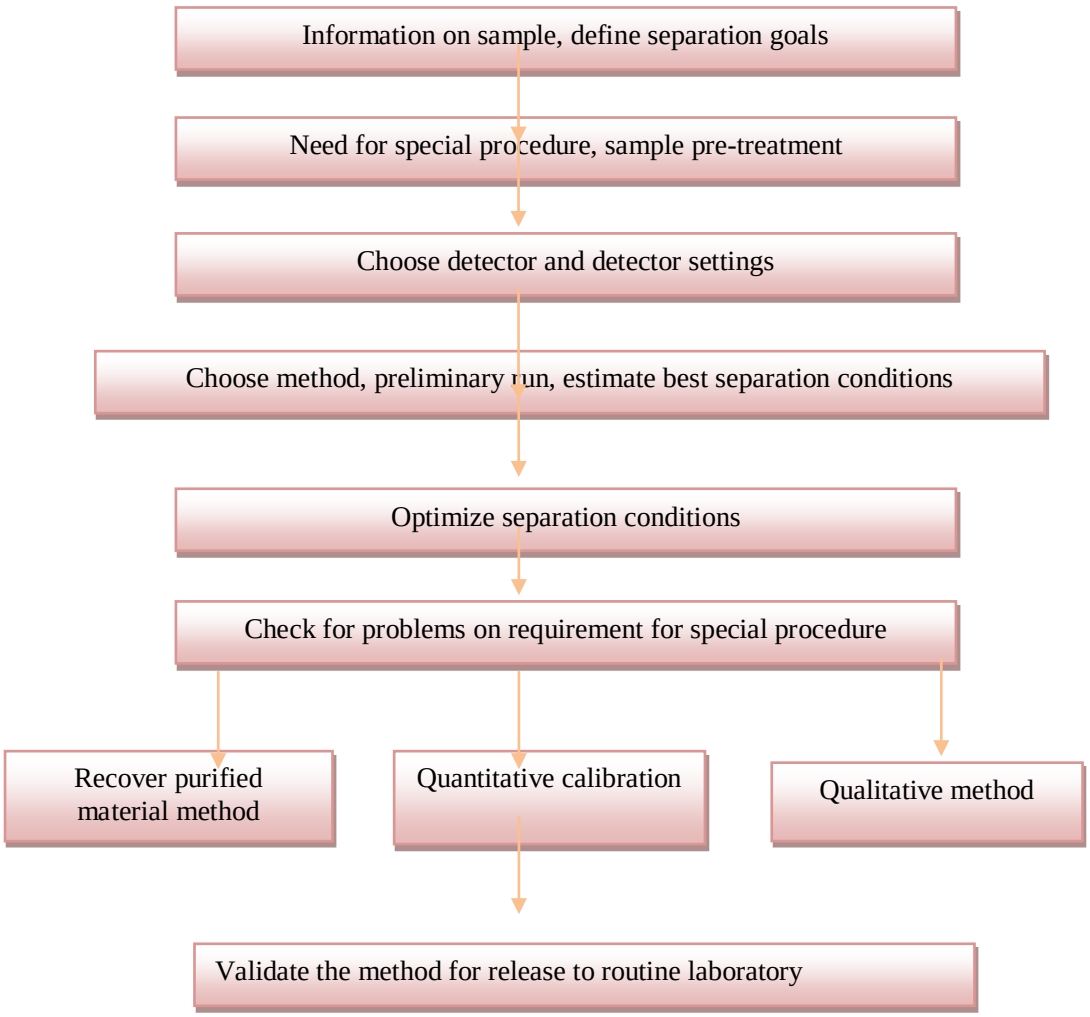
Evaluation of method development with actual samples

The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

Determination of percent recovery of actual sample and demonstration of quantitative sample analysis

Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average +/- standard deviation) from sample to sample and whether recovery has been optimized has been shown.

Strategy for Method Development



The typical HPLC separation is based on the selective distribution of analytes between a liquid mobile phase and an immiscible stationary phase. The sample is first introduced by means of an injection port into the mobile phase stream that is delivered by a high-pressure pump. Next, the components of this sample mixture are separated on the column, a process monitored with a flow-through detector as the isolated components emerge from the column.

The method is popular because it is non-destructive and may be applied to thermally labile compounds (unlike GC); it is also a very sensitive technique since it incorporates a wide choice of detection methods. With the use of Post-column derivatization methods to improve selectivity and detection limits, HPLC can easily be extended to trace determination of compounds that do not usually provide adequate detector response. The wide applicability of HPLC as a separation method makes it a valuable separation tool in many scientific tools.

Objectives

Following are the objectives of present work

- To perform method development for the drugs
- To study effect of various mobile phases used in method development
- To determine the drug content in given pharmaceutical dosage form
- To validate analytical methods as per ICH guidelines
- To demonstrate that it is suitable for its intended purpose
- To establish identity, detect and quantitate impurities and to assess characteristics

Following parameters according to ICH Guidelines to be validated

- Specificity
- Linearity
- Range
- Accuracy
- Precision
- Limit of Detection
- Limit of Quantification
- Robustness

Aim and Plan of Work

The concept of analytical chemistry lies in the precise and accurate measurements. This determination requires highly sophisticated instruments and methods like HPLC, gas chromatography, HPTLC, spectrophotometry, fluorimetry etc. Instrumental methods are sensitive, accurate, precise and desirable for regular determination of drugs in formulations, thereby is advantageous than the conventional volumetric methods. On the literature survey it was found that Ofloxacin was estimated independently and in combination with other drugs by several chromatographic, spectrometric and flourimetric methods in pharmaceutical formulations and in biological samples. Similarly Flavoxate was estimated by HPLC. And one analytical method was found for simultaneous estimation of Ofloxacin and Flavoxate in combination.

In view of the need analytical method in the quality control laboratories for routine analysis of Ofloxacin and Flavoxate in formulations, the present work aims to develop simple and accurate instrumental methods for simultaneous estimation of Ofloxacin and Flavoxate and extend it for their determination in formulation and in laboratory prepared synthetic mixture.

The plan of work is as follows:

i) Study of physicochemical properties of drug, (pH, pKa, solubility and molecular weight)
ii) Preparation of drug standard and sample,
iii) Simultaneous estimation of Flavoxate Hydrochloride and Ofloxacin from formulation by UV spectrophotometric method using Simultaneous Equation Method
iv) Optimization chromatographic conditions like,
   a. Selection of wavelength
   b. Selection of initial separation conditions
   c. Nature of stationary phase
   d. Nature of mobile phase (pH, solvent strength, solvent ratio and flow rate)
v) Study of system suitability parameters,
vi) Validation of proposed method by RP-HPLC.
vii) Applying developed method to marketed formulation.

Materials and Instruments

a) Pure drug samples

Both the drug samples of Flavoxate Hydrochloride and Ofloxacin were received as a gift samples from AMIS Pharmaceuticals - Vadodhara and Dr. MACS

BIO PHARMA., Hyderabad respectively.

b) Chemicals and solvents used

- Acetonitrile : HPLC grade, Qualigens Fine Chemicals, Mumbai.
- HPLC water : Qualigens Fine Chemicals, Mumbai.
- Orthophosphoric acid : 88% GR, Merck, Mumbai.
- Triethylamine : S.D. Fine Chemicals Ltd., Mumbai.

c) Instruments

- Schimadzu Digital Electronic Balance- BL 220H.
- Lab India SAB 5000, pH meter.
- Value Vaccum pump.
- Schimadzu UV-1800, UV/Vis-Spectrophotometer.
- Schimadzu HPLC, BDS Hypersil C18 column, UV detector.
- Ultrasonic cleaner, Life care equipments pvt.ltd.

Selection of chromatographic method for separation

Reverse phase chromatographic technique is selected since both drugs are polar in nature.

Selection of wavelength

Sensitivity of HPLC method that uses ultraviolet (UV) detector depends upon the proper selection of wavelength. An ideal wavelength is the one that gives maximum absorbance and good response for the drug detected at lower concentration also.

From the UV spectra obtained for both drugs, 301nm was selected as the wavelength for study.[16]

Initial chromatographic conditions:

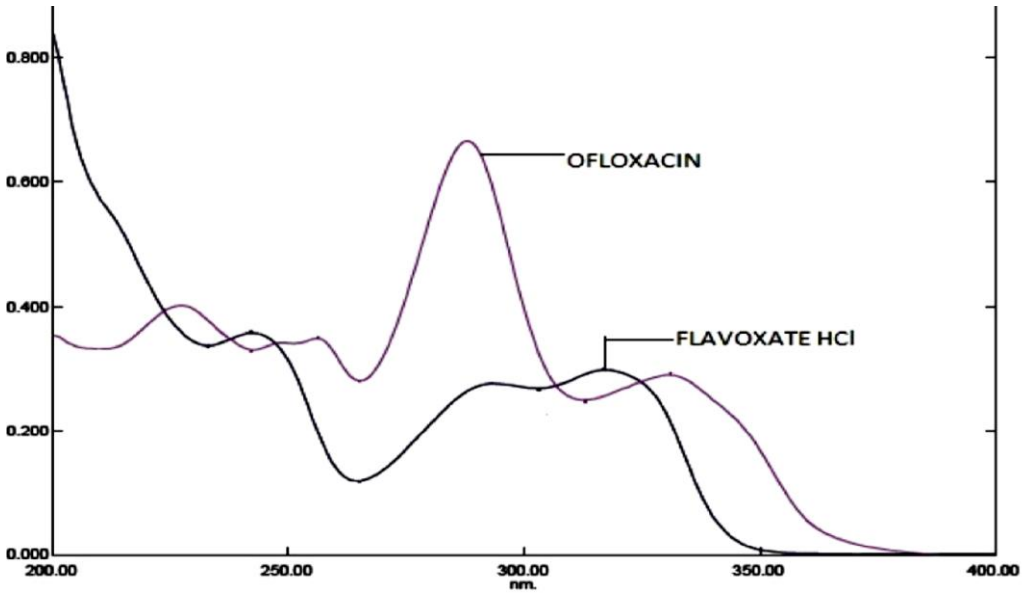
Selection of Mobile phase:

For developing RP – HPLC method, different mobile phase systems with different ratios were tried, among which 0.1% triethylamine buffer and acetonitrile (pH adjusted to 4 using 1% orthophosphoric acid) gave symmetrical peaks with good resolution (Ofloxacin R_f – 3.10 minutes, Flavoxate Hydrochloride R_f – 4.615 minutes), and hence fixed for further studies.

Different mobile phases were tried and their observations are given below in the table.

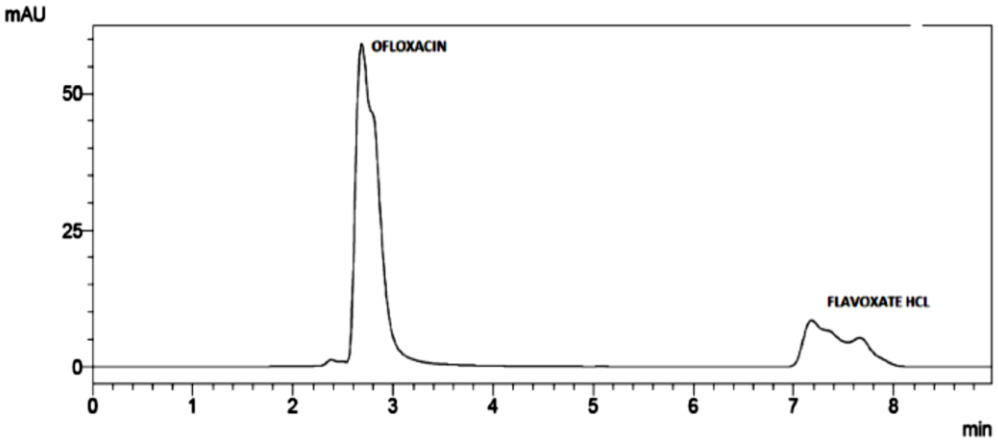
Selection of Mobile Phase

S.no	Mobile phase conditions	Observation
1.	Water : acetonitrile (30:70, v/v)	Tailing for Ofloxacin and Flavoxate peak
2.	0.1% triethylamine buffer: acetonitrile pH 8(30:70,v/v)	Splitting of Ofloxacin and Flavoxate peak
3.	0.1% triethylamine buffer: acetonitrile pH 6 (30:70,v/v)	Good, symmetrical peaks and less resolution.
4.	0.1% triethylamine buffer: acetonitrile pH 4 (40:60,v/v)	Good, symmetrical peaks and good resolution.

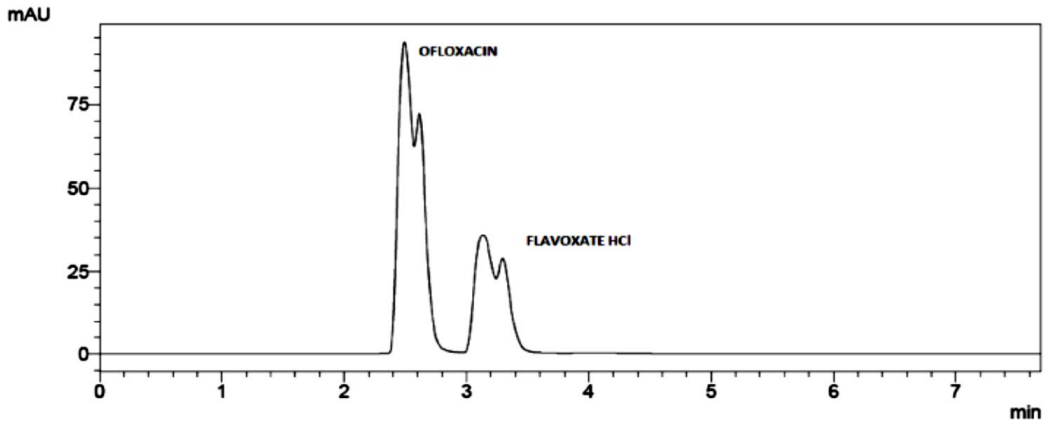


UV spectra of Flavoxate Hydrochloride and Ofloxacin.

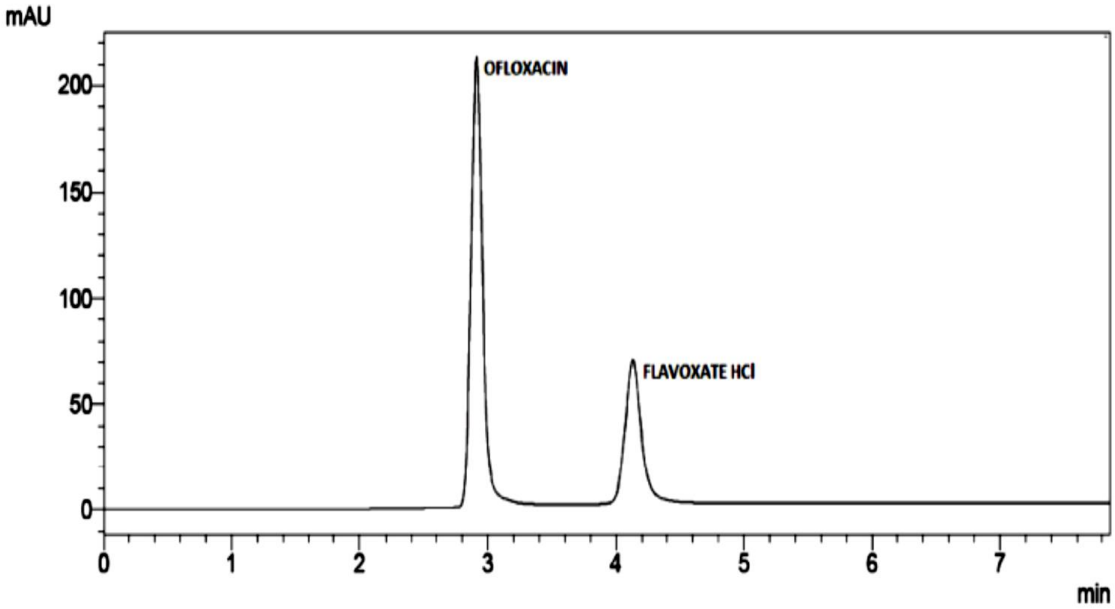
Selection of mobile phase



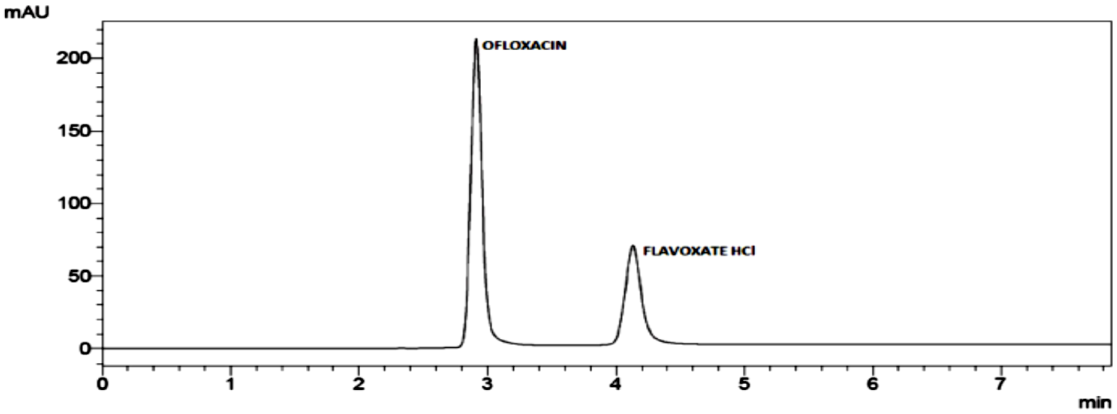
Water and Acetonitrile (40:60)



0.1% Triethylamine buffer: Acetonitrile (40:60,v/v) pH 8



0.1% Triethylamine buffer: Acetonitrile (40:60, v/v), pH 6



0.1% Triethylamine buffer: Acetonitrile (40:60,v/v), pH 4

Optimization of separation Condition

Selection of wavelength

Different wavelengths i.e., 254nm, 301nm, 322nm were taken into consideration from the UV spectra obtained. The wavelength 301nm was finally selected as the impurities were less intensified and the peak intensities were good.

Effect of strength of Triethylamine [17]

Various strengths of triethylamine such as 0.5%, 0.2% and 0.1% were taken into consideration and the chromatograms were recorded at 301nm at a flow rate of 1ml/min. the strength of 0.1% gave good separation and good symmetrical peaks as compared to other strengths of Triethylamine and hence the strength of 0.1% Triethylamine was selected for the study

Effect of ratio of mobile phase

The mobile phase system consisting of triethylamine and Acetonitrile in different ratios such as 30:70, 35:65, 40:60 % v/v, adjusted to pH 4 using 1% Orthophosphoric acid were tried and the chromatograms were recorded at 301 nm at the flow rate of 1 ml/min. The ratio of 40:60 % v/v, gave good separation and symmetrical peaks and hence the ratio of 40:60% v/v was selected for the study.[18]

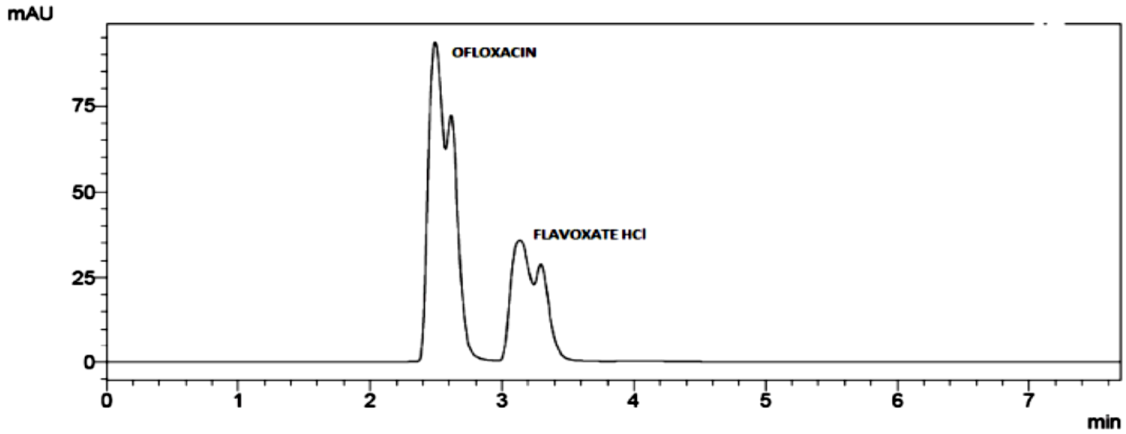
Ratio (% v/v)	Retention time	
0.1% TEA:ACN	Flavoxate Hydrochloride Phosphate	Ofloxacin
70:30	4.08	2.13
65:35	3.53	2.16
60:40	2.08	2.08

Effect of pH

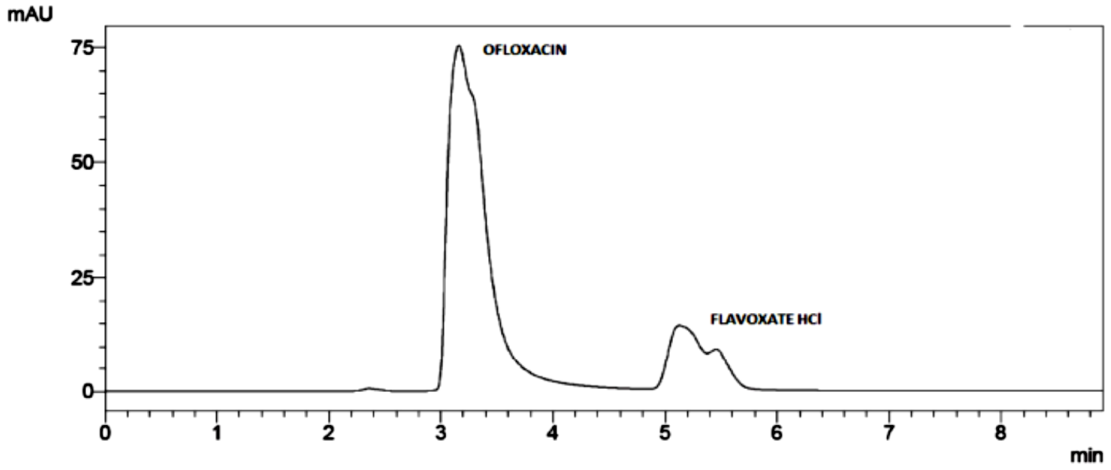
Keeping other conditions constant, chromatograms were recorded with different pH such as 4,5,6,8 etc, adjusted using 1% Orthophosphoric acid. At the pH of 4, the peak shapes of both drugs were good and hence selected for further study.

pH	Observation
3	Broad split peak
4	No tailing, Symmetrical peaks
5	Slight Tailing
6	Split peaks

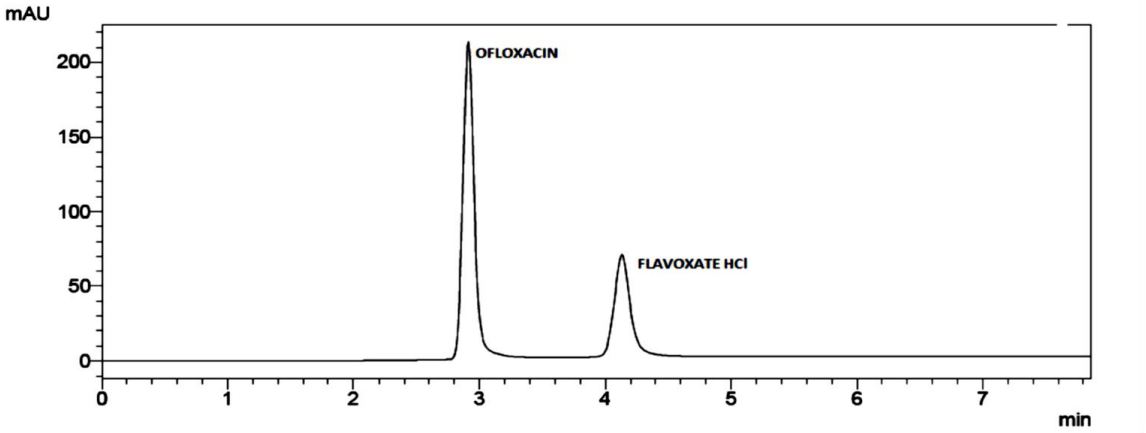
Effect of strength of Triethylamine



Triethylamine (0.5%): Acetonitrile (40: 60)

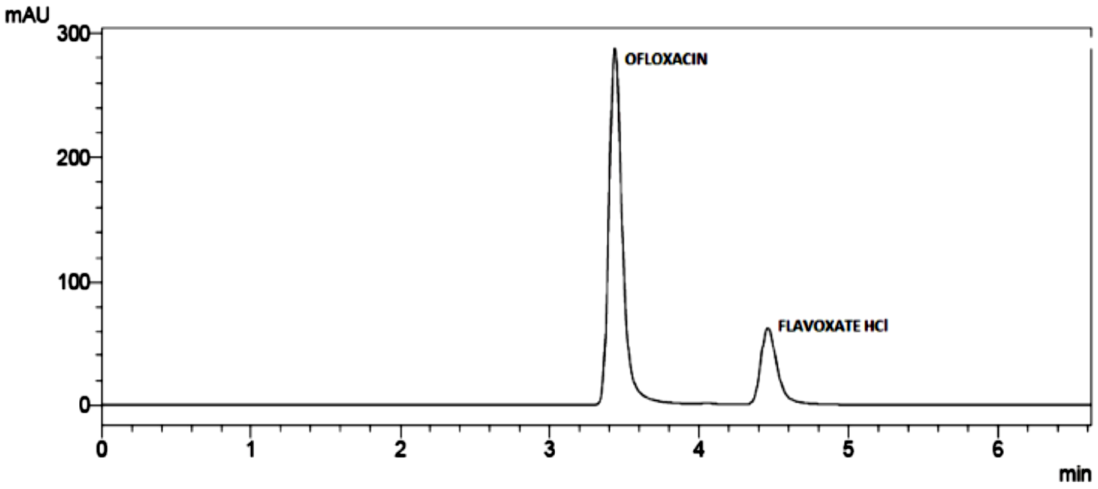


Triethylamine (0.2%): Acetonitrile (40: 60)

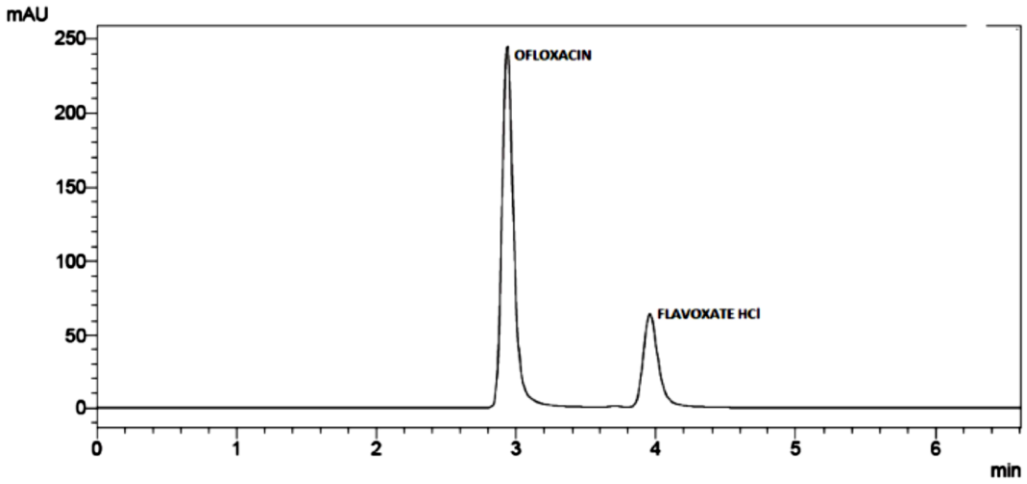


Triethylamine (0.1%): Acetonitrile (40: 60)

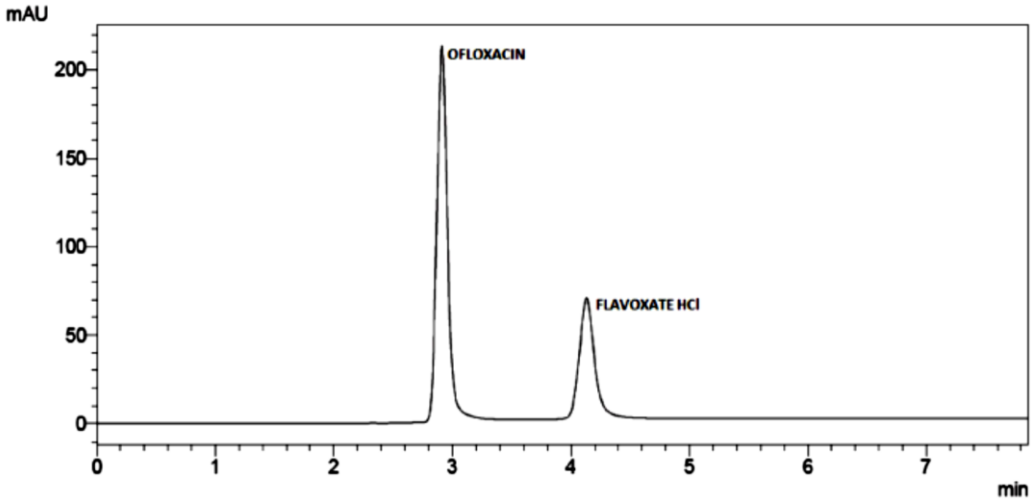
Effect of ratio of mobile phase



Triethylamine (0.1%, v/v): Acetonitrile (30:70)

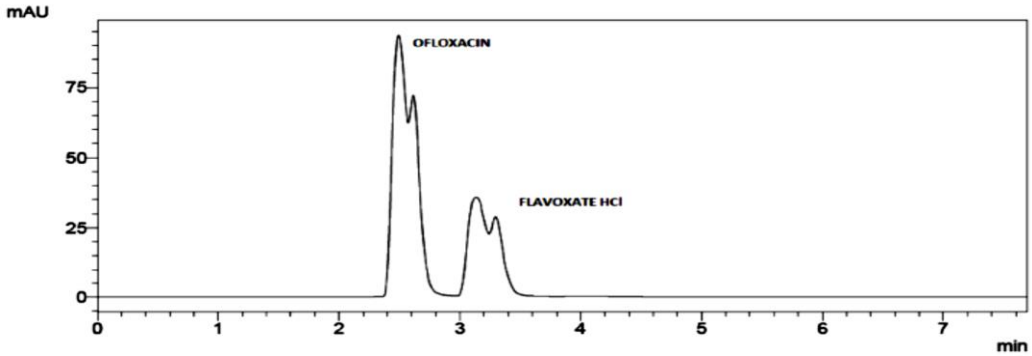


Triethylamine (0.1%, v/v): Acetonitrile (35: 65)

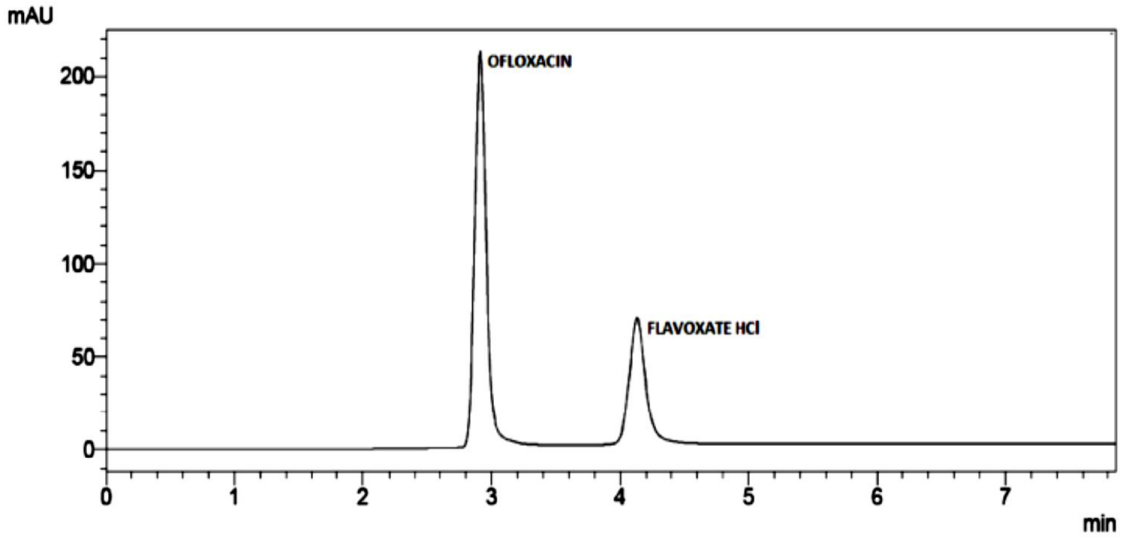


Triethylamine (0.1% v/v): Acetonitrile (40: 60)

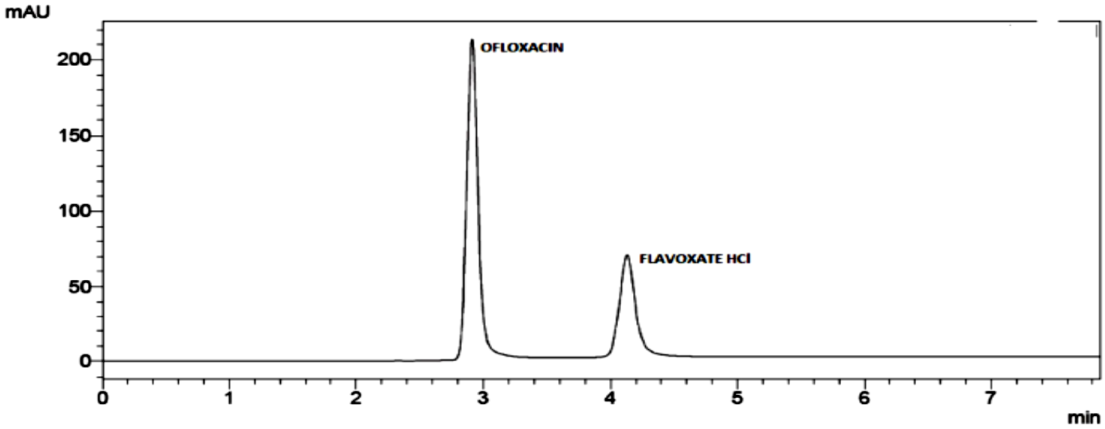
Effect of pH



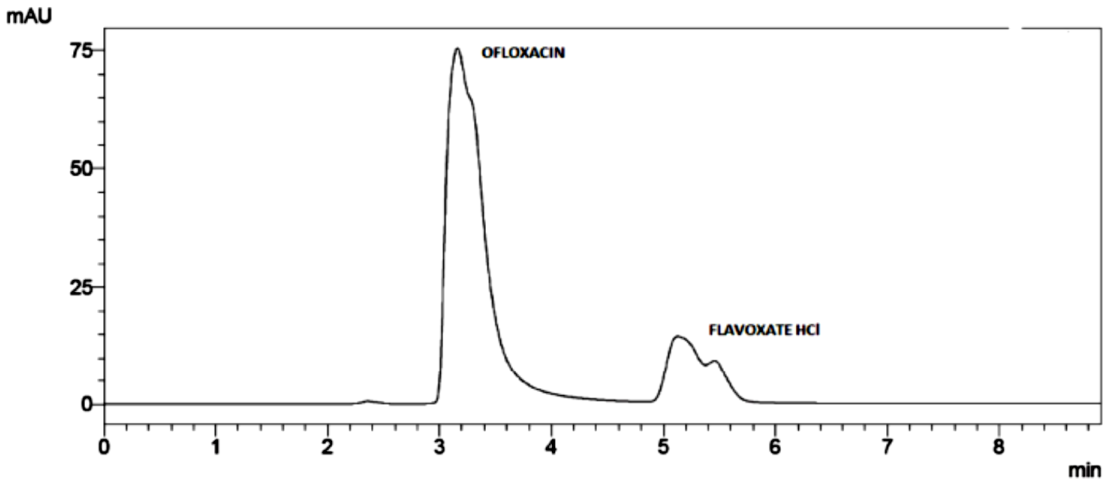
0.1% v/v Triethylamine (pH 6): Acetonitrile (40:60)



0.1% v/v Triethylamine (pH 5): Acetonitrile (40:60)



0.1% v/v Triethylamine (pH 4): Acetonitrile (40:60)



0.1% v/v Triethylamine (pH 3): Acetonitrile (40:60)

Fixed chromatographic Conditions [19]

Stationary Phase	BDS Hypersil, C18 Column, (250 x 4.6mm , 5µ)
Mobile phase	0.1% Triethylamine (pH 4): Acetonitrile
Solvent ratio	60:40
Detection wavelength	301 nm
Flow rate	1.0 ml/ml
Operating Pressure	60 kgf
Operating Temperature	Room Temperature
Injection volume	20µl
Run time	10 min
Mode of operation	Isocratic elution

Validation of RP – HPLC Method [20]

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ of Flavoxate Hydrochloride and Ofloxacin were calculated mathematically. The LOD of Flavoxate Hydrochloride and Ofloxacin were found to be 0.029µg/ml and 0.023µg/ml. The LOQ of Flavoxate Hydrochloride and Ofloxacin was found to be 0.07µg/ml and 0.09µg/ml respectively.

Linearity and Range

Flavoxate Hydrochloride and Ofloxacin were found to be linear in the range of 100-1000 ng/ml. Calibration graphs were plotted using peak areas of standard drugs vs. concentration of standard solutions, The slope, intercept and correlation coefficient values were found to be 6178, 191.6 and 0.997 respectively, for Flavoxate Hydrochloride and 15962, 587.9 and 0.997 respectively, for Ofloxacin.

Precision

Precision of method was demonstrated by

- Intraday precision
- Inter day precision

Intraday precision

Intraday precision was found out by carrying out analysis of standard drug solutions at two different concentrations in the linearity range for three times on the same day and % RSD was calculated

Intraday Precision

Level	Concentration (ng/ml)		Peak Area		%RSD*	
	FX	OX	FX	OX	FX	OX
1	400	400	23339	59899	0.80	0.21
			23465	59645		
			23709	59749		
2	600	600	37018	97709	0.67	0.14
			37216	97556		
			37520	97429		

Analysis of Formulation [21]

Fixed Chromatographic conditions were applied for the analysis of formulation.

Preparation of Standard Solution

Stock solutions containing concentrations of 10µg/ml of Flavoxate Hydrochloride and Ofloxacin were prepared using mobile phase. This solution was suitably diluted to get aliquots of standard solutions containing 100 to 1000ng/ml of Flavoxate Hydrochloride and Ofloxacin.

Preparation of Sample Solution

Twenty tablets (ZENFLOX – UTI) are powdered and the average weight was calculated. A quantity equivalent to 12 mg of drug was dissolved in mobile phase and to it known quantity of standard Flavoxate Hydrochloride and Ofloxacin were added. Finally the volume was made up to get a working concentration of 600 ng/ml each of Flavoxate Hydrochloride and Ofloxacin.

Recording of Chromatograms

A steady baseline was recorded with the fixed chromatographic conditions. Standard drug solutions containing 100 to 1000ng/ml of Flavoxate Hydrochloride and Ofloxacin were injected and chromatograms were recorded.

Retention times of Flavoxate Hydrochloride and Ofloxacin were found to be 4.615 and 3.100 minutes. This was followed by injection of sample solution obtained from the formulation.

Calibration curves were plotted using peak areas of standard drugs vs. concentration of corresponding standard solutions. Peak areas of the sample chromatograms were compared and amount of Flavoxate Hydrochloride and Ofloxacin were calculated and tabulated.[22]

Analysis of Formulation

Drug	Label claim (mg)	Amount found (mg)	%Label claim	%RSD*
	200	201.5	100.75	0.175
Ofloxacin		201.0	100.5	
	200	203.0	101.5	0.349
Flavoxate hydrochloride		202.6	101.6	

* RSD of three observations.

RESULTS AND DISCUSSION:

HPLC Method

In this method, optimization of different chromatographic parameters

like selection of

- Chromatographic method for separation
- Detection wavelength
- Different ionic strengths of mobile phase
- Mobile phase ratio
- Mobile phase pH
- Flow rate etc., were done.

A binary mixture of triethylamine buffer and acetonitrile was selected as the initial mobile phase system for the determination of both drugs. A wavelength of 301 nm was selected for present study. Firstly, various concentrations of Triethylamine buffer were tried. From this, 0.1% Triethylamine buffer was found to be ideal for the work. Then different pH's of buffer were tired, out of which Triethylamine adjusted to pH 4 with Orthophosphoric acid gave good peaks. Then the ratio of mobile phase was determined by varying the proportion of Triethylamine and Acetonitrile. Finally, the mixture of 0.1% Triethylamine buffer adjusted to pH 4 with Orthophosphoric acid and Acetonitrile (40:60% v/v) was employed for the simultaneous determination of both drugs. The retention times of Ofloxacin and Flavoxate were found to be 3.10 and 4.61 minutes respectively.

The developed method was validated as per ICH guidelines. Calibration graphs were potted using standard peak areas vs. concentration of standard solutions. The slope, intercept and correlation coefficient values were found to be 6178, 191.6 and 0.997 for Flavoxate and 15962, 587.9, 0.997 for

Ofloxacin respectively. Ofloxacin and Flavoxate HCl were found to be linear in the range of 100 to 1000ng/ml. The LOD of Ofloxacin and Flavoxate were found to be 0.023µg/ml and 0.029µg/ml respectively. The LOQ of Ofloxacin and Flavoxate were found to be 0.07 µg/ml and 0.09 µg/ml respectively. Precision of the developed method was studied under intraday precision and interday precision of the injection. Low % RSD values indicate that the method is precise. The developed method was found to be robust. The validated liquid chromatographic method was applied to simultaneous determination of Ofloxacin and Flavoxate.

CONCLUSION:

The developed LC method offers simplicity, selectivity, precision and accuracy. In proposed method symmetrical peaks with good resolution were obtained.

Out of all the methods developed, the RP-HPLC method was more sensitive and precise. However, all these methods can be used for the simultaneous analysis of Ofloxacin and Flavoxate HCl in formulation.

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