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Review Paper

A Novel Valiated RP-HPLC Method Developmet for Estimation of Ciprofloxcin in Its Pure and Tablet Dosage Form

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ABSTRACT

A new method was established for ciprofloxacin by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of ciprofloxacin by using Agilent c18 column (4.6×150mm)5.0µm, flow rate was 0.8ml/min, mobile phase ratio was (40:60v/v) Methanol:phosphate buffer pH2.5 (pH was adjusted with orthophosphoric acid), detection wave length was 270nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, UV detector 2487, Empower-software version-2. The retention times were found to be 4.449mins. The % purity of ciprofloxacin was found to be 100.24% respectively. The system suitability parameters for ciprofloxacin such as theoretical plates and tailing factor were found to be 2499 and 1.2, the resolution was found to be 5.3. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study for ciprofloxacin was found in concentration range of 0.2ml-0.6ml and correlation coefficient (r2) was found to be 0.9983% recovery was found to be 100.24%, %RSD for repeatability was 0.1, % RSD for intermediate precision was 0.06 respectively. The precision study was precise, robust, and repeatable. LOD value was 0.43, and LOQ value was 1.3 respectively.Hence the suggested RP-HPLC method can be used for routine analysis of ciprofloxacin in API and Pharmaceutical dosage form.

INTRODUCTION

Chromatography is defined as a process by which solutes are separated by a dynamic differential migration in a system consisting of two or more phases, one of which moves continuously in a given direction. The individual substances exhibit different mobilities by reason of differences in

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adsorption, partition, molecular size, ion-exchange or charge density. The individual substances thus obtained can be identified or determined by analytical methods.The importance of chromatography is increasing rapidly in pharmaceutical analysis for the exact differentiation, selective identification and quantitative determination of structurally closely related compounds. Another important field of application of chromatographic methods is the purity testing of final products and the intermediates (detection of decomposition products and byproducts). As the consequence of the above points, chromatographic methods are given important place in testing of the standards of the drugs. [6] performance liquid chromatography have more elaborate apparatus and usually provide high-resolution methods that will identify and quantitate very small amounts of material. The modern form of column chromatography has been called as high performance, high pressure, high-resolution and high-speed liquid chromatography. Huber, J. F. K., and Hulsman, J.A.G. first presented HPLC, in 1967.

Fig: 1. HPLC SYSTEM

The applications of chromatography have grown extensively in the last fifty years owing not only to the development of several new types of chromatographic techniques but also to the growing need by scientists for getting better methods for characterizing complex mixture.

HPLC is a separation technique based on the difference in the distribution of components between two immiscible phases of which one is called as liquid mobile phase and the other is a solid support called as stationary phase. [7]

Methods of Chromatography (Modes of chromatography)

The principles of different chromatography are based on the nature of interactions between the solute and the stationary phase, which may arise from hydrogen bonding, Vander Walls force, electrostatic forces and hydrophobic forces. Different modes of Chromatography are

(1) Adsorption chromatography
- Normal phase chromatography
- Reverse phase chromatography
- Ion exchange chromatography

(2) Partition chromatography
- Gas chromatography

- Liquid-Liquid partition chromatography

(3) Size exclusion chromatography (SEC)
- Gel permeation chromatography
- Gel filtration chromatography

(4) Affinity chromatography

(5) Hydrophobic interaction chromatography (HIC).[8]

Most commonly used methods in chromatography

Reserve phase chromatography

In 1960s, chromatographers started modifying the polar nature of silanol group by chemically reacting silicon with organic silanes.

The object was to make silica less polar or non-polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of chemically modified silica is now reserved i.e., it is non- polar or the nature of the phase is reverted, the chromatographic separation carried out with such silica is referred as reverse-phase chromatography. large number of chemically bonded silica based stationary phases are available commercially. Silica based stationary phases are still more popular in reverse phase chromatography, however other adsorbents based on polymer (styrene divinyl benzene copolymer) are slowly gaining ground. The less water soluble (i.e., the more non polar) sample compounds are better retained by the reverse phase surface. The retention time decreases in following order: Aliphatic > Induced dipoles (Ex: CCL_4) > Permanent dipoles (Ex: $CHCL_3$), weak Lewis bases (ethers, aldehydes, ketones) > strong Lewis bases (amines) > weak Lewis acids (alcohols, phenols) > strong Lewis acids (carboxylic acids). Also the retention increases as the number of carbon atoms increases. As a general rule the retention increases with an increase in the contact area between sample molecule and stationary phase i.e., with an increase in the number of water molecules, which are released during the adsorption of a compound. Branched chain compounds are eluted more rapidly than their corresponding normal isomers.

In reverse phase system the strong attractive forces between water molecules arising from the 3-dimensional intermolecular hydrogen bonded network present in the structure of water must be distorted or disrupted when a solute is dissolved. Only higher polar or ionic solutes can interact with the water structure. Now polar solutes are squeezed out of the mobile phase and are relatively insoluble in it but with hydrogen carbon moieties of the stationary phase. Chemically bonded octadecyl silane (ODS) and alkane with 18 carbon atoms is the most popular stationary phase used in pharmaceutical industry. Since most pharmaceutical compounds are polar and water soluble, the majority of HPLC method used for quality assurance, decomposition studies, quantitative analysis of both bulk drug and their formulationa use ODS HPLC columns. The solvent strength in reverse phase chromatography is reversed from that of adsorption chromatography (silica gel) as started earlier. Water interacts strongly and highly with silanol groups, so that, adsorption sample molecules become highly restricted and they are rapidly eluted as a result. Exactly opposite applies in reverse phase system, water cannot wet the non polar hydrophobic alkyl group such as C_{18} of ODS phase and therefore does not interact with the bonded moiety. Hence water is the weakest solvent of all and gives slowest elution rate. The elution time (retention time) in reverse phase chromatography increases with increasing amount of water in the mobile phase.[9]

Adsorption chromatography or Normal phase chromatography

In normal phase chromatography, the stationary phase is polar adsorbent. The mobile phase is generally a mixture of non-aqueous solvents. The silica structure is saturated with silanol group at the end in normal phase separations. These OH groups are statistically distributed over the whole of the surface. The silanol groups represent the active sites (very polar) in the stationary phase.

This forms a weak bond with many molecules in the vicinity when any of the the following interactions are present. Dipole-induced dipole, dipole-dipole, hydrogen bonding, π-complex bonding. These situations arise when the molecule has one or several atoms with lone pair electrons or a double bond. The adsorption strengths and hence 'K' value (elution series) increase in the following order. Saturated hydrocarbon < Olefins < Aromatic < Organic < Halogen compounds < Sulphides < Ethers < Esters < Aldehydes and Ketones < Amines < Sulphones < Amides < Carboxylic acids. The strength of interactions depends not only on the functional group in the sample molecule but also on stearic factors. If a molecule has several functional groups, then the most polar one determines the reaction properties.[10] Chemically modified silica, such as aminopropyl, cyanopropyl and diol phases are the stationary phases alternative to silica gel in normal phase chromatography The aminopropyl and cyanopropyl phases provide opportunities for specific interactions between the analyte and the statinory phase and thus offer additional options for the optimizations of separations. Other advantages of bonded phases lie in the increased homogeneity of the stationary phase surface.

Polar modifiers such as acetic acid or triethylamine (TEA) are added to the mobile phase, to deactivate the more polar adsorption sites on the surface of stationary phase, which in turn will improve peak shape as well as the reproducibility of the retention times.[11]

Instrumentation

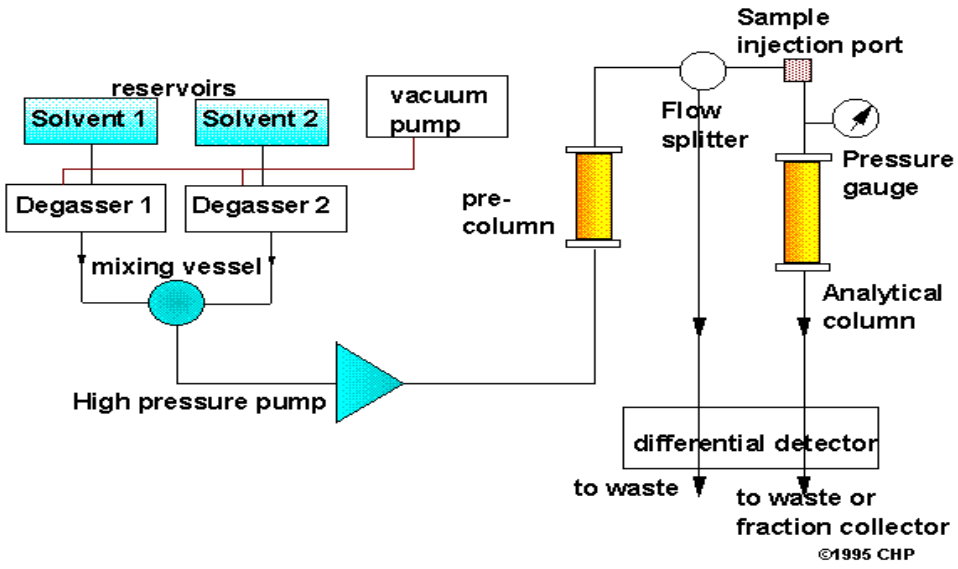


Fig: 2 HPLC Basic instrumentation

Mobile phase reservoir

In HPLC the mobile phase can be an aqueous-organic mixture or buffer solution or a mixture of organic solvents. The mobile phase is pumped under pressure from one or several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a high-pressure

drop across a chromatography column. Eluting power of the mobile phase is determined by its overall polarity, the polarity of the stationary phase and the nature of the sample components. For normal phase separations eluting power increases with increasing polarity of solvent but for reversed phase separations, eluting power decreases with increasing solvent polarity. Optimum separating conditions can be achieved by making use of mixture of two solvents. Some other properties of the solvents, which need to be considered for a successful separation, are boiling point, viscosity, detector compatibility, flammability and toxicity.

Solvent degassing system

The constituents of the mobile phase should be degassed and filtered before use. Several methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vaccum degassing with an aspiratior, filtration through 0.45 filters, vaccum degassing with an air-soluble membrane, helium purging, ultra sonication or purging or combination of this methods. HPLC systems are also provided an online degassing system, which continuously removes the dissolved gases from the mobile phase.

Gradient elution devices

HPLC columns may be run isocratically i.e., with constant eluent or they may be run in the gradient elution mode in which the mobile phase composition varies during the run. Gradient elution is a means of overcoming the problem of dealing with a complex mixture of solutes.

Pump

The most important component of HPLC in delivery system is the pump, because its performance directly effects the retention time, reproducibility and detector sensitivity. Among the several solvent delivery systems (direct gas pressure, pneumatic intensifier, reciprocating etc.), the reciprocating pump with twin or triple position is widely used, as this system gives less baseline noise, good flow rate, reproducibility etc.

Sample introducing systems

Two means for analyte introduction on the column are injection into a flowing stream and a stop flow injection. These techniques use a syringe or an injection valve. Automatic injector is a microprocessor-controlled version of a manual universal injector. Usually, up to 100 samples can be loaded into the auto injector tray. The system parameters such as flow rates, gradient, run time, volume to be injected, etc., are chosen, stored in memory and sequentially executed on consecutive injections.[12]

Characteristics of columns and column packing

The column is the 'HEART' of HPLC separation processes. The availability of a stable high performance column is essential in developing a rugged reproducible method. Most column packings used for HPLC separations make use of silica particle ($SIO_2 \times H_2O$). It consist of a network of siloxane linkages (Si-O-Si) in rigid three-dimensional structure containing inter connecting pores. Thus wide ranges of commercial products are available with surface areas ranging from 100 to 800 m/g and particle sizes from 3 to 50 µm. The silanol groups on the surface of silica give it a polar character, which is exploited in the adsorption chromatography using non-polar organic eluents. Silica can be drastically altered by reaction with organo chloro silanes or organo alkoxy silanes giving Si-O-Si-R linkages with surface. The attachment of hydrocarbon chain to silane produces a non-polar surface suitable for reverse phase chromatography where mixture of water and organic solvents are used as an eluents.

The most popular material is octadecyl silica (ODS- Silica), which contains C_{18} chains, but materials C_2, C_6, C_8, and C_{22} chains are also available. During the manufacturing, such materials may be reacted with a small mono functional silane (e.g., trimethyl chloro silane), which reduce the number of silane groups remaining on the surface (end-capping). There is a vast range of materials which have intermediate surface polarities arising from the bonding of silica with other organic compounds which contain phenyl, nitro, amino and hydroxyl groups. Strong ion exchangers are also available in which sulfonic acid groups or quarternary ammouniun groups are bonded to silica. The useful pH range for columns is 2 to 8, since siloxane linkages are altered below pH 2 while at pH values above 8 silica may dissolve.

Fig: 3 Standard HPLC Column

In HPLC, generally two types of columns are used, normal phase columns and reserve phase columns. Using normal phase chromatography, particularly of non-polar and moderately polar drugs can make excellent separation. It was originally believed that separation of compounds in mixture takes place slowly by differential adsorption on stationary silica phase. However, it now seems that partition plays an important role, with the compounds interacting with the polar silianol groups on the silica or with bound water molecules. Normal phase involves the passage of a relatively non-polar mobile phase over a polar stationary phase, reversed phase chromatography is carried out using a polar stationary phase. A range of stationary phase (C_{18}, C_8, -NH_2, -CN, -phenyl etc) is available and very selective separations can be achieved. The pH of the mobile phase can be adjusted to suppress the ionization of drug and thereby increase the retention on the column. For highly ionized drugs ion-pair chromatography is used.

Detectors

The purpose of the detector is to monitor the eluent coming of the column. Generally two types of detectors are used in the HPLC bulk property and solute property detectors.

I) Bulk property detectors: These detectors are based on differential measurement of a property, which is common to both the sample and the mobile phase. Examples of such detectors are refractive index, conductivity and dielectric constant detectors. II) Solute property detectors: Solute property detectors respond to a physical property of the solute, which is not exhibited by the pure mobile phase.These detectors measure a property, which is specific to the sample, either with or without the removal of the mobile phase prior to the detection. Solute property detectors which do not require the removal of the mobile phase before detection include spectrophotometric (UV and UV-VIS) detector, fluorescence detectors, palarographic, electro-chemical and radioactive detectors, while the moving wire flame ionization detector and electron capture detector, UV-VIS and fluorescent detectors are suitable for gradient elution, because many solvents used in HPLC do not absorb to any significant extent.

Derivatization

In HPLC derivatization is used to enhance the sensitivity and selectivity of detection when available detectors are not satisfactory for the un-derivatized compounds. Both ultra violet absorbing and fluorescence derivatives have been widely used, for the above said purpose. Ultra violet derivatization reagents include N-succinimidyl p-nitro phenyl acetate, phenyl hydrazine and 3, 5-dinitro benzyl chloride, while fluorescent derivatives can be formed with reagents such as dansyl chloride. 4-bromomethyl-7-methoxy-coumarin and fluorescamine. Derivative formation can be carried out before the sample is injected into the column or by online chemical reactions between the column outlet and the detector.[14]

Gradient elution

Gradient elution or solvent programming is the change of solvent composition during a separation in which strength increases from the beginning to the end of the separation. It is well suited to the analysis of the samples of unknown complexity since good resolution is automatically provided for a wide range of sample polarities.

There are two types of gradient systems: low-pressure gradient mixtures and high-pressure gradient mixtures. In the former the solvents are mixed at atmosphere pressure and then pumped into the column, where as in the later, solvents are pumped into a mixing chamber at high pressure before going into the column.

General methodology for the development of new HPLC methods

A good method development strategy should require only as many experimental runs as are necessary to achieve the desired final result. Finally method development should be as simple as possible, and it should allow the use of sophisticated tools such as computer modeling.

The important factors, which are to be taken into account to obtain reliable quantitative analysis, are

1. Careful sampling and sample preparation.
2. Precise sample injection.
3. Appropriate choice of the column.
4. Choice of the operating conditions to obtain the adequate resolution of the mixture.
5. Reliable performance of the recording and data handling systems.
6. Suitable integration/peak height measurement technique.
7. The mode of calculation best suited for purpose.
8. Validation of the developed method.

Choice of the column

The selection of the column in HPLC is somewhat similar to the selection of columns in G.C, in the sense that, in the adsorption and partition modes, the separation mechanism is based on inductive forces, dipole-dipole interactions and hydrogen bond formation. In case of ion-exchange chromatography, the separation is based on the differences in the charge, size etc., of the ions generated by sample molecules and the nature of ionizable group on the stationary phase. In the case of size-exclusion chromatography the selection of column is based on the molecular weight and size of the sample components.

Selection of columns based on the method is briefly summarized in the table below:

TABLE -1

Method/ Description/ Columns	Preferred method
Reverse-phase HPLC	
Uses water-organic mobile phase Columns: C_{18} (ODS), C_8 phenyl, trimethylsilyl (TMS), and cyano.	First choice for most samples, specially neutral or non-ionized compounds that dissolve in water-organic mixtures.
Ion-pair HPLC	
Uses water-organic mobile phase, a buffer to control pH, and an ion-pair reagent. Columns: C_{18}, C_8, cyano.	Accepectable choice for ionic or ionisable compounds, especially bases or cations.
Normal-phase HPLC	
Uses mixtures of organic solvents as mobile phase. Columns: cyano, diol, amino, silica.	Good second choice when reverse phase or ion-pair HPLC is ineffective, first choice for lipophilic samples that don't dissolve well in water-organic mixtures, first choice mixtures of isomers and for preparative HPLC.

Preferred experimental conditions for the initial HPLC separation

TABLE -2

Separation variable	Preferred initial choice
Column	
Dimensions(length, ID)	15X0.46 cm
Particle size	5µm[a]
Stationary phase	C_8 or C_{18}
Mobile phase	
Solvent A and B	Buffer -ACN
%B	80-100%[b]
Buffer (compound, pH, concentration)	25mM potassium phosphate, 2.0<pH<3.0[c]
Additives(e.g., amine modifiers, ion-pair reagent)	Don't use initially
Flow rate	1.5-2.0 ml/ min
Temperature	35-45^{0}C
Sample size	
volume[d]	<25µL
Weight[d]	<100µg

Polar solvent

a) 3.5 µm particles are an alternative, using a 7.5 cm column.

b) For an initial isocratic run, an initial gradient run is preferred.

c) No buffer required for neutral samples, for pH <2.5, pH-stable columns are recommended.

d) Smaller values required for smaller-volume columns (e.g., 7.5 X 0.46-cm, 3.5-µm column).

Using these conditions, the first exploratory run is carried and then improved systematically. On the basis of the initial exploratory run isocratic or gradient elution can be selected as most suitable.

If typical reverse-phase conditions provided cause is inadequate sample retention, suggesting the use of either ion-pair or normal phase HPLC. Alternative, the sample may be strongly retained with 100% ACN as mobile phase suggesting the use of non-aqueous reverse phase chromatography or normal phase HPLC.[17]

One approach is to use an isocratic mobile phase of some average solvent strength (50%) organic solvent. A better alternative is to use a very strong mobile phase first (80-100%) then reduce %B as necessary. The initial separation with 100% B results in rapid elution of the entire sample, but few groups will separate. Decreasing the solvent strength shows the rapid separation of all components with a much longer run time, with a broadening of latter bands and reduced retention sensitivity.

Goals that are to be achieved in method development are briefly summarized below

Getting started on method development

TABLE-3

Goal	Comment
Resolution	Precise and rugged quantitative analysis requires that R_S be greater than 1.5.
Separation time	<5-10 min is desirable for routine procedures.
Quantitation	≤ 2% (1 SD) for assays; ≤5% for less- demanding analysis ≤15% for trace analysis.
Pressure	<150 bar is desirable, <200 bar is usually essential (new column assumed).
Peak height	Narrow peaks are desirable for large signal/noise ratios.
Solvent Consumption	Minimum mobile-phase use per run is desirable.

Roughly in order of decreasing importance but may vary with analysis requirements. Separation or resolution is a primary requirement in quantitative HPLC. The resolution (R_s) value should be maximum (R_s>1.5) favors maximum precision. Resolution usually degrades during the life of the column and can vary from day to day with minor fluctuations in separation conditions.

Therefore, values of R_s=2 or greater should be the goal during method development for sample mixtures. Such resolution will favor both improved assay precision and greater method ruggedness. Some HPLC assays do not require base line separation of the compounds of interest (qualitative analysis). In such cases only enough separation of individual components is required to provide characteristic retention times for peak identification. The time required for a separation (runtime = retention time for base line) should be as short as possible and the total time spent on method development is reasonable (runtimes 5 to 10 minutes are desirable). Conditions for the final HPLC method should be selected so that the operating pressure with a new column does not exceed 170 bar (2500 psi) and an upper pressure limit below 2000 psi is desirable. There are two reasons for this pressure limit, despite the fact that most HPLC equipment can be operated at much higher pressures. First, during the life of a column, the back pressure may rise by

a factor of as much as 2 due to the gradual plugging of the column by particulate matter. Second, at lower pressures (<170 bars) pumps, sample valves and especially auto samplers operate much better, sales last longer, columns tend to plug less and system reliability is significantly improved. For these reasons, a target pressure of less than 50 % of the maximum capability of the pump is desirable. When dealing with more challenging samples or if the goals of separation are particularly stringent, a large number of method development runs may be required to achieve acceptable separation.

Repeatable separation

As the experimental runs described above are being carried out, it is important to confirm that each chromatogram can be repeated. When we change conditions (mobile phase, column, and temperature) between method development experiments, enough time must elapse for the column to come into equilibrium with the new mobile phase and temperature. Usually column equilibration is achieved after passage of 10 to 20 column volumes of the new mobile phase through the column. However, this should be confirmed by repeating the experiment under the same conditions. When constant retention times are observed in two such back–to–back repeat experiments (± 0.5% or better), it can be assumed that the column is equilibrated and the experiments are repeatable.

Completing the HPLC method development

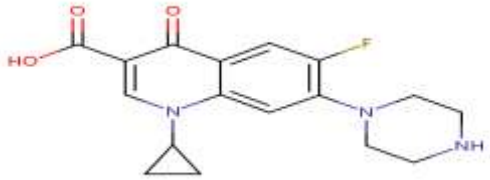
The final procedure should meet all the goals that were defined at the beginning of method development. The method should also be robust in routine operation and usable by all laboratories and personnel for which it is intended.

System suitability

System suitability experiments can be defined as tests ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validations have been completed. The criteria selected will be based on the actual performance of the method as determined during its validation. For example, if sample retention times form part of the system suitability criteria, their variation (SD) can be determined during validation. System suitability might then require that retention times fall within a ± 3 SD range during routine performance of the method. The USP (2000) defines parameters that can be used to determine system suitability prior to analysis. These parameters include plate number (N), tailing factor, k and/or α, resolution (R_s) and relative standard deviation (RSD) of peak height or peak area for respective injections. The RSD of peak height or area of five injections of a standard solution is normally accepted as one of the standard criteria. For an assay method of major component, the RSD should typically be less than 1% for these five respective injections. The plate number and /or tailing factor are used if the run contain only one peak. For chromatographic separations with more than one peak, such as an internal standard assay or an impurity method expected to contain many peaks, some measure of separations such as R_s is recommended. Reproducibility of t_R or k value for a specific compound also defines system performance.

2. DRUG PROFILE

CIPROFLOXACIN



Chemical Data

IUPAC Name : 1-cyclopropyl-fluoro-4-oxo-7- (piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid

Chemical formula : $C_{17}H_{18}FN_3O_3$

Molecular weight : 331.34

PHYSICAL DATA:-

Description : A broad-spectrum antimicrobial carboxyfluoroquinoline

Solubility : Soluble in all organic solvents and water.

P ka : 6.09

Mechanism Of Action : The bactericidal action of ciprofloxacin results from inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, strand supercoiling repair, and recombination.

3. LITERATURE REVIEW

1. Sani A. Ali et al, A new simple, rapid, selective, precise and accurate isocratic reverse phase high performance liquid chromatography assay has been developed for the estimation of Ciprofloxacin Hydrochloride in tablet formulation. The separation was achieved by using C-18 column (LichroCART® 125x4mm, 5µm) coupled with a guard column of silica in mobile phase methanol: buffer (0.025M Orthophosphoric acid with the pH adjusted to 3.0±0.1 with triethylamine) (40:60v/v). The flow rate was 2.0ml/min and the drug was detected using UV detector at the wavelength of 278nm. The retention time was within 1.753 – 1.757 minutes. The method was validated as per ICH guidelines. The proposed method was found to be accurate, repeatability and consistent. It was successfully applied for the analysis of the drug in marketed formulation and could be effectively used for the routine analysis of formulation containing the drug without any alteration in the chromatography conditions.

2. Patel, S.A et al, A simple, sensitive, specific, economic, accurate and precise reverse phase high performance liquid chromatographic (RP-HPLC) method was developed for simultaneous estimation of ciprofloxacin and ornidazole in pharmaceutical formulations. The separation was achieved on Phenomenex C18 Keywords: Ciprofloxacin; Ornidazole; RP-HPLC; Validation; Simultaneous; Pharmaceutical dosage form column (250mm i.d., 4.6mm,5µm particle size) using water: acetonitrile: triethylamine (80:20:0.1,v/v/v) and final pH adjusted to 3.06±0.02 with 5%v/v ortho-phosphoric acid as the mobile phase at a flow rate of 1.5ml/min at an ambient temperature. The quantification was achieved with UV detection at 318 nm. The injection volume was 20 µl. The retention times of ciprofloxacin and ornidazole were 4.02 min and 9.13min, respectively. The method was validated for linearity, precision, recovery, specificity, limit of detection, limit of quantification and robustness. The linearity was obtained in the concentration range of 1-16µg/ml for each ciprofloxacin and ornidazole with mean recovery of 99.41±1.30 and

99.87±1.10 for ciprofloxacin and ornidazole, respectively. The limit of detection and quantification for ciprofloxacin were 0.169 and 0.512µg/ml, respectively and for ornidazole were 0.283 and 0.858µg/ml, respectively. The method was found to be simple and sensitive and can be useful for the routine quality control testing of ciprofloxacin and ornidazole combined pharmaceutical dosage forms.

3. Murugan S et al, A facile and rapid isocratic reverse phase high performance liquid chromatography assay method has been developed for simultaneous estimation of Ciprofloxacin Hydrochloride and Tinidazole in tablet formulation. The column was equilibrated for at least 30 min and separation was achieved by using inerstil- BDS C18 (250×4.6nm, 5µ). The column was maintained at ambient temperature (27°C). The mobile phase employed was Methanol: Orthophosphoric acid (0.28ml) in 1000mL of water (55:45 v/v). The eluent was monitored using PDA detector at 245nm. A volume of 20 µL of standard and sample solutions was injected in to the HPLC. The flow rate was 1.0ml /min. The retention times were 2.955 min and 3.539 for Tinidazole and ciprofloxacin HCl respectively. The developed method was validated as per ICH guidelines. The developed method was found to be accurate, precise and reproducible.

4. Nimila et al, A high performance liquid chromatographic method has been proposed for the simultaneous determination of ciprofloxacin hydrochloride and ornidazole in pure and dosage form. The HPLC instrument of Agilent technologies (Agilent 1120 LC Germany) model was used where the drug was chromatographed on a C-18 Zorabax column (4.6mm x 250mm) using a mixture of acetonitrile and water (Millipore Q, pH 3.0 adjusted with O-phosphoric acid) in the ratio of (45:55v/v) as the mobile phase at a flow rate of 1ml/min and the detection was done at 299nm (isobestic point). The retention time for ciprofloxacin HCL and ornidazole were 1.96 and 4.33 min respectively. The calibration curves were found to be linear in the range of 12 to 20Î¼g/ml with a correlation coefficient of 0.9998 and 0.9997 respectively. The results of analysis have been validated statistically and by recovery studies. The percentage recovery was obtained for ciprofloxacin HCL and ornidazole of 99.86 to 100.14% and 100.40 to100.53% respectively. The simplicity and accuracy of the proposed method ensures its use in routine quality control analysis of pharmaceutical formulations.

5. Urvish H. Desai et al, High performance liquid chromatography method was applied to the simultaneous determination of ciprofloxacin and dexamethasone. The chromatographic separation was achieved on reversed-phase C18 column(25cm × 4.6mm id, 5 µm) in the isocratic mode using methanol-water-triethylamine (55:45:0.6, v/v/v), pH adjusted to 3.0±0.05 with orthophosphoric acid as the mobile phase at a flow rate 0.8ml/min. Quantitation was achieved with UV detection at 254 nm. In the HPLC method, quantification was achieved over the concentration range of 3-18 and 1-6µg/ml, with mean recoveries of 99.94±1.51 and 100.28±1.25% for ciprofloxacin and dexamethasone respectively. The proposed methods were successfully applied for the analysis of synthetic mixtures and pharmaceutical formulations of ciprofloxacin and dexamethasone without any interference from common excipients.

6. Dhavani Kanikanti et al, A simple, rapid, precise and accurate reverse phase high performance liquid chromatography has been developed and validated for simultaneous estimation of Ciprofloxacin Hydrochloride and Tinidazole in tablet formulations. HPLC Waters

e2695 system equipped with Empower 2 Software with PDA Detector and INERSIL ODS C18 column (4.6x 250*5µm particle size) was operated in isocratic mode using water and methanol (60:40v/v) as mobile phase and pumped at rate of 0.8ml/min and eluents were monitored using UV-Visible detector at 316nm .The linearity was found in the range of 50-150 µg/ml and shows a correlation coefficient of 0.999. The retention time of Ciprofloxacin Hydrochloride and Tinidazole was noted to be 5.6 and 4.6 mins, respectively. This study concluded that the proposed method was found to be accurate, reproducible, and consistent and could be effectively used for the routine analysis of these drugs in marketed formulations.

4. AIM AND OBJECTIVE

Literature review reveals that there is few analytical method reported for the analysis of Ciprofloxacin estimation by RP-HPLC. Spectrophotometer, HPLC and HPTLC are the reported analytical methods for compounds either individually or in combination with other dosage form. Hence, it was felt that, there is a need of new analytical method development for the Ciproflaxacin in pharmaceutical dosage form.

Present work is aimed to develop a new, simple, fast, rapid, accurate, efficient and reproducible RP-HPLC method for the simultaneous analysis of Ciprofloxacin. The developed method will be validated according to ICH guidelines.

Objective of the work

- The analytical method for the estimation of Ciprofloxacin will be developed by RP-HPLC method by optimizing the chromatographic conditions.
- The developed method is validated according to ICH guidelines for various parameters specified in ICH guidelines, Q2 (R1).

5. EXPERIMENTAL WORK

Materials and Methods

Chemicals and standards used

Table.No.10.List of chemicals and standards used

S.No	Chemicals	Manufacturer Name	Grade
1.	Water	Merck	HPLC grade
2.	Methanol	Merck	HPLC grade
3.	Acetonitrile	Merck	HPLC grade
4.	Ortho phosphoric acid	Merck	G.R
5.	KH_2PO_4	Merck	G.R
6.	K_2HPO_4	Merck	G.R
7.	0. 22µ Nylon filter	Advanced lab	HPLC grade
8.	0.45µ filter paper	Millipore	HPLC grade
9.	Ciprofloxacin	In – House	In- House

Instruments used

Table.No.11. List of instruments used

S.No	Instrument name	Model number	Soft ware	Manufacturers Name
1	HPLC-auto sampler –UV detector	Separation module2695, UV.detector2487	Empower-software version-2	Waters
2	U.V double beam spectrometer	UV 3000+	U.V win soft ware	Lab India
3	Digital weighing balance(sensitivity 5mg)	ER 200A	-	Ascoset
4	pH meter	AD 102U	-	ADWA
5	Sonicator	SE60US	-	Enertech

Method development for the estimation of Ciprofloxacin by using RP-HPLC.

1. Selection of mobile phase
2. Selection of detection wavelength
3. Selection of column
4. Selection of solvent delivery system
5. Selection of flow rate
6. Selection of column temperature
7. Selection of diluent
8. Selection of test concentration and injection volume

1. Selection of mobile phase

- pH 2.5 phosphate buffer : Methanol (60 : 40% v/v)
- Buffer pH should be between 2 to 8.
- Below 2: siloxane linkages are cleaved.
  Above 8: dissolution of silica.
- pH selected: 3 ±0.05
- pH controls the elution properties by controlling the ionization characteristics.
- Reasons: To decrease the retention and improve separation. Good Response, Area, Tailing factor, Resolution.

2. Selection of wavelength: 10 mg of Ciprofloxacin was dissolved in mobile phase. The solution was scanned from 200-400 nm the spectrum was obtained. The overlay spectrum was used for selection of wavelength for Ciprofloxacin . The isobestic point was taken as detection wavelength.

Selection of column

- Heart of HPLC made of 316 grade stainless steel packed with stationary phase.

Silica based columns with different cross linking's in the increasing order of polarity are as follows:

←------- Non-polar----------moderately polar-------Polar----------→

C_{18}< C_8< C_6< Phenyl < Amino <Cyano< Silica

- In reverse phase chromatography, hydrophobic interaction between drug molecule and the alkyl chains on the column packing material.
- Column is selected based on solubility, polarity and chemical differences among analysts and Column selected: i.e. Agilent C18 column (4.6 x150 mm)5 µ.

4. Selection of solvent delivery system

- Always preferable solvent delivery system.
- More chance of getting reproducible result on retention time of analytes.
- More economic than gradient technique.

5. Selection of flow rate

Acceptable limit: - Not more than 2.5 ml/min

- Flow rate selected was 1ml/min

- Flow rate is selected based on

Reasons:

- For earlier elution of analyte and elution of all impurities within 6.0 min.
- Information from the reference method in literature.

6. Selection of diluent

- Selection of diluent is based on the solubility of the analyte
- Diluent selected: Methanol : phosphate buffer pH 2.5 (40 : 60v/v)

7 Selection of column temperature:

- Preferable temperature is ambient or room temperature.

Reasons:

- To elute all impurities along with analyte with in 10.0 min of run time.
- Less retention time
- Good peak shape
- Higher theoretical plates.
- Good resolution.

8. Selection of test concentration and injection volume

Test concentration is finalized after it is proved that API is completely extractable at the selected test concentration.

- Test concentration is fixed based upon the response of API peak at selected detector wavelength.
- And the test concentration selected is 10ppm.
- Injection volume selected was 10µL.

Reason: good peak area, retention time, peak symmetry.

9. Procedure

Preparation of phosphate buffer

0.698 grams of K_2HPO_4 was weighed and taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water and pH was adjusted to 2.5 with orthophosphoric acid. The resulting solution was sonicated and filtered.

Preparation of mobile phase

Mix a mixture of above buffer 400 ml (40%) and 600 ml of methanol (HPLC grade 60%) and degassed in ultrasonic water bath for 5 minutes. Filter through 0.22µ filter under vacuum filtration.

Diluents preparation

Mobile phase was used as the diluent.

Preparation of the Ciprofloxacin standard preparation

10 mg of Ciprofloxacin working standard was accurately weighed and transferred into a 10 ml clean dry volumetric flask and add about 2 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette out 0.4 ml from the above stock solution into a 10 ml volumetric flask and was diluted up to the mark with diluent. Final concentration is 40µg/ml.

Preparation of the Ciprofloxacin standard and sample solution preparation:

An equivalent tablet power such that 10 mg of Ciprofloxacin and tablet powder were accurately weighed and transferred into a 10 ml clean dry volumetric flask, add about 2ml of diluent and sonicate to dissolve it completely and making volume up to the mark with the same solvent(Stock solution). Further pipette 10ml of the above stock solution into a 100ml volumetric flask and was diluted up to the mark with diluent.

Standard solution preparation

10 mg Ciprofloxacin working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette out 1ml of the

above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

Procedure

10µL of the blank, standard and sample were inje cted into the chromatographic system and areas for the Ciprofloxacin the peaks were used for calculating the % assay by using the formulae.

System suitability

- Tailing factor for the peaks due to Ciprofloxacin in standard solution should not be more than 1.5.
- Theoretical plates for the Ciprofloxacin peaks in standard solution should not be less than 2000.

Assay calculation

$$\textbf{Assay}\ \% = \frac{\textbf{sample area}}{\textbf{Standard area}} \times \frac{\textbf{dilution sample}}{\textbf{dilution of standard}} \times \frac{\boldsymbol{P}}{\mathbf{100}} \times \frac{\boldsymbol{Avg.wt}}{\boldsymbol{Lc}} \times \mathbf{100}$$

Where:

Avg.wt = average weight of tablets

P= Percentage purity of working standard

LC= Label Claim of Ciprofloxacin mg/ml.

ANALYTICAL METHOD VALIDATION

Validation parameters

- Specificity
- Linearity
- Range
- Accuracy
- Precision
- Repeatability
- Intermediate Precision
- Detection Limit
- Quantitation Limit
- Robustness

Specificity

The system suitability for specificity was carried out to determine whether there is any interference of any impurities in retention time of analytical peak. The specificity was performed by injecting blank.

2. Linearity

Preparation of stock solution

10 mg of Ciprofloxacin working standard were accurately weighed and were transferred into a 10ml clean dry volumetric flask, add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Preparation of Level–I (20µg/ml of Ciprofloxacin)

0.2ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level–II (30µg/ml of Ciprofloxacin)

0.3 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level–III (40µg/ml of Ciprofloxacin)

0.4 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level–IV (50µg/ml of Ciprofloxacin)

0.5 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

Preparation of Level–V (60µg/ml of Ciprofloxacin)

0.6 ml of stock solution was taken in to 10ml of volumetric flask and diluted up to the mark with diluent.

Procedure

Each level was injected into the chromatographic system and peak area was measured. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and the correlation coefficient was calculated.

Acceptance criteria

- Correlation coefficient should be not less than 0.999.

3. Range

Based on precision, linearity and accuracy data it can be concluded that the assay method is precise, linear and accurate in the range of 20µg/ml-60µg/ml and 20µg/ml-60µg/ml of Ciprofloxacin respectively.

4. Accuracy

Preparation of standard stock solution

10mg of Ciprofloxacin working standard were accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette out 1 ml of the above stock solution into a 10 ml volumetric flask and was diluted up to the mark with diluent.

Preparation of sample solutions

For preparation of 50% solution (with respect to target assay concentration)

5mg of Ciprofloxacin working standard were accurately weighed and transferred into a 10 ml clean dry volumetric flask add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock Solution).Further pipette out 10 ml of the above stock solution into a 100ml volumetric flask and was diluted up to the mark with diluent.

For preparation of 100% solution (with respect to target assay concentration)

10 mg of Ciprofloxacin working standards were accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2 ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette out 1ml of above stock solution into a 10 ml volumetric flask and was diluted up to the mark with diluent.

For preparation of 150% solution (with respect to target assay concentration)

15 mg of Ciprofloxacin working standards into a 10ml clean dry volumetric flask add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette out 1ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

Procedure

The standard solutions of accuracy 50%, 100% and 150%were injected into chromatographic system. Calculate the amount found and amount added for Ciprofloxacin and calculate the individual % recovery and mean % recovery values.

Acceptance criteria

- The % recovery for each level should be between 98.0 to 102.0%

5. Precision

5.1 Repeatability

Preparation of stock solution

10 mg of Ciprofloxacin working standard were accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2ml of diluent

and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette out 0.4ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

Procedure: The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Acceptance criteria

- ❖ The % RSD for the area of five standard injections results should not be more than 2.

5.2 Intermediate Precision/Ruggedness

To evaluate the intermediate precision (also known as ruggedness) of the method, precision was performed on different days by using different make column of same dimensions.

Preparation of stock solution

10 mg of Ciprofloxacin working standard were accurately weighed and transferred into a 10ml clean dry volumetric flask add about 2ml of diluent and sonic ate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette out 0.4ml of the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

Procedure

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

Acceptance criteria

- ❖ The % RSD for the area of five sample injections results should not be more than 2%.

6. Limit of detection (LOD)

LOD's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula. The standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$\mathbf{LOD = 3.3\ X\ \frac{\sigma}{S}}$$

Where

σ - Standard deviation (SD)

S - Slope

7. Limit of quantification

LOQ's can be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula. Again, the standard deviation of the response can be determined based on the standard deviation of y-intercepts of regression lines.

Formula:

$$\mathbf{LOQ = 10\ X\ \frac{\sigma}{S}}$$

Where

σ - Standard deviation

S - Slope

8. Robustness

As part of the robustness, deliberate change in the flow rate, mobile phase composition was made to evaluate the impact on the method.

a) The flow rate was varied at 0.8ml/min to 1.2ml/min. Standard solution 40ppm of Ciprofloxacin was prepared and analysed using the varied flow rates along with method flow rate.

b) The organic composition in themobile phase was varied from 65% to75 %standard solution 40 µg/ml of Ciprofloxacin were prepared and analysed using the varied mobile phase composition along with the actual mobile phase composition in the method.

9. System suitability

10 mg of Ciprofloxacin working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and add about 2ml of diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette out 0.4ml of Ciprofloxacin from the above stock solution into a 10ml volumetric flask and was diluted up to the mark with diluent.

RESULTS AND DISCUSSIONS

The present investigation reported in the thesis was aimed to develop a new method development and validation for the estimation of Ciprofloxacin by RP-HPLC method. Literature reveals that there are no analytical methods reported for the estimation Ciprofloxacin by RP-HPLC method. Hence, it was felt that, there is a need of new analytical method development for the simultaneous estimation of Ciprofloxacin in pharmaceutical dosage form.

Method Development

The chromatographic method development for the estimation of Ciprofloxacin were optimized by several trials for various parameters as different column, flow rate and mobile phase, finally the following chromatographic method was selected for the separation and quantification of Ciprofloxacin in API and pharmaceutical dosage form by RP-HPLC method.

Chromatographic trials for estimation of Ciprofloxacin by RP- HPLC.

Trail	Mobile phase composition	Flow rate(mL/min)	Observation
Trail 1	Methanol: water(50:50)	1.0	No proper separation
Trail 2	Acetonitile:water(50:50)s	1.0	Broad peak, poor resolution
Trail 3	Acetonitrile pH8:phosphate buffer(50:50)	1.0	No proper separation
Trail 4	Acetonitrile pH3:phosphate buffer(65:35)	1.0	Peak shape is not good
Trail 5(optimized)	Methanol: phosphate buffer pH2.5(40:60)	1.0	Symmetric peak,Rt=4.449min

Optimized Chromatographic conditions

Column : Agilent C18 column (4.6×150mm)5µ
Mobile phase ratio : Methanol:pH2.5 phosphate buffer(40:60% v/v)
Detection wavelength : 270 nm
Flow rate : 1.0ml/min
Injection volume : 10µl
Column temperature : Ambient
Auto sampler temperature : Ambient
Run time : 8min
Retention time : 4.449mins

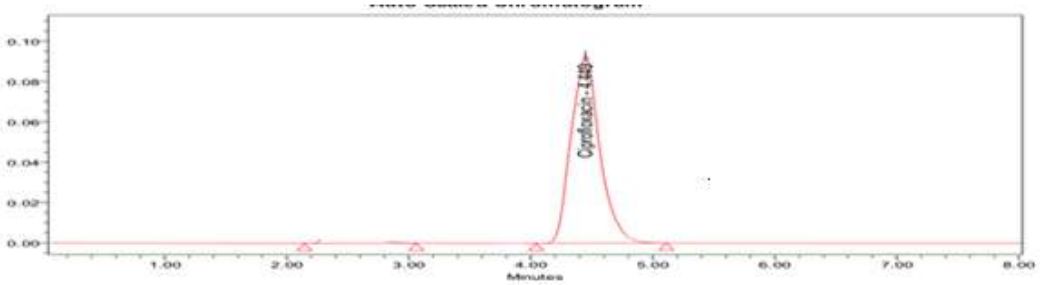
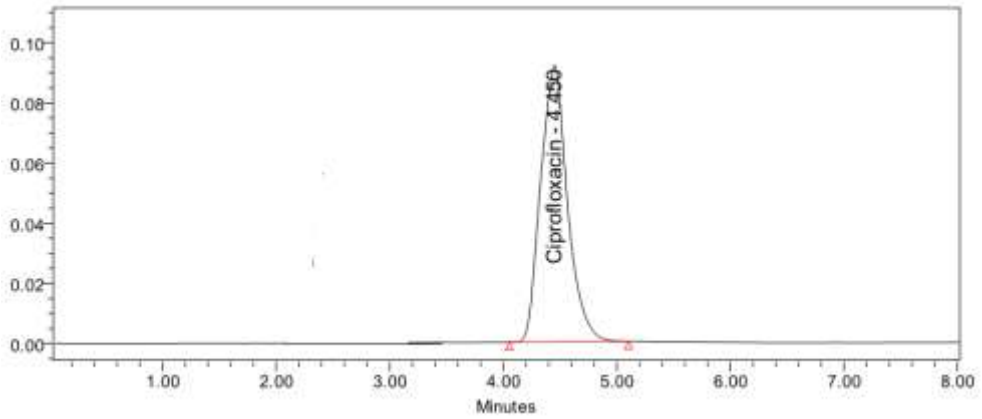


Figure: Representative RP-HPLC chromatogram of ciprofloxacin showing sharp, symmetric peak at Rt=4.449min

S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailig	USP Resolution
1	Ciproflaxacin	4.499	1497470	87075	2499	1.2	5.3

Observation

The separation was good, peak shape was good, so we conclude that there is no required for reduce the retention times of peaks, so it is taken as final method.



S.No	Peak Name	R_t	Area	Height	USP Plate Count	USP Tailing	USP Resolution
1	Ciproflaxacin	4.450	1507743	92010	2575	1.2	5.3

Figure: Representative RP-HPLC chromatogram of ciprofloxacin standard peak

Table. Showing linearity results for ciprofloxacin level -1 to level-5

S.No	Concentration	Peak Name	R_t	Area	Height
1	20µg/ml	Ciprofloxacin	4.420	623631	77864
2	30µg/ml	Ciprofloxacin	4.422	1001364	65557
3	40µg/ml	Ciprofloxacin	4.422	1333201	94130
4	50µg/ml	Ciprofloxacin	4.422	1623245	105760
5	60µg/ml	Ciprofloxacin	4.422	1977034	129435

Correlation Coefficient	0.9983

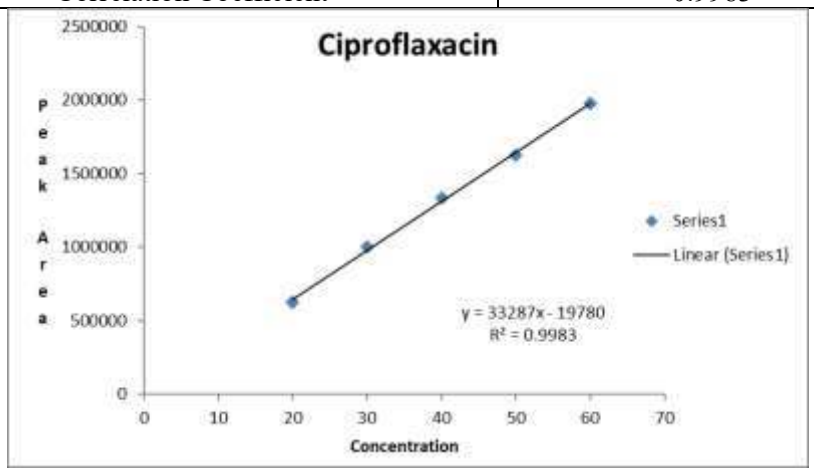


Fig. Showing calibration graph for CIPROFLAXACIN

The linearity study was performed for concentration range of 20µg-60µg and 20µg-60µg of ciprofloxacin and the correlation coefficient was found to be 0.998.(NLT 0.999).

Table : Chromatogram Values for Accuracy of Ciprofloxacin

Sample No.	Spike Level	Amount (mg) added	Amount (mg) found	% Recovery	Mean % Recovery
1	50 %	5	5.005	100.1%	100.24%
2	100 %	10	10.0	100.0%	
3	150 %	15	15.09	100.63%	

Acceptance Criteria:

- The % Recovery for each level should be between 98.0 to 102.0%.

SUMMARY AND CONCLUSION

A new method was established for ciprofloxacin by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of ciprofloxacin by using Agilent c18 column (4.6×150mm)5.0µm, flow rate was 0.8ml/min, mobile phase ratio was (40:60v/v) Methanol:phosphate buffer pH2.5 (pH was adjusted with orthophosphoric acid), detection wave length was 270nm. The instrument used was WATERS HPLC Auto Sam pler, Separation module 2695, UV detector 2487, Empower-software version-2. The retention times were found to be 4.449mins. The % purity of ciprofloxacin was found to be 100.24% respectively. The system suitability parameters for ciprofloxacin such as theoretical plates and tailing factor were found to be 2.499 and 1.2, the resolution was found to be 5.3. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study for ciprofloxacin was found in concentration range of 0.2ml-0.6ml and correlation coefficient (r^2) was found to be 0.9983% recovery was found to be 100.24%, %RSD for repeatability was 0.1, % RSD for intermediate precision was 0.06 respectively. The precision study was precise, robust, and repeatable. LOD value was 0.43, and LOQ value was 1.3 respectively.

- Hence the suggested RP-HPLC method can be used for routine analysis of ciprofloxacin in API and Pharmaceutical dosage form.

Table- Overall summarized method validation results

S. No	Parameter	Requirement	Results	Acceptance criteria
			CIPROFLOXACIN	
1.	**System suitability**	R_t	4.449 mins	
2.		Tailing factor	1.2	NMT 2
3.		Resolution	5.3	NLT 2
4.		Plate count	2499.0	NLT 2000
5.		Assay value	100.9%	100 ± 2.0%
6.	**Accuracy**	% recovery	100.24%	100 ± 2.0%
7.	**Precision**	%RSD	0.1	NMT 2%
8.	**Intermediate precision**	%RSD	0.06	NMT 2%
9.	**Linearity**	Correlation coefficient	0.998	NLT 0.999
10.	**LOD**		0.43	LOQ is three times more than LOD
11.	**LOQ**		1.3	
12	**Robustness**	More flow	R_t=3.788	Robust even by change in the flow rate ±0.2ml/min
		Less flow	R_t=5.572	
		More organic	R_t=3.788	Robust even by change in the mobile phase ±5%.
		Less organic	R_t=5.572	

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