“DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD AND UV-SPECTROPHOTOMETRIC SIMULTANEOUS EQUATION METHOD OF ACECLOFENAC AND DIACEREIN IN COMBINED DOSAGE FORM”

‘DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD AND UV-SPECROPHOTOMETRIC SIMULTANEOUS EQUATION METHOD OF MOSAPRIDE AND PANTOPRAZOLE IN COMBINED DOSAGE FORM”

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CERTIFICATE

This is to certify that the Dissertation entitled **"Development and Validation of RP-HPLC method and UV -Spectrophotometric Simultaneous equation method of Aceclofenac and Diacerein in combined dosage form", "Development and Validation of RP-HPLC method and UV- Spectrophotometric Simultaneous equation method of Mosapride and Pantoprazole in combined dosage form"** by **Mr.K.ALAGURAJ** in the department of Pharmaceutical Chemistry, Madurai Medical College, Madurai - 625 020, in partial fulfillment of the requirements for the **Degree of Master of Pharmacy** in Pharmaceutical Chemistry under my guidance and supervision during the academic year **2008-2009**.

This dissertation is forwarded to The Controller of Examination, The Tamilnadu Dr. MGR Medical University, Chennai.

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INDEX

INTRODUCTION

Analytical chemistry is the study of the chemical composition of natural and artificial materials.Unlike other major sub disciplines of chemistry such as inorganic chemistry and organic chemistry, analytical chemistry is not restricted to any particular type of chemical compound or reaction.Properties studied in analytical chemistry include geometric features such as molecular morphologies distributions of species, as well as features such as composition and species identity. The contributions made by analytical chemists have played critical roles in the sciences ranging from the development of concepts and theories (pure science) to a variety of practical applications, such as biomedical applications, environmental monitoring, quality control of industrial manufacturing and forensic science

.

Modern analytical chemistry is dominated by instrumental analysis. Many analytical chemists focus on a single type of instrument.Academics tend to either focus on new applications and discoveries or on new methods of analysis.

An effort to develop a new method might involve the use of a tunable laser to increase the specificity and sensitivity of a spectrometric method. Many methods, once developed,are kept purposely static so that data can be compared over long periods of time.

Traditionally, analytical chemistry has been split into two main types

[1] Qualitative

Qualitative inorganic analysis seeks to establish the presence of a given element or inorganic compound in a sample.

Qualitative organic analysis seeks to establish the presence of a given functional group or organic compound in a sample.

[2] Quantitative

Quantitative analysis seeks to establish the amount of a given element or compound in a sample.

Most modern analytical chemistry is categorized by two different approaches such as analytical targets or analytical methods .

By Analytical Targets

Bioanalytical chemistry

Material analysis

Chemical analysis

Environmental analysis

Forensics

By Analytical Methods

Spectroscopy

Mass Spectrometry

Spectrophotometry and Colorimetry

Chromatography and Electrophoresis

Crystallography

Microscopy

Electrochemistry

Traditional analytical techniques

Although modern analytical chemistry is dominated by sophisticated instrumentation, the roots of analytical chemistry and some of the principles used in modern instruments are from traditional techniques many of which are still used today. These techniques also tend to form the backbone of most undergraduate analytical chemistry educational labs.

Titration

Titration involves the addition of a reactant to a solution being analyzed until some equivalence point is reached. Often the amount of material in the solution being analyzed may be determined. Most familiar to those who have taken college chemistry is the acid-base titration involving a color changing indicator. There are many other types of titrations, for example potentiometric titrations. These titrations may use different types of indicators

to

reach some equivalence point.

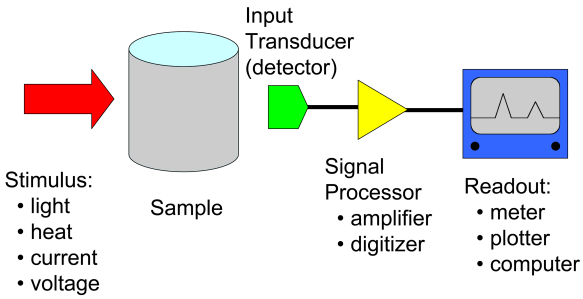
Gravimetry.

Gravimetric analysis involves determining the amount of material present by weighing the sample before and/or after some transformation. A common example used in undergraduate education is the determination of the amount of water in a hydrate by heating the sample to remove the water such that the difference in weight is due to the water lost.

Inorganic qualitative analysis

Inorganic qualitative analysis generally refers to a systematic scheme to confirm the presence of certain, usually aqueous, ions or elements by performing a series of reactions that eliminate ranges of possibilities and then confirms suspected ions with a confirming test. Sometimes small carbon containing ions are included in such schemes. With modern instrumentation these tests are rarely used but can be useful for educational purposes and in field work or other situations where access to state-of-the-art instruments are not available or expedient.

Instrumental Analysis



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

Spectroscopy[1,2,3]

Spectroscopy measures the interaction of the molecules with electromagnetic radiation. Spectroscopy consists of many different applications such as atomic absorption spectroscopy, atomic emission spectroscopy, ultraviolet-visible spectroscopy, x-ray fluorescence spectroscopy, infrared spectroscopy, Raman spectroscopy, nuclear magnetic resonance spectroscopy, photoemission spectroscopy, Mössbauer spectroscopy and so on.

Mass Spectrometry

Mass spectrometry measures mass-to-charge ratio of molecules using electric and magnetic fields. There are several ionization methods: electron impact, chemical ionization, electrospray, fast atom bombardment, matrix assisted laser desorption ionization, and others. Also, mass spectrometry is categorized by approaches of mass analyzers: magnetic-sector,quadrupole mass analyzer, quadrupole ion trap, Time-of-flight, Fourier transform ion cyclotron resonance, and so on.

Crystallography

Crystallography is a technique that characterizes the chemical structure of materials at the atomic level by analyzing the diffraction patterns of usually x-rays that have been deflected by atoms in the material. From the raw data the relative placement of atoms in space may be determined.

Electrochemical Analysis

Electroanalytical methods measure the potential (volts) and/or current (amps) in an electrochemical cell containing the analyte.[5][6] These methods can be categorized according to which aspects of the cell are controlled and which are measured. The three main categories are potentiometry (the difference in electrode potentials is measured), coulometry (the cell's current is measured over time), and voltammetry (the cell's current is measured while actively altering the cell's potential).

Thermal Analysis

Calorimetry and thermogravimetric analysis measure the interaction of a material and heat.

Separation

Separation processes are used to decrease the complexity of material mixtures. Chromatography and electrophoresis are representative of this field.

Hybrid Techniques

Combinations of the above techniques produce "hybrid" or

"hyphenated" techniques. Several examples are in popular use today and new hybrid techniques are under development. For example,

Gas chromatography-mass spectrometry, LC-MS, GC-IR, LC-NMR, LC-IR, CE-MS, and so on.

Hyphenated separation techniques refers to a combination of two (or more) techniques to detect and separate chemicals from solutions. Most often the other technique is some form of chromatography. Hyphenated techniques are widely used in chemistry and biochemistry. A slash is sometimes used instead of hyphen, especially if the name of one of the methods contains a hyphen itself.

Examples of hyphenated techniques:
LC-MS (or HPLC-MS)
HPLC/ESI-MS
LC-DAD
CE-MS
CE-UV
GC-MS
LC-IR

Microscopy

The visualization of single molecules, single cells, biological tissues and nano- micro materials is very important and attractive approach in analytical science. Also, hybridization with other traditional analytical tools is revolutionizing analytical science. Microscopy can be categorized into three

different fields: optical microscopy, electron microscopy, and scanning probe microscopy. Recently, this field is rapidly progressing because of the rapid development of computer and camera industries.

Lab-on-a-chip

Devices that integrate (multiple) laboratory functions on a single chip of only millimeters to a few square centimeters in size and that are capable of handling extremely small fluid volumes down to less than pico liters.

Methods and data analysis

Standard Curve

A standard method for analysis of concentration involves the creation of a calibration curve. This allows for determination of the amount of a chemical in a material by comparing the results of unknown sample to those of a series known standards.If the concentration of element or compound in a sample is too high for the detection range of the technique, it can simply be diluted in a pure solvent. If the amount in the sample is below an instrument's range of measurement, the method of addition can be used. In this method a known quantity of the element or compound under study is added, and the difference between the concentration added, and the concentration observed is the amount actually in the sample.

Internal Standards

Sometimes an internal standard is added at a known concentration directly to an analytical sample to aid in quantitation. The amount of analyte present is then determined relative to the internal standard as a calibrant.

Analytical chemistry research is largely driven by performance (sensitivity, selectivity, robustness, linear range, accuracy, precision, and speed), and cost (purchase, operation, training, time, and space). Among the main branches of contemporary analytical atomic spectrometry, the most widespread and universal are optical and mass spectrometry (see Prospects in Analytical Atomic Spectrometry). In the direct elemental analysis of solid samples, the new leaders are laser-induced breakdown and laser ablation mass spectrometry, and the related techniques with transfer of the laser ablation products into inductively coupled plasma.

Advances in design of diode lasers and optical parametric oscillators promote developments in fluorescence and ionization spectrometry and also in absorption techniques where uses of optical cavities for increased effective absorption pathlength are expected to expand. Steady progress and growth in applications of plasma- and laser-based methods are noticeable. An interest towards the absolute (standardless) analysis has revived, particularly in the

emission spectrometry.

A lot of effort is put in shrinking the analysis techniques to chip size. Although there are few examples of such systems competitive with traditional analysis techniques, potential advantages include size/portability, speed, and cost. (micro Total Analysis System (μTAS) or Lab-on-a-chip). Microscale chemistry reduces the amounts of chemicals used.

Much effort is also put into analyzing biological systems. Examples of rapidly expanding fields in this area are:

1)Genomics - DNA sequencing and its related research. Genetic fingerprinting and DNA microarray are very popular tools and research fields.

2)Proteomics - the analysis of protein concentrations and modifications, especially in response to various stressors, at various developmental stages, or in various parts of the body.

3)Metabolomics - similar to proteomics, but dealing with metabolites.

4)Transcriptomics- mRNA and its associated field

5)Lipidomics - lipids and its associated field

6)Peptidomics - peptides and its associated field

7)Metalomics - similar to proteomics and metabolomics, but dealing with metal concentrations and especially with their binding to proteins and other molecules.

Analytical chemistry has played critical roles in the understanding of basic science to a variety of practical applications, such as biomedical applications, environmental monitoring, quality control of industrial manufacturing, forensic science and so on.

The recent developments of computer automation and information technologies have innervated analytical chemistry to initiate a number of new biological fields.

For example, automated DNA sequencing machines were the basis to complete human genome projects leading to the birth of genomics. Protein identification and peptide sequencing by mass spectrometry opened a new field of proteomics. Furthermore, a number of ~omics based on analytical chemistry have become important areas in modern biology.

Also, analytical chemistry has been an indispensable area in the development of nanotechnology. Surface characterization instruments, electron microscopes and scanning probe microscopes enables scientists to

visualize atomic structures with chemical characterizations.

Analytical chemistry is pursuing the development of practical applications and commercial instruments rather than elucidating scientific fundamentals. This may be an arguable difference from overlapping science areas such as physical chemistry and biophysics, although there isn't any distinct

boundaries among disciplines in contemporary science and technology.

However, this aspect may attract many engineers' interest; thus, it is not difficult to see papers from engineering departments in analytical chemistry journals.

Among active contemporary analytical chemistry research fields, micro total analysis system is considered as a great promise of revolutionary technology. In this approach, integrated and miniaturized analytical systems are being developed to control and analyze single cells and single molecules. This cutting-edge technology has a promising potential of leading a new revolution in science as integrated circuits did in computer developments

SPECTROSCOPY

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

It is of two types,

Absorption Spectroscopy:

Where absorption of electro magnetic radiation (EMR) takes place.

(eg.) Colorimetry, UV spectroscopy, IR spectroscopy, etc.

Emission Spectroscopy:

Where emission of radiation is being studied.

(eg.) Fluorimetry, Flame Photometry.

Quantitative Spectrophotometric Assay:

The assay of an absorbing substance may be quickly carried out by preparing a solution in a transparent solvent and measuring its absorbance at a

suitable wavelength.

The wavelength normally selected is a wavelength of maximum absorption (λmax).

UV – SPECTROSCOPY [4,5]

It involves the measurement of amount of ultra-violet radiation absorbed by a substance in the solution. The wavelength between 190 – 390 nm (practically 200 – 400 nm) is considered to be UV radiations/ region. Coloured compounds absorb in visible range i.e. 400-800 nm.

The assay of an absorbing substance can be carried out by using

Standard absorptivity Value.

Use of calibration graph.

Single point standardisation.

Standard absorptivity value:

This procedure is adopted by official compendia for the stable substance that have reasonably broad absorption bands and which are practically unaffected by variation of instrumental parameters. The use of standard A (1%, 1cm) value avoids the need to prepare a standard solution of

the reference substance in order to determine its absorptivity.

Use of calibration graph:

In this procedure, the absorbance of a number (typically 4-6) of standard solution of the reference substance at concentrations encompassing

sample concentration are measured and a calibration graph is constructed. The concentration of the analyte in the sample solution is read from the graph as a concentration corresponding to absorbance of the solution .

Single point standardization

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

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

Where C test and C standard are the concentrations in the sample and standard solutions and A test and A standard are the absorbances of sample and standard solutions respectively. Important characteristics of spectrophotometric methods

include.

High sensitivities

Moderate to high selectivity

Good accuracy

Wide applicability to both organic and inorganic systems.

Easy and convenience of data acquisition.

The use of UV and visible spectroscopy for quantitative analysis employs the method of comparing the absorbance of standards and samples at a selected wavelength. The analysis of mixtures of two or more components is facilitated by activity of absorbance. Other applications include measurement of absorption of complexes to establish their composition. All chromogenic compounds are not suitable for quantitative measurements, i.e. the choice of the system and procedure depends largely on the chemistry of the species to be determined.

ASSAY OF SUBSTANCES IN MULTI COMPONENT SAMPLES

They are

Simultaneous equation method

Absorbance ratio method

Derivative spectroscopy method.

Chemical Derivatisation Methods.

Multi-component mode of analysis.

SIMULTANEOUS EQUATION METHOD:[6]

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

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

$$\frac{A2 / A1}{ax2 / ax1} \quad \text{and} \quad \frac{ay2 / ay1}{A2 / A1}$$

Where ax1, ax2 = Absorptivities of X at λ1 and λ2

ay1, ay2 = Absorptivities of X at λ1 and λ2

A1, A2 = Absorbances of the diluted sample at λ1 and λ2.

The ratio should lie outside the range of 0.1 – 2.0 for the precise determination

of (Y and X) two drugs respectively.

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

ABSORBANCE RATIO METHOD

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

DERIVATIVE SPECTROSCOPIC METHOD:

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

For the purpose of spectral analysis to relate chemical structure to electronic transition and for analytical situations in which mixtures contribute interfering absorption, a method of manipulating the spectral data called derivative spectroscopy was developed.

In this technique spectra are obtained by plotting the first or higher derivation of absorbance or transmittance with respect to wavelength versus wavelength. Often these plots reverse spectral details, with respect to wavelength versus wavelength. Often these plots reverse spectral details, which is lost in an ordinary spectrum. In addition concentration measurements of an analyte in the presence of their interference can sometimes be made easily or more accurately.

Enhanced resolution, band width discrimination are the advantages of derivative spectrophotometry that permit the selective discrimination of certain absorbing substances in samples in which non specific interferences may prohibit the application of simple spectrophotometric methods.

CHEMICAL DERIVATISATION METHOD:

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

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

The interference from irrelevant absorption may be avoided by converting the

analyte to its derivative which absorbs in the visible region where irrelevant absorption is negligible.

Indirect spectrophotometric procedure is also used to improve the selectivity of the assay of an ultra violet absorbing substance in a sample that contains other UV-absorbing components.

Assay as single component sample:-

The concentration of a component in a sample which contains other absorbing substances may be determined by a simple spectrophotometric measurement of absorbance.

Assay as a single component sample for interference :-

If the identityl, concentration and absorptivity of the absorbing interferents are known. It is possible to calculate their contribution to the total absorbance of a mixture.

The concentration of the absorbing component of interest is then calculated from the corrected absorbance, (total absorbance minur the absorbance of the interfering substances) in the usual way.

Difference spectrophotometry:-

Difference spectrophotometric assay is that the measured value is the difference absorbance (AA) between two equimolar solutions of the analyte in

different chemical forms which exhibit different spectral characteristics.

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

1) reproducible changes may be induced in the spectrum of the analyte by the addition of one or more reagents.
2) The absorbance of the interfering substances not altered by reagents.

CHROMATOGRAPHY [7,8]

. Modern pharmaceutical formulations are complex mixtures containing one or more therapeutically active ingredients. to a number of inert materials like diluents, disintegrants, clourants and flavours. In order to ensure quality and stability of the final product, the pharmaceutical analyst must be able to seperate the mixutres into individual components prior to quantitative analysis.

Amongst the most powerful techniques available to the analyst for the seperation of these mixutres, a group of highly efficient methods which are collectively called as chromatography.

It's a group of technique which works on the principle of seperation of compounents of a mixutre into individual components, depending on their effinities for the soluties between two immicible phases. one of the phases is affixed bed of large surface area, white the other is a fluid, which moves through the surface of the fixed phase. The fixed phase is called statinary phase

and the other is termed as the mobile phase.

Depending on the type of chromatography employed, the mobile phase may be a pure liquid or a mixture of solutions (eg. Buffer) or it may be gas (pure or homogenous mixture)

Classification of chromatograpy:-

It can classified into five types based on the type of equilibrium process. The different type of chromatography are

1. Adsorption chromatography
2. Partition chromatography
3. Ion exchange chromatography
4. Size exclusion or gel permeation chromatography
5. Affinity chromatography

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

Adsorption chromatography:-

In adsorption chromatography, the mobile phase containing the dissolved solutes passes over the surface of the stationary phase. Retention of the component and their consequent separation depends on ability of the solutes from the mobile phase and adsorb them temporarily by means of electrostatic forces. Usually silica or alumina is utilized as the adsorbent with relatively non

polar solvents such as hexane as the mobile phase in normal phase adsorption non where as in reversed beds with relative polar solvents such as water, acetonitile and methamol are used as mobile phase.

Partition chromatography:-

In partition chromatography an inert solid material such as silica get or diatomaceous earth serves to support a thin layer of liquid which is the effective stationary phase. As the mobile phase containing the solutes parses in close proximity to this liquid phase, retention and sepration occurs due to the solubility of the analytes in the two fluids as determined by their partition co efficients.

The method sffers from dis advantage due to some solubility of stationary phase in the mobile phase. Hence precautions must be taken to limit dissolution of stationary phase.

Ion Exchange chromatography:-

In ion exchange chromatography the stationary phase consist of a polymeric resinmatrix on the suface of ionic functional groups, eg. Carboxylic acids or quaternany ammes have been bonded chemically. As the mobile phase passes over this surface, ionic solutes are retained by forming electrostatic

chemical bonds with the functional groups. The mobile phase used in this type is always liquid.

Size exclusion chromatography:-

In size exclusion chromatography the stationary phase is a polymeric substance containing numerous pores of mole cular dimensions. Solutes whose molecular size is sufficiently small will leave the mobile phase to diffrue into the pores. Large, remain in the mobile phase and are not retained. This method is mostly suitable for the separation of mixtures in which the solutes vary considerably in molecular size. The mobile phase in this type may either liquid or gaseous.

Affinity chromatography:-

This technique utilizes highly specific interaction between one kind of Solute molecule and a second molecule covalently attached to the stationary phase.

High performance liquid chromatography: [9, 10]

The technique of high performance liquid chromatography (HPLC) was developed in the late 1960s and early 1970s.The technique is based on the same modes of separation as classical column chromatography, i.e. adsorption, partition (including reversed-phase partition), ion exchange and gel permeation.

Apparatus:

The mode of operation of this system is isocratic, i.e.one particular solvent or mixture is pumped throughout the analysis. For some determinations the solvent composition may be altered gradually to give gradient elution.

Pumps:

Pumps are required to deliver a constant flow of mobile phase at pressures ranging from 1 to 550 bar. Mechanical pumps of the reciprocating piston type give a pulsating supply of mobile phase. A damping device is therefore required to smooth out the pulses so that excessive noise at high levels of sensitivity or low pressures does not detract from detection of small

quantities of sample. This type of pump is extremely useful, however, in that a constant volume of liquid is delivered, the actual value being set by adjustment of piston stroke.

Dual-piston reciprocating pumps produce an almost pulse-free flow because the two pistons are carefully phased so that as one is filling the other is pumping. These pumps are more expensive than single piston pumps but are of benefit when using a flow-sensitive detector such as an ultraviolet or refractive index detector.

Modern injectors are based on injection valves which allow the sample at atmospheric pressure to be transferred to the high-pressure mobile phase immediately before the column inlet. The design of different valves varies widely but a typical arrangement is with the injector in the LOAD position, the sample is injected from a syringe through a needle port into the loop. The valve lever is then turned through 600 to the INJECT position and the sample is swept into the flowing mobile phase. If an excess of sample is flushed through the loop in the LOAD position, the volume injected is the volume of the loop, which is typically 10-20 μl for analytical separations and 0.1-1 ml for semi-preparative or preparative separations. This complete filling procedure offers

the analyst the highest reproducibility, and is capable of giving relative standard deviations of less than 0.2%.

Detectors:

It should be sensitive to at least 10-8g ml-1 and give a linear response over a wide concentration range. It should also have a low dead volume to reduce further band-broadening of the components in the detector and

good stability to prevent fluctuations of the response. The most commonly used detectors in the HPLC analysis of pharmaceutical substances are described below.

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

Single wavelength detectors: Equipped with a low-pressure mercury discharge lamp. The absorbance is measured at the most intense resonance wavelength of mercury at 254nm.

Multi-wavelength detectors:

Employ mercury and other discharge sources which, when used in

combination with interference filters allow a number of monochromatic wavelengths to be selected, e.g. 206, 226, 280, 313, 340 or 365 nm.

Variable wavelength detectors: use a deuterium light source and a grating monochromator to allow selection of any wavelength in the deuterium (190-360nm)

Programmable detectors:

Allow the automatic change of wavelength between and during chromatographic analysis.

Diode array detectors:

Are microprocessor-controlled photodiode array spectrophotometers in which light from an ultraviolet source passes through the flow cell into a polychromator which disperses the beam so that the full spectrum fall on the array of diodes. Each diode detects light at a discrete wavelength and the array provides an almost instantaneous absorption spectrum of the solute in the eluate.

Fluorescence detectors:

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

Refractive index detectors:

These are differential refractometers which respond to the change in

the bulk property of the refractive index of the solution of the component in the mobile solvent system. The refractive index detector is the closest approach to the universal detector in that some solvent is usually available in which the sample gives rise to a measurable difference in refractive index between solvent and solution.

Electrochemical detectors:

These are based on standard electrochemical principles involving amperometry, voltametry and polarography.

Columns:

HPLC columns are made of high quality stainless steel , polished internally to a mirror finish. Standard analytical columns are 4-5mm internal diameter and 10-30 cm in length. Shorter columns (3-6cm in length) containing a small particle size packing material (3 or 5μm) produce similar or better efficiencies, in terms of the number of theoretical plates(about 7000), that those of 20 cm columns containing 10μm irregular particles and are used when short analysis times and high throughput of samples are required.

Micro bore columns of 1-2 mm internal diameter and 10-25cm in length have certain advantages of lower detection limits and lower consumption of solvent, the latter being important if expensive HPLC-grade solvents are used. HPLC may also be carried out on the semi- preparative or preparative

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

PRINCIPLES OF OPERATION:

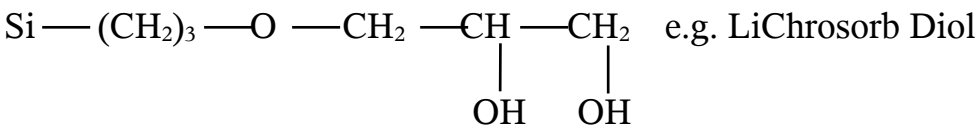
Adsorption HPLC:

Unmodified silica has proved to be the most widely used in HPLC. It offers high efficiency and a high permeability which allows normal operating pressures of less than 2000 psi to be used. The functional group responsible for adsorption is the silanol (Si-OH) group, which interacts with the sample solutes by hydrogen bonding. There is therefore increasing retention of solutes with increasing solute polarity. Alumina is used as an adsorbent less frequently than silica, although for some separations, in particular of aromatic substances and of structural isomers, greater selectively is obtained with alumina.

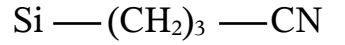
Partition HPLC:

Packing materials based on silica are also used in partition chromatography. Early applications of partition HPLC involved coating the silica (in this case acting as a support) with a polar liquid stationary phase, e.g.

ethane-1, 2-diol, and using as the mobile phase a less polar solvent or mixture of solvents saturated with the stationary phase to avoid the loss of the stationary phase by dissolution in the mobile phase. Such packing materials have now been replaced by silica to which polar phases are chemically bonded. Examples of the functional groups in these chemically bonded partition HPLC packing materials are:



e.g. LiChrosorb Diol



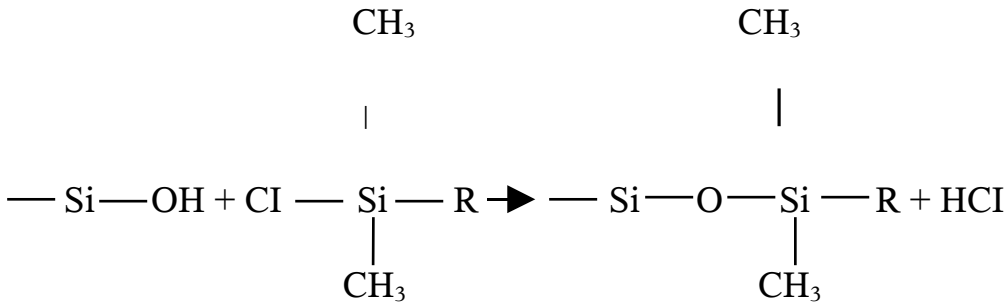
e.g. μ Bondpak CN

e.g. Polygosil NH2

The order of increasing polarity of these is diol: cyano : amino. This type of chromatography, in which the mobile phase is less polar than the stationary phase, is called normal-phase partition chromatography. As the adsorption chromatography, substances are eluted in the order of their increasing polarities, i.e. the least polar substance is eluted first.

In reversed-phase partition HPLC the relative polarities of the stationary and mobile phases are the opposite to those in normal-phase HPLC, i.e. the stationary phase is less polar than the mobile phase, and consequently the solutes are eluted in the order of their decreasing polarities. The stationary phase

is silica, chemically bonded through a siloxane (Si O Si C) linkage to a low polar functional group. These phases are prepared by treating the surface silanol groups of silica with an organochlorosilane reagent:



where R = C6H13 (hexyl), C8H17 (octyl) or C18H37 (octadecyl).

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

Mobile phase in RP-HPLC:

The mobile phase in reversed-phase HPLC generally comprises water and a less polar organic solvent modifier, e.g. methanol or acetonitrile. Separations in these systems are considered to be due to different degrees of hydrophobicity of the solutes, the less polar solutes partitioning to a greater extent into the non-polar stationary phase and consequently being retained on the column longer

than the more polar solutes. The rate of elution of the components is controlled by the polarity of the organic modifier and its proportion in the mobile phase. The rate of elution is increased by reducing the polarity, e.g. by increasing the proportion of the organic solvent or by using acetonitrile instead of methanol. The simple alteration of the composition of the mobile phase or of the flow rate allows the rate of elution of the solutes to be adjusted to an optimum value and permits the separation of a wide range of chemical types.

For example, the retention time of carboxylic acids is increased by reducing the pH with phosphoric acid, acetic acid or buffers. Conversely, the retention of nitrogenous bases is increased by raising the pH. As the pH limits for reversed-phase columns are pH 2-7.5, the overall retention of substances with pKa values near the extremes of this range is made by adjusting both pH and solvent polarity ion pair RP-HPLC.

Ion-pair rp-hplc:

The partition coefficients of ionic and ionisable species between the non-polar stationary phase and the polar mobile phase can also be markedly affected by the presence of certain oppositely charged ions (counter ions) in the mobile phase. This effect is used in ion-pair reversed-phase HPLC. The principle, which is the same as that of the acid-dye technique in spectrophotometric analysis, is that a counter ion is added to the mobile phase, which forms an ion-pair renders it more hydrophobic and results in an increased

retention of the solute. Tetramethylammonium or tetrabutylammonium ions are used as counter ions for organic acids, and heptane sulphonate or dodecyl sulphonate (C12H25So3-) ions are used as counter ions for nitrogenous bases.

Ion exchange HPLC:

Packings for ion exchange HPLC are based on the cross-linked polystyrene-divinyl benzene resins or on ion exchange residues chemically bonded to silica.

Most ion exchange HPLC is carried out in aqueous media although the bonded phases can be used with mixtures of aqueous buffers and organic solvents if the solutes show poor water solubility.

Size exclusion HPLC:

Cross-linked polystryrene-divinylbenzene resins or silica microspheres (5-15 μm in diameter) are used to fractionate materials of high molecular weight. A single aqueous or organic solvent is used as the mobile phase and the desired retention of solutes on the column is achieved by the choice of the appropriate grade of the packing material for the molecular weight range of the solutes in the sample.

VALIDATION [11,12,13,14]

"Validation of an analytical method is the process **by** which it is established **by** laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application .

Validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation programme required depends entirely on the particular method and its proposed applications.

Typical analytical parameters used in assay validation include:

1. Precision
2. Accuracy
3. Linearity
4. Range
5. Ruggedness
6. Limit of detection
7. Limit of quantitation
8. Selectivity
9. Specificity

Precision

The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample .

Precision is a measure of the reproducibility of the *whole* analytical method (including sampling, sample preparation and analysis) under normal operating circumstances.

Precision is determined by using the method to assay a sample for a sufficient number of times to obtain statistically valid results (ie between 6 - 1 0). The precision is then expressed as the relative standard deviation

$$\%RSD = \frac{\text{std dev} \times 100\%}{\text{Mean}}$$

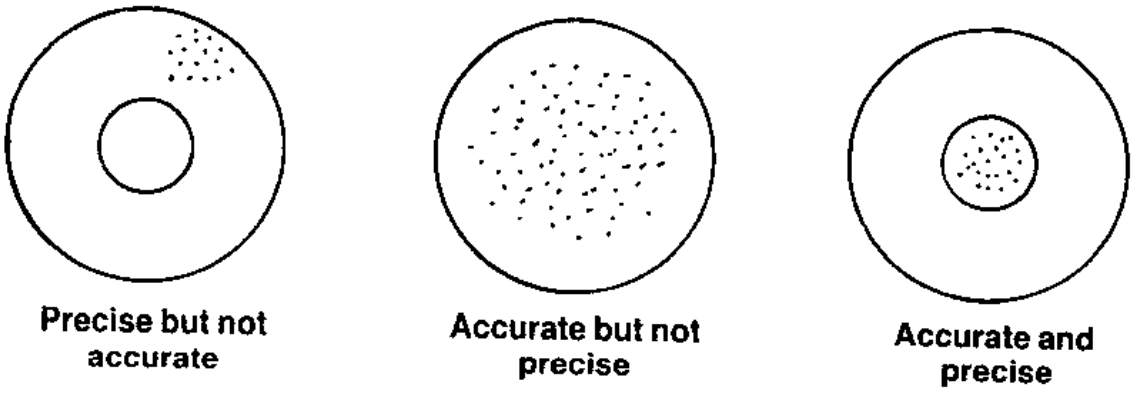
An example of precision criteria for an assay method is that the instrument precision (RSD) will be 1% and the intra-assay precision will be,, 2%.

2.2 Accuracy

Accuracy is a measure of the closeness of test results obtained by a method to the true value.

Accuracy indicates the deviation between the mean value found and the true value. lt is determined by applying the method to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay.

Accuracy and precision are not the same, as the diagram below indicates. A method can have good precision and yet not be accurate.



Errors in measurement can be divided into two general categories: systematic errors and random errors.

Systematic errors result from sources that can be traced to the methodology, the instrument or the operator, and affect both the accuracy and the precision of the

measurement.

Random errors only affect the precision, and are difficult to eliminate, because they are the result of random fluctuations in the measured signal, due to noise and other factors.

Whilst systematic errors are proportional to the sum of individual contributions, random errors are proportional to the root of the sum of the squares of the individual contributions.Thus, the imprecision of the entire procedure is often dominated by the random errors of the most imprecise step.

Accuracy is usually determined in one of four ways. First, accuracy can be assessed by analyzing a sample of known concentration and comparing the measured value to the true value. National Institute of Standards and Technology (NIST) reference standards are often used; however, such a well characterized sample is usually not available for new drug-related analytes. The second approach is to compare test results from the new method with results from an existing alternate method that is known to be accurate. Again, for pharmaceutical studies, such an alternate method is usually not available. The third and fourth approaches are based on the recovery of known amounts of analyte spiked into sample matrix.

The third approach, which is the most widely used recovery study, is performed by spiking analyte in blank matrices. For assay methods, spiked samples are prepared in triplicate at three levels over a range of 50-150% of the target concentration. If potential impurities have been isolated, they should be added to the matrix to mimic impure samples.

The fourth approach is the technique of standard additions, which can also be used to determine recovery of spiked analyte. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte. This can occur, for example, with lyophilized material, in which the speciation in the lyophilized material is significantly different when the analyte is absent.

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

Linearity

This is the method's ability to obtain results which are either directly, or after mathematical transformation proportional to the concentration of the analyte within a given range.

Linearity is determined by calculating the regression line using a mathematical treatment of the results (ie least mean squares) vs analyte concentration.

A linearity study verifies that the sample solutions are in a concentration range where analyte response is linearly proportional to concentration. For assay methods, this study is generally performed by preparing standard solutions at five concentration levels, from 50 to 150% of the target analyte concentration. Five levels are required to allow detection of curvature in the plotted data. The standards are evaluated using the chromatographic conditions determined during the specificity studies.

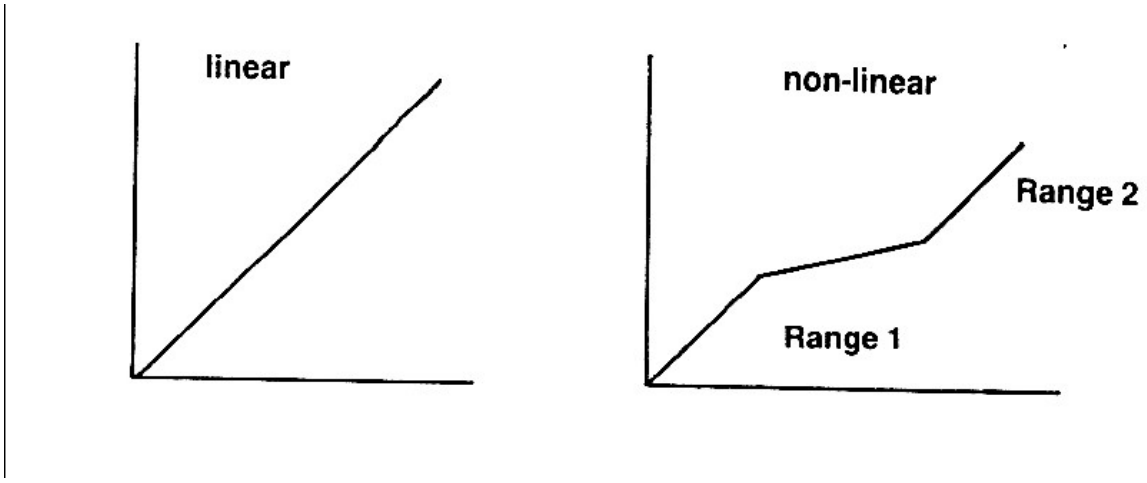
Standards should be prepared and analyzed a minimum of three times. The 50 to 150% range for this study is wider than what is required by the FDA guidelines. In the final method procedure, a tighter range of three standards is generally used, such as 80, 100, and 120% of target; and in some instances, a single standard concentration is used.

Validating over a wider range provides confidence that the routine standard levels are well removed from nonlinear response concentrations, that the method covers a wide enough range to incorporate the limits of content uniformity testing, and that it allows quantitation of crude samples in support of process development.

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

Range

The range of the method is the interval between the upper and lower levels of an analyte that have been determined with acceptable precision, accuracy and linearity. It is determined on either a linear or nonlinear response curve (ie where more than one range is involved, as shown below) and is normally expressed in the same units as the test results.



Limit of Detection

This is the lowest concentration in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The limit of detection is important for impurity tests and the assays of dosages containing low drug levels and placebos.

The limit of detection is generally quoted as the concentration yielding a signal-to-noise ratio of 2:1 and is confirmed by analyzing a number of samples near this value (6) using the following equation.

Based on the standard deviation of the response and the slope, the detection limit (DL) may be expressed as

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

Where
σ = standard deviation of the response
S= slope of the calibration curve (of the analyte)

.

Since the limit of detection is dependant on the signal-to-noise ratio, it can be improved by enhancing the analyte signal and reducing the detector noise. The signal (ie peak height) can be increased by selecting the optimum monitoring wavelength, increasing the injection volume or mass (below signal or column saturation), increasing the peak sharpness with high efficiency

columns and by optimizing the mobile phase. For absorbance detectors, longer path lengths in the flow cell enhances sensitivity though often to the detriment of post column dispersion.

Noise can be reduced by using high sensitivity detectors with low noise and drift characteristics, slower detector response time, mobile phases with low absorbance and pumps with low pulsation.

Limit of Quantitation

This is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.

lt is quoted as the concentration yielding a signal-to-noise ratio of 1 0: 1 and is confirmed by analyzing a number of samples near this value.

Based on the standard deviation of the response and the slope, quantitation limit may be expressed as

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

Where
σ = standard deviation of the response
S = slope of the calibration curve (of the analyte)

Stability:

During the earlier validation studies, the method developer gained some information on the stability of reagents, mobile phases, standards, and sample solutions. For routine testing in which many samples are prepared and analyzed each day, it is often essential that solutions be stable enough to allow for delays such as instrument breakdown or overnight analyses using auto samplers. At this point, the limits of stability should be tested. Samples and standards should be tested over a least a 48-hrs period, and quantitation of components should be determined by comparison to freshly prepared standards. If the solutions are not stable over 48-hrs, storage conditions or additives should be identified that can improve stability.

An example of stability criteria for assay methods is that sample and standard solutions and the mobile phase will be stable for 48 hrs under defined storage conditions. Acceptable stability is 2% change in standard or sample response, relative to freshly prepared standards. The mobile phase is considered to have acceptable stability if aged mobile phase produces equivalent chromatography (capacity factors, resolution, or tailing factor) and assay results are within 2% of the value obtained with fresh mobile phase.

Specificity

Specificity for an assay ensures that the signal measured comes from the substance of interest, and that there is no interference from excipient and/or degradation products and/or impurities.

For chromatographic methods, developing a separation involves demonstrating specificity, which is the ability of the method to accurately measure the analyte response in the presence of all potential sample components. The response of the analyte in test mixtures containing the analyte and all potential sample components (placebo formulation, synthesis intermediates, excipients, degradation products, process impurities, etc.) is compared with the response of a solution containing only the analyte. Other potential sample components are generated by exposing the analyte to stress conditions sufficient to degrade it to 80-90% purity. For bulk (0.1 N NaOH), and oxidant (3% H2O2) are typical. For formulated products, heat, light, and humidity (85%) are often used.

The resulting mixtures are then analyzed, and the analyte peak is evaluated for peak purity and resolution from the nearest eluting peak. If an alternate chromatographic column is to be allowed in the final method procedure, it should be identified during these studies.

Once acceptable resolution is obtained for the analyte and potential sample components, the chromatographic parameters, such as column type, mobile-phase composition, flow rate, and detection mode, are considered set.

Specificity criteria for an assay method is that the analyte peak will have baseline chromatographic resolution of at least 1.5 from all other sample components. If this cannot be achieved the unresolved components at their maximum expected levels will not affect the final assay result by more than 0.5%.

Determination of this can be carried out by assessing the peak identity and purity.

The purity index is a measure of the peak's relative purity, measured using a full comparison of spectral data for the leading and training edge of the peak Figure 4. A value of 1.5 is commonly accepted to indicate a pure peak but >1.5 would indicate the presence of an impurity, (9) as shown in Figure 5.

Figure 4: Diagram indicating the leading and trailing spectra used for peak purity

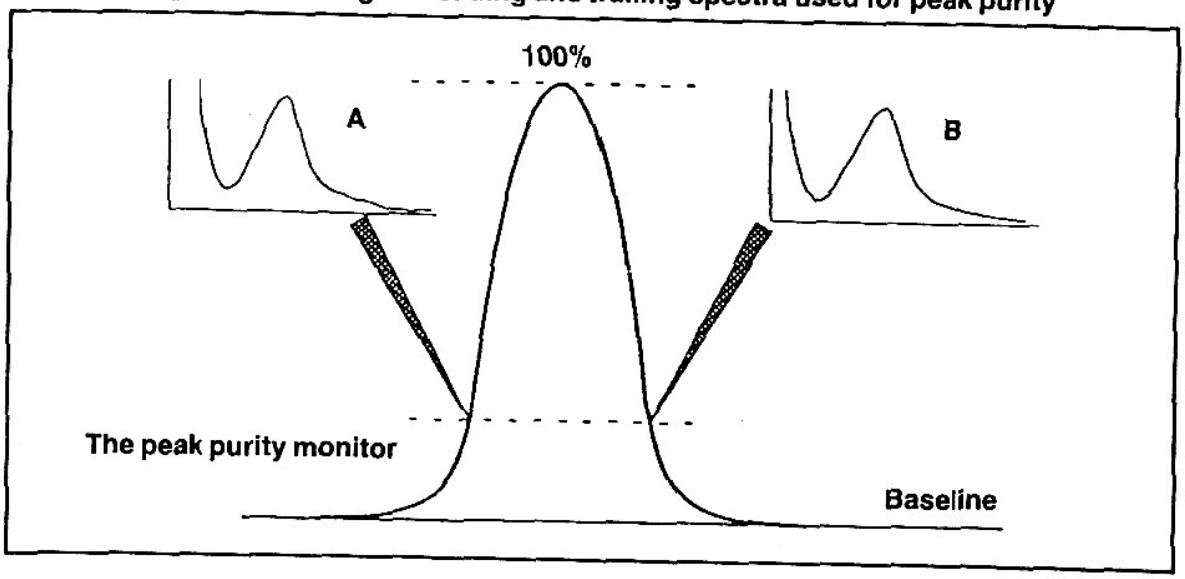


Figure 5:Spectral comparison showing peak purity

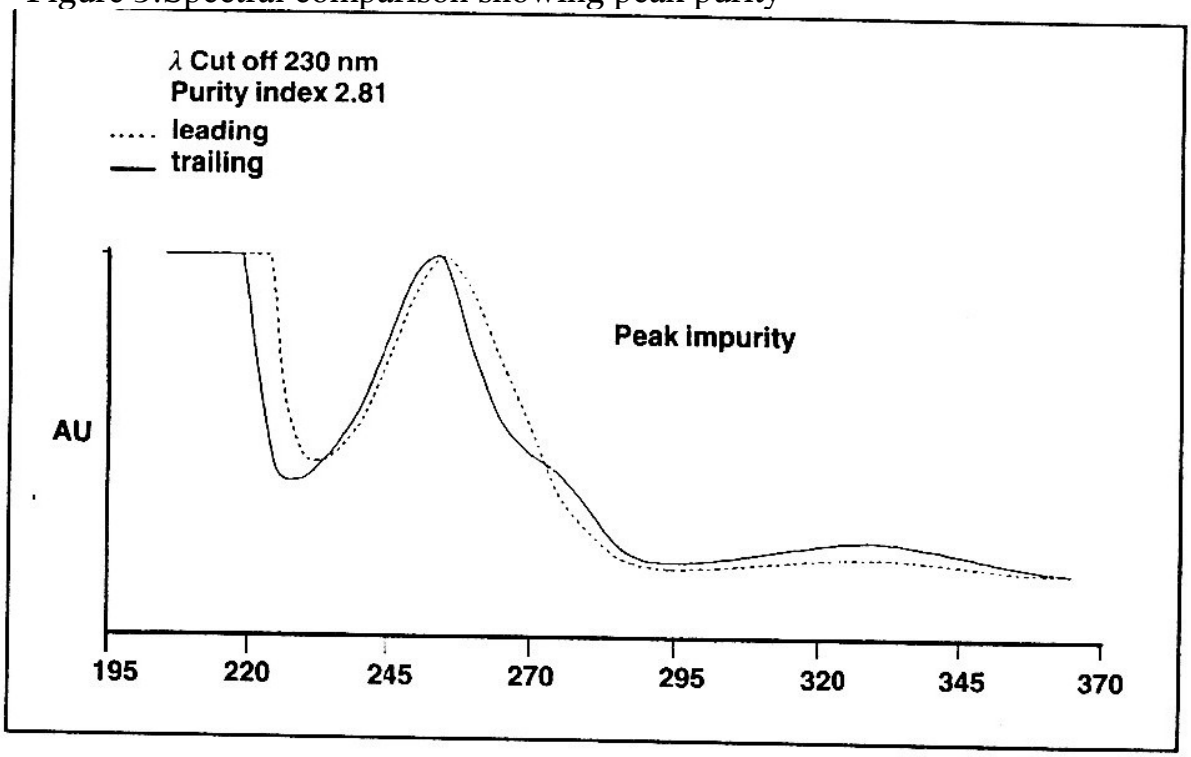
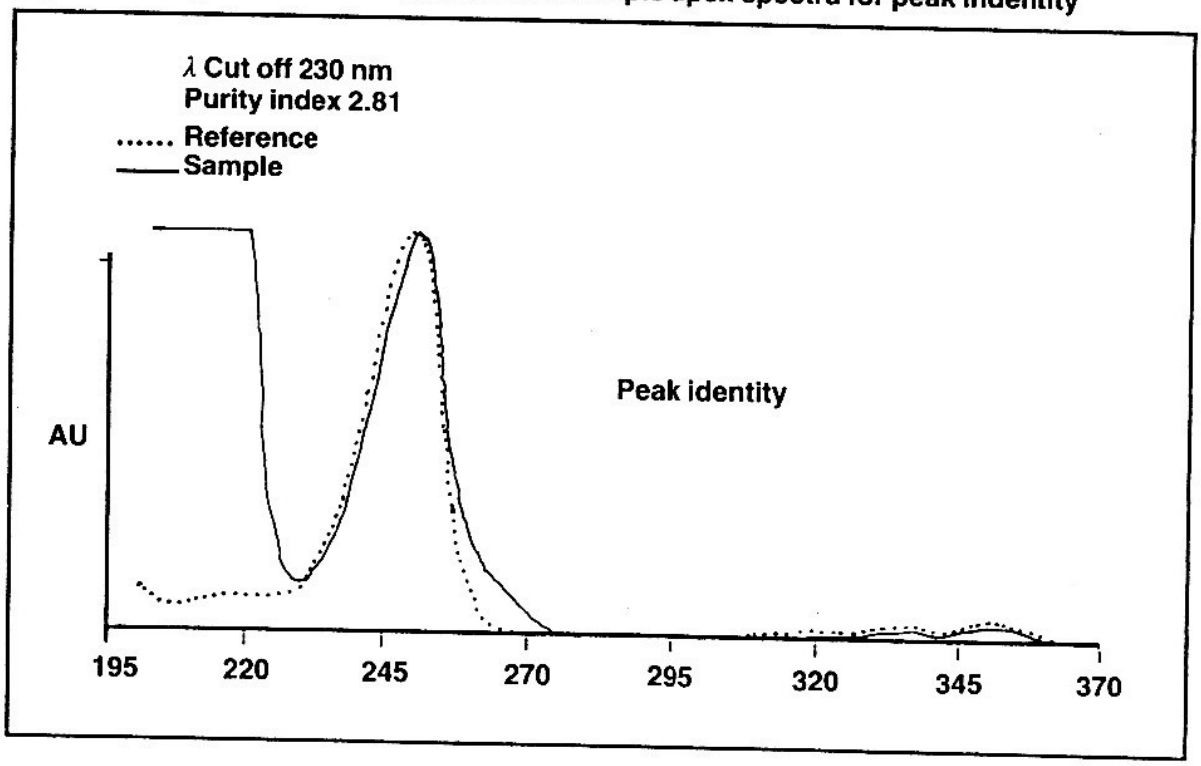


Figure 6: Comparison of reference versus sample apex spectra for peak indentity



SYSTEM SUITABILITY TESTS (SST)

Once a method or system has been validated the task becomes one of routinely checking the suitability of the system to perform within the validated limits. The simplest form of an HPLC system suitability test involves a comparison of the chromatogram trace with a standard trace (as shown below). This allows a comparison of the peak shape, peak width, baseline resolution.

Alternatively these parameters can be calculated experimentally to provide a quantitative system suitability test report:

Number of theoretical plates (efficiency)

Capacity factor,

Separation (relative retention)

Resolution,

Tailing factor

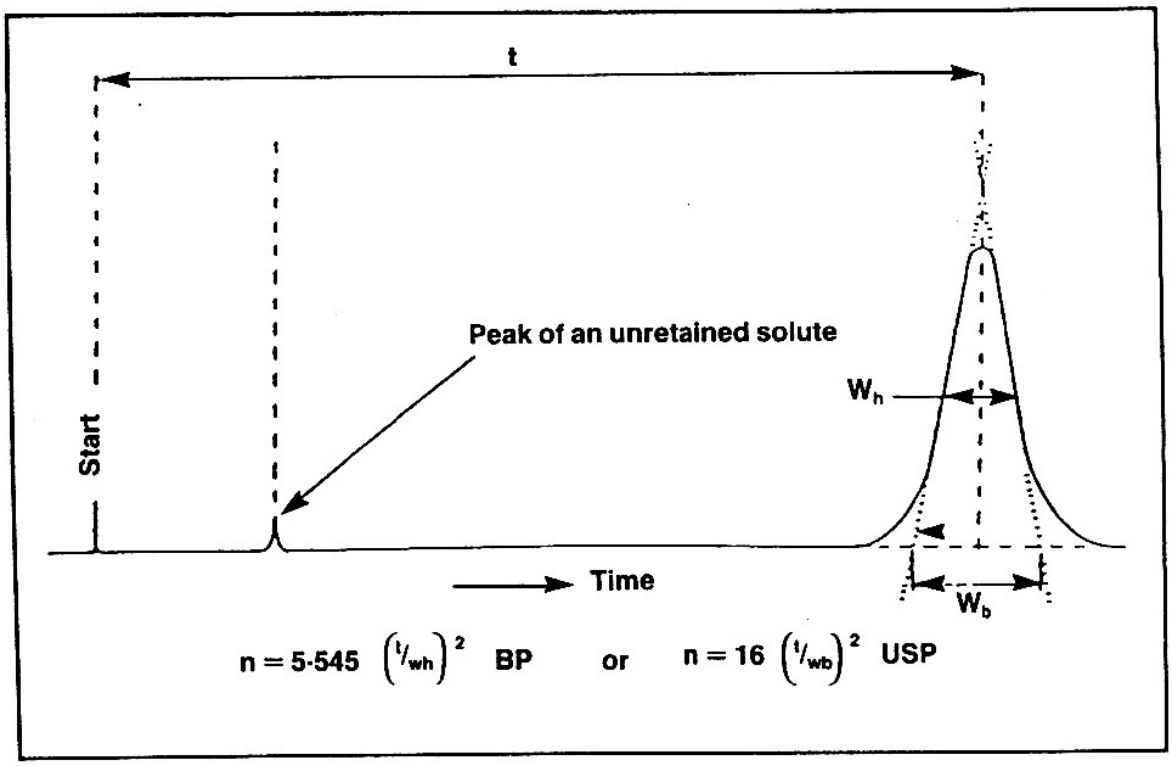
Relative Standard Deviation (Precision)

These are measured on a peak or peaks of known retention time and peak width.

Plate number or number of theoretical plates (n)

This a measure of the sharpness of the peaks and therefore the efficiency of the column. This can be calculated in various ways, for example the USP uses the peak width at the base and the BP at half the height.

Plate number calculation



where

Wh = peak width at 1/2 peak height

Wb = peak width at base

t = retention time of peak

Therefore the higher the plate number the more efficient the column. The plate number depends on column length - ie the longer the column the larger the plate number.

Therefore the column's efficiency can also be quoted as:

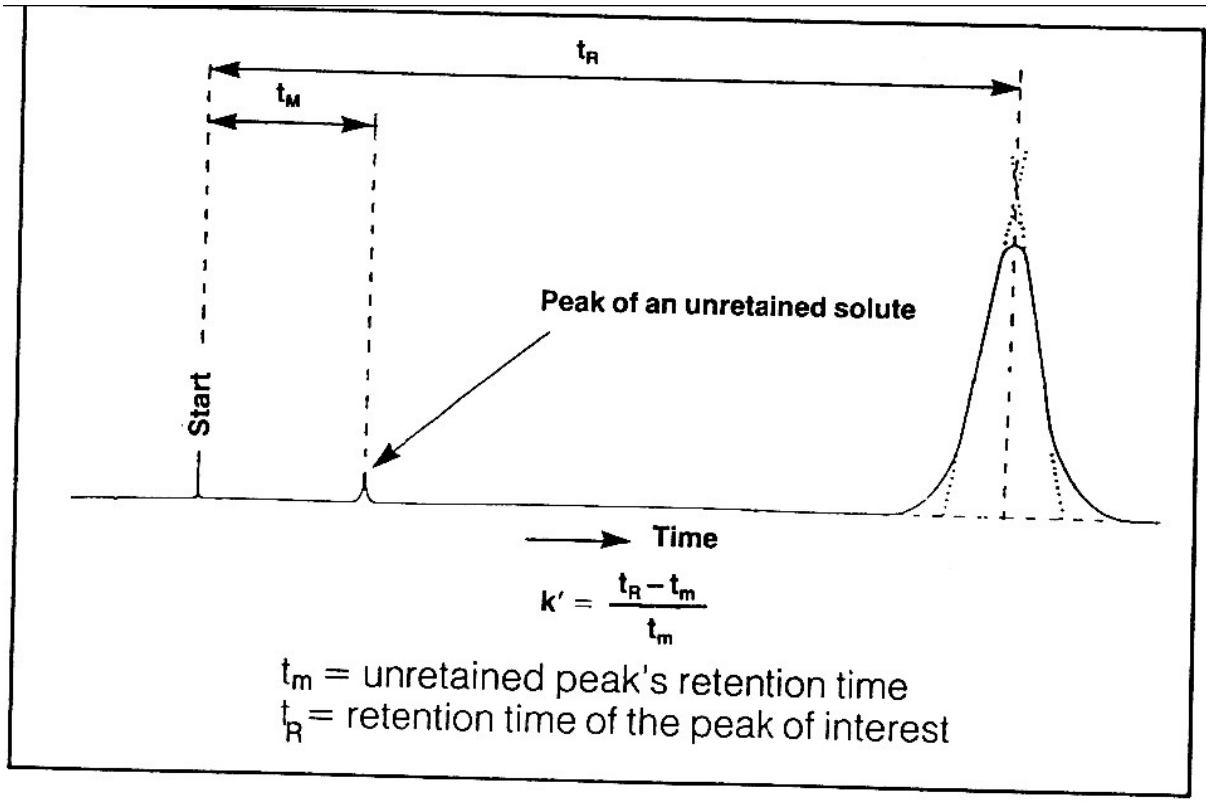
Either- as the plate height (h), **or** the height equivalent to one theoretical plate (HETP).

$$h = \frac{L}{n}$$ where L = length of column n *Or*- as plates/meter

.

Capacity factor (capacity ratio) k

This value gives an indication of how long each component is retained on the column (ie how many times longer the component is retarded by the stationary phase than it spends in the mobile phase).



k' is used in preference to retention time because it is less sensitive to fluctuations in chromatographic conditions (ie flow rate) and therefore ensures greater reproducibility from run to run.

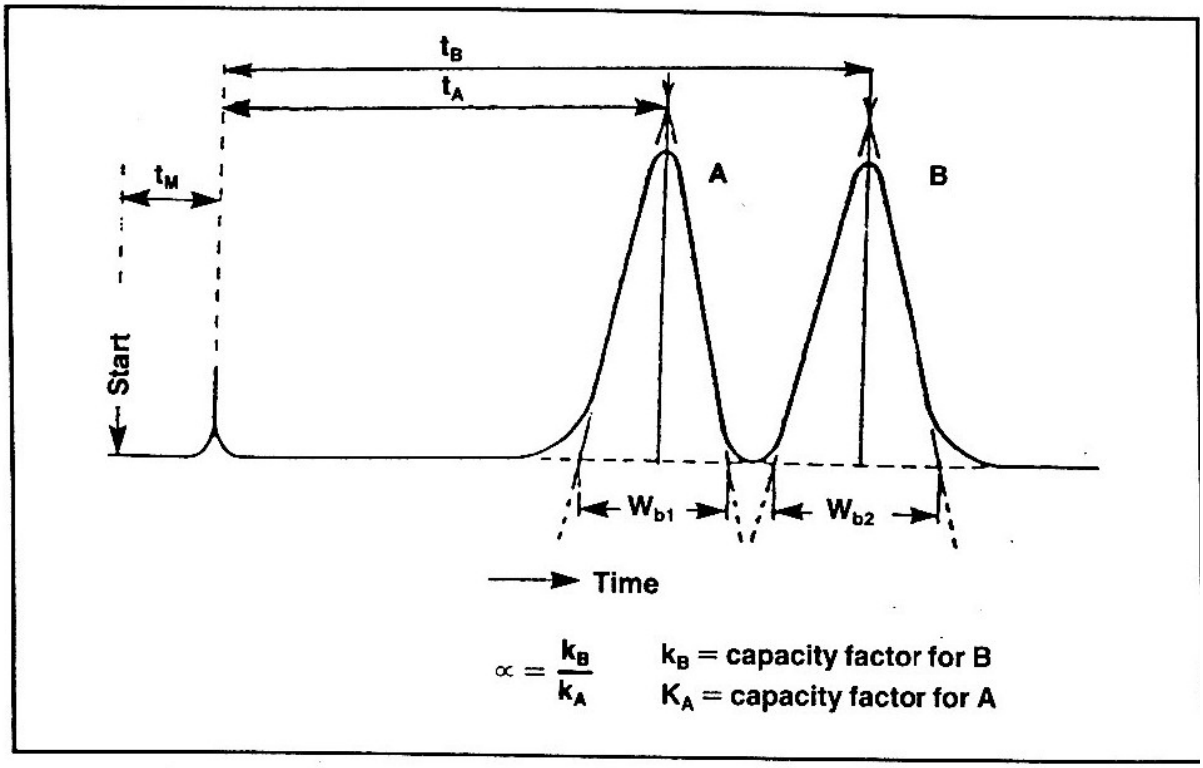
In practice the k value for the first peak of interest should be >l to assure that it is separated from the solvent.

3.3 Separation Factor (relative retention)

This describes the relative position of two adjacent peaks. ldeally, it is calculated using the capacity factor because the peaks' separation depends on the components' interaction with the stationary phase.

Therefore considering peaks A and B

Separation factor calculation



k for the later peak is always placed in the numerator to assure a value >l.

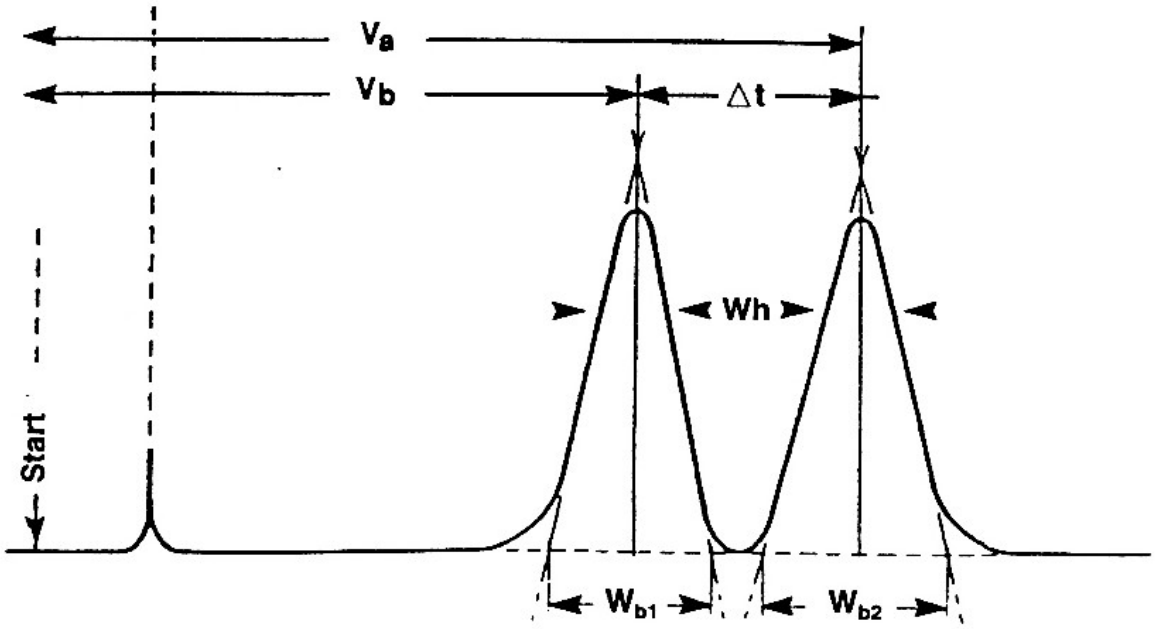
If the capacity factor is used then the separation factor should be consistent for a given column, mobile phase, composition and specified temperature, regardless of the instrument used.

NB. The separation factor gives no indication of the efficiency of the column.

Peak Resolution R

This is not only a measure of the separation between two peaks, but also the efficiency of the column. It is expressed as the ratio of the distance between the two peak maxima. (At) to the mean value of the peak width at base (Wb).

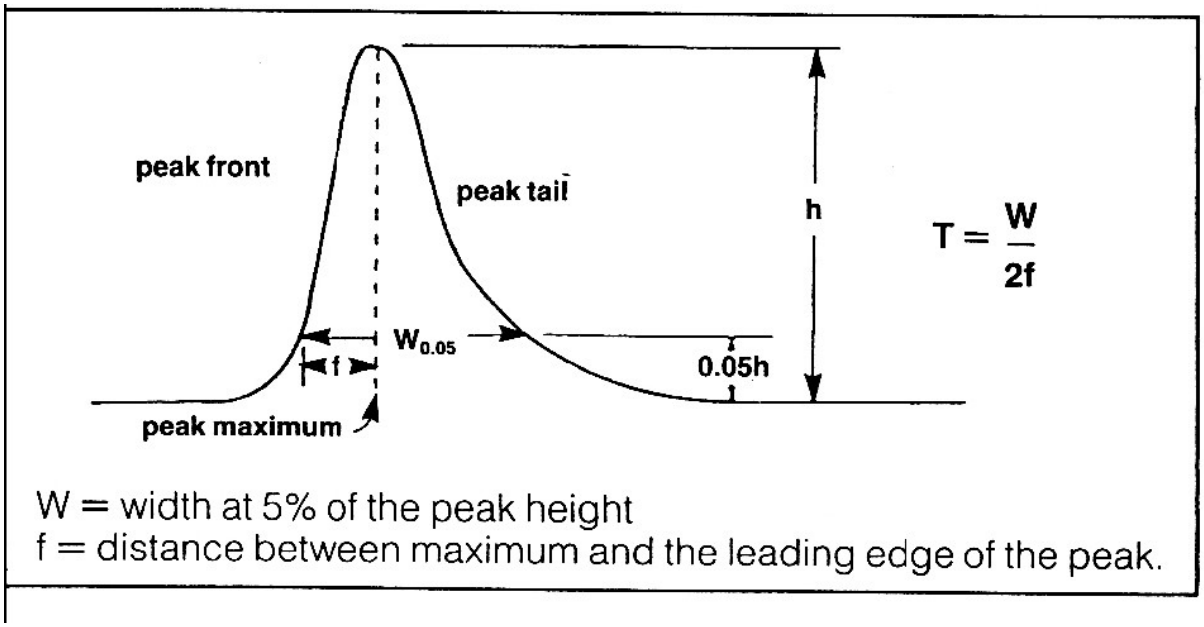
Peak resolution calculation



Tailing factor T

This is a measure for the asymmetry of the peak.

Tailing Factor calculation



Ruggedness

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions ie different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (ie from laboratory to laboratory, from analyst to analyst.)

Reproducibility:

The last type of precision study is reproducibility which is determined by testing homogeneous samples in multiple laboratories, often as part of inter laboratory crossover studies. The evaluation of reproducibility results often focuses more on measuring bias in results than on determining differences in precision alone. Statistical equivalence is often used as a measure of acceptable inter laboratory results. An alternative, more practical approach is the use of "analytical equivalence" in which a range of acceptable results is chosen prior to the study and used to judge the acceptability of the results obtained from the different laboratories.

An example of reproducibility criteria for an assay method could be that the assay results obtained in multiple laboratories will be statistically equivalent or the mean results will be within 2% of the value obtained by the primary testing lab.

Robustness:

The robustness of a method is its ability to remain unaffected by small changes in parameters such as percent organic content and pH of the mobile phase, buffer concentration, temperature, and injection volume. These method parameters may be evaluated one factor at a time or simultaneously as part of a

factorial experiment. Obtaining data on the effects of these parameters may allow a range of acceptable values to be included in the final method procedure. For example, if column performance changes over time, adjusting the mobile-phase strength to compensate for changes in the column may be allowed if such data are included in the validation.

Performing a thorough method validation can be a tedious process, but the quality of data generated with the method is directly linked to the quality of this process. Time constraints often do not allow for sufficient method validations. Many researchers have experienced the consequences of invalid methods and realized that the amount of time and resources required to solve problems discovered later exceeds what would have been expended initially if the validation studies had been performed properly.

STATISTICAL PARAMETERS:

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

Lineanrity coefficient (γ)

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

Slope (m)

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

Standard deviation (σ)

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

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

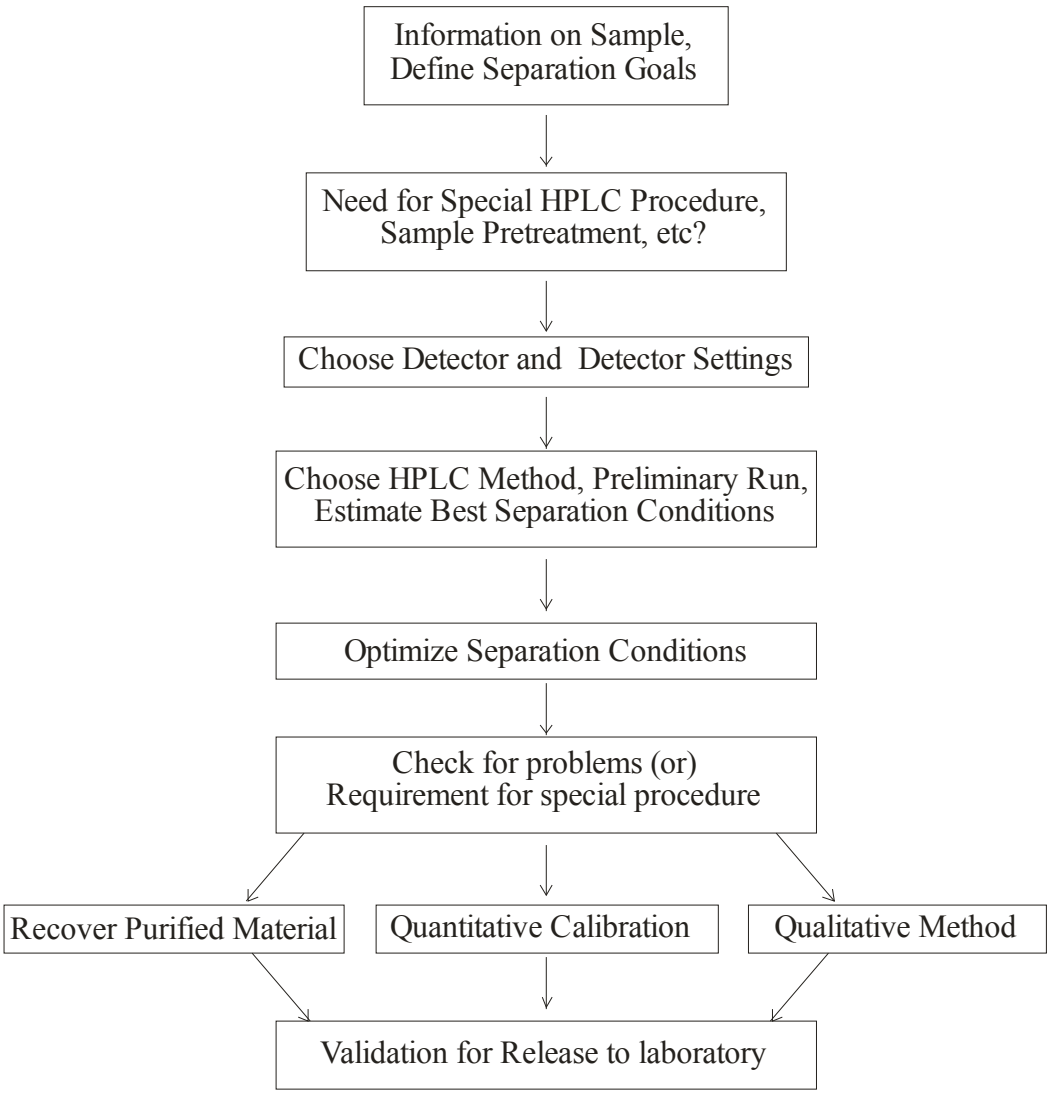
$$\text{Standard error(SE)} = \frac{\text{SD}}{\sqrt{n}}$$

$$\text{Intercept (c)} = y - m\,x$$

where
Σ= Sum of observation
X= Mean of arithmetic average (Σx/n)
X= Individual observed value.
x-x= Deviation of a value from the mean.
N= Number of observation

R.S.D=Relative standard deviation

DEVELOPMENT OF REVERSE PHASE HPLC METHOD6



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

REVIEW OF LITERATURE

REVIEW OF LITERATURE

HYE SUK LEE, et al, Simultaneous determination of aceclofenac and diclofenac in human plasma by narrowbore HPLC using column-switching using acetonitrile-potassium phosphate.[15]

Joshi, R.et al. a simple reversed-phase high-performance liquid chromatographic method that has been developed and validated for simultaneous estimation of acetaminophen, chlorzoxazone, and aceclofenac in tablet dosage form.

The estimation was carried out on an Luna C18 (5 µm × 25 cm × 4.6 mm i.d.) column using a mixture of buffer, methanol, and acetonitrile in the ratio 215:130:155 with final pH of 6.5 as a mobile phase, at a flow rate of 1.5 ml/min. Ultraviolet (UV) detection was performed at 275 nm. Total run time was 10 min; these three drugs (acetaminophen, chlorzoxazone, and aceclofenac) were eluted at the retention times of 2.055, 5.096, and 7.605 min respectively.[16]

Prashant Musmade et al, A simple and sensitive high-performance liquid chromatographic (HPLC) method was developed for quantification of aceclofenac in rat plasma. Ibuprofen was used as an internal standard (IS).

The present method used protein precipitation for extraction of aceclofenac from rat plasma. Separation was carried out on reversed-phase C_{18} column (250 mm × 4.6 mm, 5 μ) and the column effluent was monitored by UV detector at 282 nm. The mobile phase used was methanol-triethylamine (pH 7.0; 0.3% v/v in Milli-Q water) (60:40%, v/v) at a flow rate of 1.0 mL min^{-1} .[17]

Shaikh,et al A simple, rapid, and precise reversed-phase liquid chromatographic method is developed for simultaneous determination of paracetamol, aceclofenac, and chlorzoxazone in their ternary mixtures of commercial pharmaceutical preparation. This method uses a Zorbax SB C18, 250 x 4.6 mm, 5 microm analytical column. Mobile phase is acetonitrile and buffer (40:60, v/v), buffer containing 50 mM orthophosporic acid; pH of the buffer is adjusted to 6 with 10% w/v sodium hydroxide solution. The instrumental settings are at a flow rate of 1 mL/min; the column temperature is 25 degrees C, and detector wavelength is 270 nm.[18]

Burkhard Hinz,et al, a liquid-liquid extraction-based reversed-phase HPLC method with UV detection was validated and applied for the analysis of aceclofenac and three of its metabolites (4′-hydroxy-aceclofenac, diclofenac, 4′-hydroxy-diclofenac) in human plasma.

The analytes were separated using an acetonitrile-phosphate buffer gradient at a flow rate of 1 mL/min, and UV detection at 282 nm. The retention times for aceclofenac, diclofenac, 4′-hydroxy-aceclofenac, 4′-hydroxy-diclofenac and ketoprofen (internal standard) were 69.1, 60.9, 46.9, 28.4 and 21.2 min, respectively.[19]

KK Srinivasan ,et al, A derivative spectrophotometric procedure has been developed for the simultaneous determination of individual combination of aceclofenac and tramadol with paracetamol in combined tablet preparation. Tablet extracts of the drugs were prepared in distilled water.

The zero crossing point technique and the compensation technique were used to estimate the amount of each drug in the combined formulations, and were compared. The results were found to be accurate and free from interferences. The procedure is rapid, simple, nondestructive, and

does not require solutions of equations. Calibration graphs are linear (r=0.9999), with a zero intercept up to 24 mg/ml of each drug in combination with paracetamol. Detection limits at the $p = 0.05$ level of significance were calculated to be 0.5 mg/ml of aceclofenac, tramadol and paracetamol respectively.[20]

R Gopinath, et al, A simple, selective, rapid, precise and economical reverse phase HPLC method has been developed for the simultaneous estimation of paracetamol and aceclofenac from pharmaceutical dosage forms. The method was carried out on a Hichrom C18 (25 cm×4.6 mm i.d., 5 µ) column with a mobile phase consisting of acetonitrile:20 mM phosphate buffer (pH 5.0) (60:40 v/v) at a flow rate of

0.8 ml/min.

Detection was carried out at 265 nm. Etoricoxib was used as an internal standard. The retention time of paracetamol, aceclofenac and etoricoxib was 4.75, 6.44 and 8.83 min, respectively. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantitation and solution stability. The proposed method can be used for the estimation of these drugs in combined dosage forms.[21]

MY Momin, et al, A simple, precise, accurate, and validated reverse phase HPLC method has been developed for the simultaneous estimation of aceclofenac and paracetamol in tablet by reverse phase C-18 column (Intersile 4.6 mm×25 cm, 10 µm) using acetonitrile: 50 mM NaH_2

PO_4 in a ratio of 65:35 (pH adjusted to 3.0 with orthophosphoric acid) as a mobile phase at a flow rate of 1.5 ml/min and detection at 276 nm. The retention time for aceclofenac and paracetamol was found to be 1.58 and 4.01 min respectively, and recoveries from tablet were between 99 and 101%. The method can be used for estimation of combination of these drugs in tablets.[22]

BHINGE J. R,et al, a stability-indicating assay method for the determination of aceclofenac after being subjected to different International Conference on Harmonization prescribed stress conditions, such as hydrolysis, oxidation, heat, and photolysis. Aceclofenac (2-[2-[2-(2,6-dichlorophenyl)aminophenyl]acetyl]oxyacetic acid) is decomposed under hydrolytic stress (neutral, acidic, and alkaline) and also on exposure to light (in solution form). The compound is stable to oxidative stress, heat, and photolytic stress (in solid form). The major degradation product is diclofenac, which is confirmed through comparison with the standard.

Separation of the drug from major and minor degradation products is achieved on a C-18 column using methanol-0.02% of ortho phosphoric acid in a ratio of 70:30.[23]

Yan,et al, A sensitive high-performance liquid chromatographic method with fluorescence detection (excitation 435 and emission 515 nm) was established and validated for quantification of five anthraquinones (aloe-emodin, rhein, emodin, chrysophanol and physcion) in rat plasma. Following a single-step liquid-liquid extraction, the analytes and internal standard (1,8-dihydroxyanthraquinone) were separated on a reversed-phase C(18) column with water-phosphoric acid-methanol as mobile phase at a flow rate of 1 mL/min.

The linear ranges of the calibration curves were 6.5-1300 ng/mL for aloe-emodin, 20-4000 ng/mL for rhein, 40-8000 ng/mL for emodin, 15-3000 ng/mL for chrysophanol and 13-2600 ng/mL for physcion.

The lower limit of quantification was 6.5 ng/mL for aloe-emodin, 20 ng/mL for rhein, 40 ng/mL for emodin, 15 ng/mL for chrysophanol and 13 ng/mL for physcion. The mean accuracy was 94.3-105.1% for aloe-emodin, 90.3-108.8% for rhein, 92.6-106.7% for emodin, 95.8-103.8% for chrysophanol and 98.7-101.2% for physcion. The within-batch and between-batch precisions were < or = 5.5% and < or = 13.4%, respectively.

This method is suitable for determining the five anthraquinones in plasma simultaneously and thus investigating the pharmacokinetics of anthraquinones from Xiexin decoction in rats.[24]

Yi, et al, A simple HPLC method using column-switching and ultraviolet detection was developed for the simultaneous determination of baicalin (BA), rhein (RH) and berberine (BE) in rat plasma. Plasma samples

were injected directly onto a Capcell Pak MF C(8) column (150 mm x 4.6 mm i.d.) to remove protein and to be pre-separated by an isocratic elution using 50 mmol/L phosphate sodium (pH 6.85)-acetonitrile (10:1, v/v). After the drug-containing fractions were transferred to a Kromasil C(18) column (150 mm x 4.6 mm i.d.) by a valve switching step, the valve position was switched back and the main separation was performed by an isocratic elution using triethylamine adjusted 20 mmol/L phosphoric acid (pH 6.78)-acetonitrile (4:1, v/v).

The flow rate was always 1.0 mL/min. The calibration curve showed excellent linear relationship (r>or=0.9997) over the concentration range of 0.4-7.9 microg/mL for baicalin, 0.2-7.8 microg/mL for rhein and 0.4-7.7 microg/mL for berberine in rat plasma. The intra- and inter-day assay precisions (R.S.D.) of three analytes were in the range of 0.34-4.3% and the accuracies were between 98.0% and 102.4%.

Their recoveries were all greater than 95%. The method was successfully applied to the multi-constituents plasma concentration-time curve study after oral administration of a traditional Chinese medicine prescription Xiexin-Tang in rats.[25]

Sílvia H. M. BORGMANN, et al, develop and validate a dissolution test for diacerhein in capsules using spectrophotometric method. The dissolution established conditions were: 900 mL of sodium phosphate buffer pH 7.0 with 0.75% of sodium lauryl sulphate as dissolution medium, using a basket apparatus at a stirring rate of 50 rpm.

The drug release was evaluated by UV spectrophotometric method at 258 nm. The method was validated to meet requirements for a global regulatory filing. The validation included specificity, linearity, precision and accuracy.[26]

NAGESHWARA RAO. R, et al an isocratic reversed-phase high-performance liquid chromatographic (RP-HPLC) method for determination and evaluation of purity of mosapride citrate in bulk drugs and pharmaceuticals has been developed using Waters Symmetry C18 column with acetonitrile:0.024 M orthophosphoric acid (28:72, v/v) adjusted to pH 3.0 with triethylamine and photodiode array detector set at 276 nm. The

method is simple, rapid, selective and capable of detecting all process related impurities at trace levels in the finished products with detection limits ranging in between 0.2 x 10-8g and 6.4 x 10-8g.

The method has been validated with respect to accuracy, precision, linearity, ruggedness, and limit of detection and quantification. The linearity range was 125-1000 µg/ml.

The percentage recoveries from pharmaceutical dosages were ranged from 95.53 to 100.7. The method was found to be suitable not only for monitoring the reactions during the process development but also quality assurance of mosapride citrate.[27]

N. V. S. Ramakrishna,et al ,simple, rapid, sensitive and specific liquid chromatography-tandem mass spectrometry method was developed and validated for quantification of mosapride (I), a novel and potent gastroprokinetic agent that enhances the upper gastrointestinal motility by stimulating 5-HT4 receptor. The analyte and internal standard, tamsulosin (II), were extracted by liquid-liquid extraction with diethyl ether-dichloromethane (70:30, v/v) using a Glas-Col Multi-Pulse Vortexer. The chromatographic separation was performed on a reversed-phase Waters symmetry C18 column with a mobile phase of 0.03% formic acid-acetonitrile (10:90, v/v).

The protonated analyte was quantitated in positive ionization by multiple reaction monitoring with a mass spectrometer. The mass transitions m/z 422.3 →198.3 and m/z 409.1 →228.1 were used to measure I and II, respectively. The assay exhibited a linear dynamic range of 0.5-100.0 ng/mL for mosapride in human plasma. The lower limit of quantitation was 500 pg/mL with a relative standard deviation of less than 15%. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. A run time of 2.0 min for each sample made it possible to analyze a throughput of more than 400 human plasma samples per day. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability or bioequivalence studies.[28]

Patel,et al , Simple, sensitive high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) methods are developed for the quantitative estimation of rabeprazole and mosapride in their combined pharmaceutical dosage forms. In HPLC, rabeprazole and mosapride are chromatographed using 0.01M 6.5 pH ammonium acetate buffer-methanol-acetonitrile (40:20:40, v/v, pH 5.70 ± 0.02) as the mobile phase at a flow rate of 1.0 mL/min. In TLC, the mobile phase is ethyl acetate-methanol-benzene (2:0.5:2.5, v/v). Both the drugs are scanned at 276 nm. The retention times of rabeprazole and mosapride are found to be 4.93 ±

0.01 and 9.79 ± 0.02, respectively. The R_f values of rabeprazole and mosapride are found to be 0.42 ± 0.02 and 0.61 ± 0.02, respectively. The linearities of rabeprazole and mosapride are in the range of 400-2000 ng/mL and 300-1500 ng/mL, respectively, for HPLC; in TLC, the linearities of rabeprazole and mosapride are in the range of 400-1200 ng/spot and 300-900 ng/spot, respectively. The limit of detection is found to be 97.7 ng/mL for rabeprazole and 97.6 ng/mL for mosapride in HPLC; in TLC the limit of detection is found to be 132.29 ng/spot for rabeprazole and 98.25 ng/spot for mosapride. The proposed methods can be applied to the determination of rabeprazole and mosapride in combined pharmaceutical products.[29]

Yoichi Aoki ,et al, A simple method for determination of mosapride citrate and its metabolite, des-p-fluorobenzyl mosapride (M-1), in equine muscle, liver, kidney, adipose tissue and intestine by liquid chromatography–tandem mass spectrometry has been developed.

(±)-4-Amino-5-chloro-2-ethoxy-N-[[4-(2-chlorobenzyl) morpholinyl]methyl]benzamide was used as an internal standard. The analytes and internal standard were spiked and extracted from tissues by acetonitrile. The chromatographic separation was performed on a reversed-phase TSK-GEL SUPER ODS column with a mobile phase of acetonitrile–0.05% (v/v) formic acid containing 5 mmol/L nonafluoropentanoic acid (2:3, v/v). The method exhibited a large linear range from 0.0005 to 0.2 μg/mL

for both mosapride citrate and M-1 ($r > 0.9976$). In the intra-day assay ($n = 5$), the relative standard deviations (RSDs) ranged from 1.1 to 7.8% for mosapride citrate and 1.6 to 7.2% for M-1. In the inter-day assay ($n = 3$), the RSDs ranged from 1.0 to 13% for mosapride citrate and 0.8 to 11% for M-1.

The extraction recovery at 1.28 μg/g of mosapride citrate from equine tissues ranged from 97 to 107%. The lower limit of quantification for mosapride citrate was found to be 0.004 μg/g. Stability studies were carried out at different storage conditions.[30]

Zarghi A, et al, A rapid, sensitive and reproducible HPLC method was developed and validated for the analysis of pantoprazole (CAS 102625-70-7) in human plasma. The separation was achieved on a monolithic silica column using acetonitrile-potassium dihydrogen phosphate buffer (25:75,v/v), pH 3.0, as the mobile phase at a flow rate of 1.5 ml min(-1). The wavelength was set at 290 nm. The assay enables the measurement of pantoprazole for therapeutic drug monitoring with a minimum quantification limit of 20 ng ml(-1).[31]

T. Sivakumar*, et al A simple reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been developed and validated for simultaneous determination of domperidone and pantoprazole in capsules. The com-pounds were separated on an ODS analytical column with a mixture of methanol, acetonitrile, and triethylamine

solution (10 mM, pH 7.0 ± 0.05 adjusted with 85% phosphoric acid) in the ratio 20:33:47 (v/v) as mobile phase at a flow rate of 1.0 mL min−1. UV detection was performed at 285 nm. The method was validated for accuracy, precision, specificity, linearity, and sensitivity.

The developed and validated method was successfully used for quantitative analysis of Pantop-D capsules. Total chromatographic analysis time per sample was approximately 10 min with pantoprazole, acetophenone (internal standard), and domperidone eluting with retention times of 4.34, 5.52, and 9.46 min, respectively.

Validation studies revea-led the method is specific, rapid, reliable, and reproducible. Calibration plots were linear over the concentration ranges 0.5–5 μg mL−1 and 1–10 μg mL−1 for domperidone and pantoprazole, respectively. The LODs were 15.3 and 3.0 ng mL−1 and the LOQs were 51.0 and 10.1 ng mL−1 for domperidone and pantoprazole, respectively. The high recovery and low relative standard deviation confirm the suitability of the method for deter-mination of domperidone and pantoprazole in capsules.[32]

KOCYIGIT-KAYMAKCOGLU ,et al, A rapid and specific high-performance liquid chromatographic method was developed and validated for the simultaneous determination of ketoprofen, valsartan and pantoprazole in human plasma. Chromatographic separation of ketoprofen, valsartan and

pantoprazole was performed using a Chromasil C18 column (250 mm x 4.6 mm i.d., 5 μm particle size).

The mobile phase consisted of a mixture of 0.02 M sodium dihydrogen phosphate buffer (pH 3.15) and acetonitrile (58:42, v/v) pumped through the chromatographic system at a flow rate of 1 mL min-1. The Diode Array detector was operated at 225 and 272 nm. Rofecoxib was used as an internal standard.

Sample treatment procedure consisted of deproteinisation with acetonitrile-methanol (50:50 v/v). Analytical recoveries were in the range of 79.00-118.00% of nominal values of valsartan, ketoprofen and pantoprazole.

The method was reproducible and accurate with lower limits of quantification 250 μg · L-1 for pantoprazole and 500 μg · L-1 for ketoprofen and valsartan. This method was relatively easy to perform and allows simultaneous determination of these three drugs in plasma at nanogram levels.[33]

B. H. Patel, et al, A simple, sensitive, and precise high performance liquid chromatographic method for the analysis of pantoprazole, rabeprazole, esomeprazole, domperidone and itopride, with ultraviolet detection at 210 nm, has been developed, validated, and used for the determination of compounds in commercial pharmaceutical products.

The compounds were well separated on a Hypersil BDS C18 reversed-phase column by use of a mobile phase consisting of 0.05 M, 4.70 pH, potassium dihydrogen phosphate buffer - acetonitrile (720:280 v/v) at a flow rate of 1.0 mL min−1.

The linearity ranges were 400–4,000 ng mL−1 for pantoprazole, 200–2,000 ng mL−1 for rabeprazole, 400–4,000 ng mL−1 for esomeprazole, 300–3,000 ng mL−1 for domperidone and 500–5,000 ng mL−1 for itopride. Limits of detection (LOD) obtained were: pantoprazole 147.51 ng mL−1, rabeprazole 65.65 ng mL−1, esomeprazole 131.27 ng mL−1, domperidone 98.33 ng mL−1 and itopride 162.35 ng mL−1. The study showed that reversed-phase liquid chromatography is sensitive and selective for the determination of pantoprazole, rabeprazole, esomeprazole, domperidone and itopride using single mobile phase.[34]

Bhavesh H. Patel et al, high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) methods for the simultaneous estimation of pantoprazole (PANT) and domperidone (DOM) in pure powder and capsule formulations.
The HPLC separation was achieved on a Phenomenex C18 column (250 mm id, 4.6 mm, 5 µm) using 0.01 M, 6.5 pH ammonium acetate buffer-methanol-acetonitrile (30 + 40 + 30, v/v/v, pH 7.20) as the mobile phase at a flow rate of 1.0 mL/min at ambient temperature. The HPTLC separation was

achieved on an aluminum-backed layer of silica gel 60F254 using ethyl acetate–methanol (60 + 40, v/v) as the mobile phase. Quantification was achieved with ultraviolet (UV) detection at 287 nm over the concentration range 400–4000 and 300–3000 ng/mL with mean recovery of 99.35 ± 0.80 and 99.08 ± 0.57% for PANT and DOM, respectively (HPLC method). Quantification was achieved with UV detection at 287 nm over the concentration range 80–240 and 60–180 ng/spot with mean recovery of 98.40 ± 0.67 and 98.75 ± 0.71% for PANT and DOM, respectively (HPTLC method). These methods are simple, precise, and sensitive, and they are applicable for the simultaneous determination of PANT and DOM in pure powder and capsule formulations.

AIM OF THE WORK

AIM OF THE WORK

1) The combination of an Anti-inflammatory drug ACECLOFENAC and an Anti-arthritic drug DIACEREIN are selected for analysis by UV spectrophotometric method and reverse phase high performance liquid chromatography.

It is estimated by simultaneous equation method as the λmax of the drugs are dissimilar and their absorbance ratio lies outside the range 0.1 to 2.

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

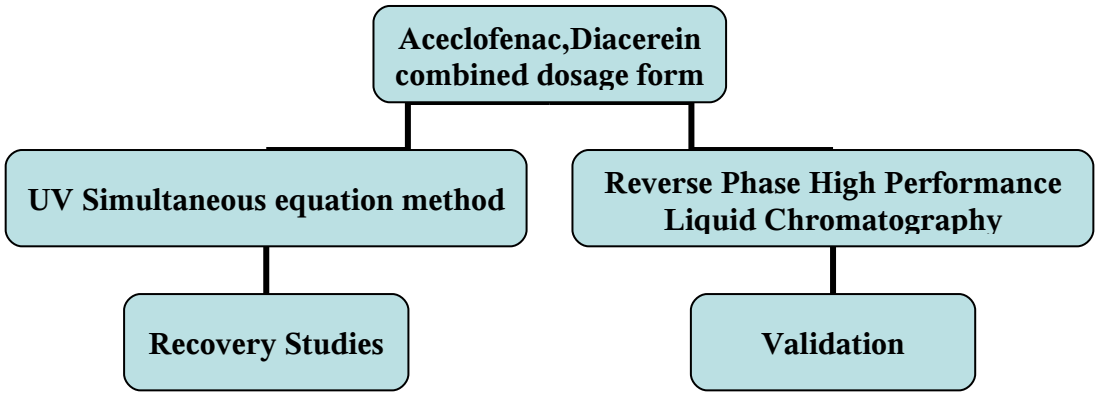
2) The combination of Gastroprokinetic agent MOSAPRIDE and Proton pump inhibitor PANTOPRAZOLE are selected for analysis by UV spectrophotometric method and reverse phase high performance liquid chromatography.

It is estimated by simultaneous equation method as the λmax of the drugs are dissimilar and their absorbance ratio lies outside the range 0.1 to 2.

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

SCHEME OF THE WORK

1.



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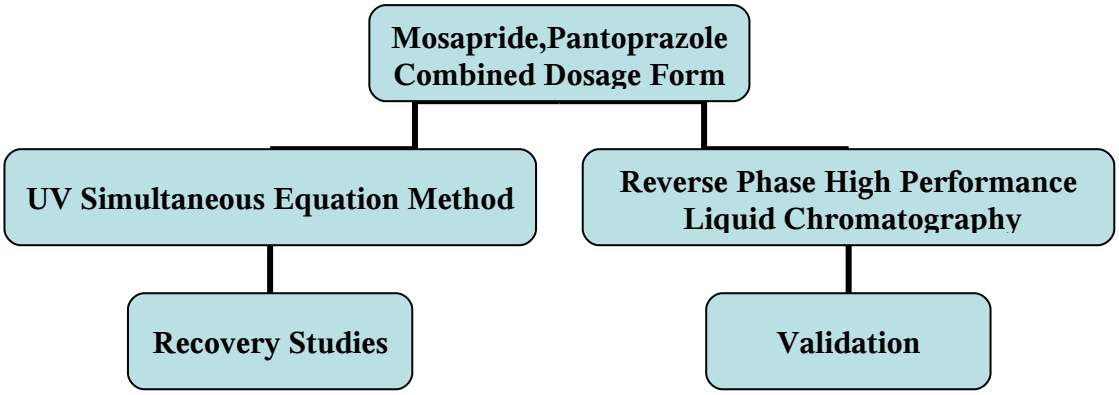
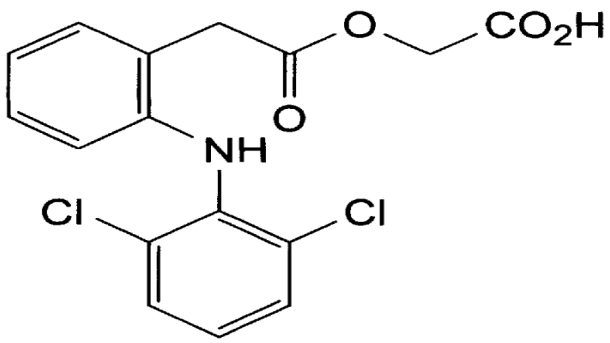


DRUG PROFILE

ACECLOFENAC [35]

Molecular structure:



Chemical Name : [[[2-[(2,6- Dichlorophenyl)amino]phenyl]acetyl] oxy]acetic acid

Molecular Formula : C16H13Cl2NO4

Molecular Weight : 354.2

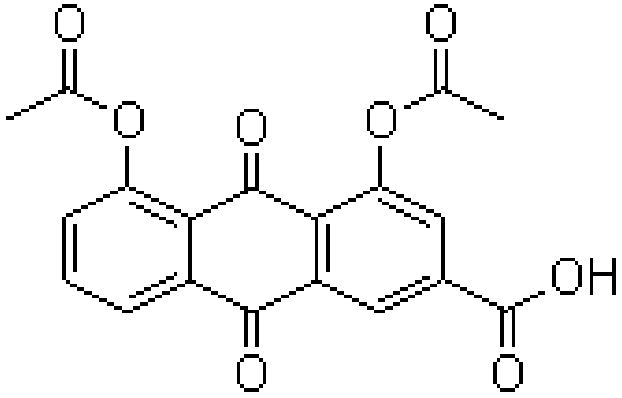
Appearance : White or almost white, crystalline powder

Solubility : Practically insoluble in water, freely soluble in acetone, ethanol.

Action and use : Analgesic; anti-inflammatory

DIACEREIN [35]

Molecular structure:



Chemical Name : 1,8-diacetoxy-3-carboxyanthraquinone

Molecular Formula : $C_{19}H_{12}O_8$

Molecular weight : 368.3

Appearance **:** light yellow colour powder

Solubility **:** soluble in ethanol

Action and use **:** Antiarthritic drug

REVERSE PHASE HIGH PERFOMANCE LIQUID CHROMATOGRAPHY AND VALIDATION IN ESTIMATION OF ACECLOFENAC AND DIACEREIN IN COMBINED TABLET DOSAGE FORM.

Instruments:

- Shimadzu liquid chromatograph LC – 20 AT VP
- Mettler Toledo AG 285 Balance CP-225D
- DIGISUN-DI-707 pH meter
- Millipore filter (10.45/ μm)
- Whatman filter paper
- Sonicator

Reagents and Chemicals

- Acetonitrile
- HPLC grade water
- Buffer

REFERENCE STANDARDS:

Aceclofenac and Diacerein:

These two reference standards were obtained as gift samples from Ceeall analytical laboratory,Chennai. The authenticity and purity of the sample was certified by the same.

Sample Tablet brand used : Dycerin-A[36]

Label claim : Aceclofenac - 100 mg
Diacerein - 50 mg

METHOD DEVELOPMENT AND OPTIMIZATION:

SELECTION OF WAVELENGTH:

The known concentration of Aceclofenac and Diacerein were taken and dissolved in THF(Tetra hydro furan). The wavelength were tried from 200nm to 400nm and the Peaks of the drugs were showing fronting and tailing. The peak areas were also found to be minimum.

Finally 270nm were selected for the analysis.

OPTIMIZATION OF CHROMATOGRAPHIC PARAMETAERS

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

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

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

DYCERIN-A is a combination of Aceclofenac 100mg and Diacerein50mg. The method development of Aceclofenac and Diacerein required adequate resolution of two drug peaks in the chromatogram.

DIFFERENT COMBINATIONS OF BUFFER AND SOLVENTS:

Buffer(potassium dihydrogen ortho phosphate pH:3) and acetonitrile (50:50)

Buffer(potassium dihydrogen ortho phosphate pH:3) and acetonitrile (20:80)

Buffer(potassium dihydrogen ortho phosphate pH:3) and acetonitrile (30:70)

Buffer(potassium dihydrogen ortho phosphate pH:3) and methanol and acetonitrile (40:30:30),finally add 2.5% v/v THF

Buffer(potassium dihydrogen ortho phosphate pH:3) and methanol and acetonitrile (45:20:35),finally add 5% v/v THF

Peaks of Aceclofenac and Diacerein were well resolved with solvent system Buffer (Potassium dihydrogen ortho phosphate pH:3) acetonitrile and methanol.(45:35:20).

SELECTION OF FLOW RATE:

The Flow rate for Aceclofenac and Diacerein were tried with 0.5ml, 1ml, 1.5ml and 2ml, the peaks of the drugs were showing fronting and tailing with 0.5ml and 2ml respectively and finally 2ml per minute was selected for the analysis.

PREPARATION OF BUFFER SOLUTION:

Buffer solution was prepared by using 0.136g of potassiumdi hydrogen ortho phosphate and 0.01M citric acid in 500ml of HPLC grade water, pH adjusted to 4 with TEA, filtered through 0.45µ nylon membrane and degassed.

PREPARATION OF MOBILE PHASE:

Mix the Buffer, acetonitrile and methanol in the ratio of 45:35:20,finally add 5% v/v THF and degass it. Filtered through 0.45µ membrane.

DILUENT

Mobile phase is used as diluent.

DETERMINATION OF RETENTION TIME:

(A) STANDARD SOLUTION OF ACECLOFENAC:

Accurately 200.2mg of Aceclofenac was taken in a 100ml volumetric flask and dissolved in 10ml THF,the volume was adjusted to 100ml with mobile phase. 5 ml was taken in a separate 50ml volumetric flask and the volume was adjusted to 50 ml with mobile phase to get concentration of 100µg/ml of Aceclofenac. 20µl of this solution was injected and chromatogram was obtained.

(B) STANDARD SOLUTION OF DIACEREIN:

Accurately 100.2mg of Diacerein was taken in a 100ml volumetric flask and dissolved in 10ml THF,the volume was adjusted to 100ml with mobile phase. 5 ml was taken in a separate 50ml volumetric flask and the volume was adjusted to 50 ml with mobile phase to get concentration of 50µg/ml of Diacerein. 20µl of this solution was injected and chromatogram was obtained

(C) PREPARATION OF MIXED STANDARD SOLUTION:

100.2mg of Diacerin and 200.2mg Aceclofenac was transferred into a 100ml dried volumetric flask. The compounds were first dissolved in 10ml of THF and it was sonicated. Then the volume was adjusted to 100ml with mobile phase. From the stock solution 5ml was transferred to a 50ml volumetric flask and the volume was adjusted to 50ml with mobile phase to get a concentration of 50µg/ml of Diacerin and 100µg/ml of Aceclofenac . 20 µl of the resulting solution was injected and chromatogram was recorded .

FIXED CHROMATOGRAPHIC CONDITIONS:

INSTRUMENT : Shimadzu liquid chromatograph
LC-20 AT VP

COLUMN : C18

WAVELENGTH : 265nm

TEMPERATURE : Ambient temperature.

FLOW RATE : 2ml/min

INJECTION VOLUME : 20µl.

MOBILE PHASE : Buffer (Potassium di hydrogen ortho phosphate pH:3) acetonitrile and methanol.(45:35:20), finally add 5%v/v THF

RETENTION TIME : 3.52 min for Diacerein ,6.2 min for Aceclofenac

QUANTITATIVE DETERMINATION OF THE DRUGS BY USING THE DEVELOPED METHOD

Sample : Dycerin-A

Label Claim : Aceclofenac – 100mg
Diacerein - 50mg

METHOD:

Twenty tablets were weighed and powdered. 512.6mg sample tablet DYCERIN-A(equivalent to 100.1mg of Aceclofenac and 50.1mg Diacerein)

was taken into 100ml dried volumetric flask. The powder was first dissolved in 10ml of THF and sonicated and finally the volume was adjusted to 100ml with mobile phase. From this solution 5ml was transferred to 50ml volumetric flask and volume was adjusted to 50ml with mobile phase to get a concentration of 100µg/ml of Aceclofenac and 50µg/ml of Diacerein. 20µl of the solution was injected and the chromatogram obtained.

The amount of Aceclofenac and Diacerein present in the tablet formulation was calculated by comparing the peak area of the standard and reports are given in Table-1

Amount of drug present in the tablet:

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

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

Table-1

Quantitative Estimation

S. No.	Brand Name	Content	Label Claim (mg)	Peak area	Amount present (mg)	Percent Purity% w/v
1.	DYCERIN-A	Diacerein	50mg	5488.995	49.89	99.78%
		Aceclofenac	100mg	4220.921	100.29	100.29%

Acceptance critearia: 98-102%w/v

Assay for Diacerein:

Amount Present =

$$\frac{\frac{5488.99}{5}}{5480.852} \times \frac{100.2/100x5/50}{512.6/100x5/50} \times \frac{99.72}{100} \times 255.6 = 49.89 \text{ mg}$$

% Label Claim =

$$\frac{49.89}{50} \times 100 = 99.78\ \%$$

Assay for Aceclofenac:

Amount Present =

$$\frac{\frac{4220.92}{1}}{4195.154} \times \frac{200.2/50 \times 5/50}{512.6/100x5/50} \times \frac{99.85}{100} \times 255.6 = 100.29 \text{ mg}$$

% Label Claim =

$$\frac{100.29}{100} \times 100 = 100.29\ \%$$

VALIDATION

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

Design of experiment:

Typical analytical parameters used in assay validation are,

- Specificity
- Linearity and range
- Limit of quantification
- Limit of detection
- Accuracy
- Precision
    - System precision
    - Method precision
- Robustness
- Ruggedness
- System suitability studies
    - Resolution
    - Number of theoretical plates
    - The tailing factor.

SPECIFICITY

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

Determination:

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

Preparation of placebo:

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

Procedure:

- 100mg placebo was accurately weighed and transferred into a 25ml volumetric flask and the volume was made to 25ml with the mobile phase. The solution was filtered through Millipore filter paper and degassed. 20µl of this solution was injected and chromatogram was recorded .
- 100.2mg of Diacerin and 200.2mg Aceclofenac was transferred into a 100ml dried volumetric flask. The compounds were first dissolved in 10ml of THF and it was sonicated. Then the volume was adjusted to 100ml with mobile phase. From the stock solution 5ml was transferred to a 50ml volumetric flask and the volume was adjusted to 50ml with mobile phase to get a concentration of 50µg/ml of Diacerin and 100µg/ml of Aceclofenac. To this solution 100mg of placebo was

added and it was sonicated ,filtered through a Millipore filter paper. 20 µl of the resulting solution was injected and chromatogram was recorded.The mixed standard solution was also injected without placebo and it was recorded and the reports are shown in Table-9 &10

Table-9

Specificity for Diacerein

S.No.	Sample	Area obtained
1.	Standarad	5480.852
2.	Standard+Placebo	5479.520
3.	Placebo	0

Table-10

Specificity for Aceclofenac

S.No.	Sample	Area obtained
1.	Standarad	4195.154
2.	Standard+Placebo	4196.246
3.	Placebo	0

LINEARITY AND RANGE:

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

Determination:

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

Method:

Preparation of mixed standard stock solution

Accurately weighed 100.2mg of Diacerin and 200.2mg Aceclofenac was transferred into a 100ml dried volumetric flask. The compounds were first dissolved in 10ml of THF and then the volume was adjusted to 100ml with mobile phase. From the From the resulting solution,4, 4.5, 5, 5.5, 6ml were transferred into 5 different 50ml volumetric flask. The volume was made with mobile phase to get a final concentration of 80.16,90.18,100.2,110.22,120.24 µg/ml of Diacerein and 160.16,180.18,200.2,220.22,240.24 µg/ml of Aceclofenac. 20µl of the resulting solution was injected and chromatogram was recorded.

Acceptance Criteria

- Correlation coefficient should not be less than 0.99

The linearity datas and analytical performance parameters of Diacerein and Aceclofenac are shown in Table-11- 13 .

Table-11

LINEARITY DATA

Diacerein

S.No.	Concentration(µg/ml)	Peak Area
1.	80.16	4360.112
2.	90.18	4907.984
3.	100.20	5492.272
4.	110.22	6112.583
5.	120.24	6448.837

Table-12

LINEARITY DATA

Aceclofenac

S.No.	Concentration(µg/ml)	Peak Area
1.	160.16	3320.230
2.	180.18	3736.739
3.	200.20	4186.285

4.	220.22	4623.256
5.	240.24	5086.729

Table-13

ANALYTICAL PERFORMANCE PARAMETERS

S. no.	Drug name	Linear dynamic range(µ/ml)	Correlation coefficient	Slope	Intercept
1.	Diacerein	(80.16-120.24)	0.996	53.71	82.615
2.	Aceclofenac	(160.16-240.24)	0.999	22.0752	-228.808

ACCURACY

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

Determination:

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

analyte recovered by the assay.

Acceptance criteria:

Percentage recovery should be within 98-102%

PROCEDURE:

Mixed standard stock solution 5ml and sample stock solution 5ml were mixed together in 50 ml volumetric flask and the volume was made upto 50ml with mobile phase to get 100% range. Similarly 80% and 120% range was prepared. 20µl of this solution was injected three times and chromatograms were shown in the following graphs and values in table 14 and 15 .

Table-14

Recovery Study of Diacerein

S.No.	RANGE	Area obtained	Amount Recovered(mg)	% Recovery
1.	80%	4436.412	50.41	100.82
		4443.341	50.49	100.98
		4439.341	50.44	100.88
2	100%	5531.448	50.28	100.56
		5517.152	50.15	100.30
		5500.326	50.00	100.00
3	120%	6537.755	49.53	99.06
		6534.844	49.50	99.00
		6601.524	50.00	100.00

Table-15

Recovery Study of Aceclofenac

S.No.	RANGE	Area obtained	Amount Recovered(mg)	% Recovery
1.	80%	3389.522	100.66	100.66
		3420.120	101.57	101.57
		3397.976	100.90	100.90
2	100%	4169.926	99.07	99.07
		4200.111	99.79	99.79
		4280.002	101.69	101.69
3	120%	5081.058	100.6	100.6
		5076.152	100.5	100.5
		5103.123	101.04	101.04

PRECISION

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

Determination:

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

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

Acceptance Criteria:

The relative standard deviation should be with in 2%

SYSTEM PRECISION :

Accurately weighed 100.2mg of Diacerin and 200.2mg Aceclofenac sodium was transferred into a 100ml dried volumetric flask. The compounds were first dissolved in 10ml of THF and then the volume was adjusted to 100ml with mobile phase. From the resulting solution 5ml was transferred into 50ml volumetric flask. The volume was made up with mobile phase to 50ml.

Method:

The system precision was evaluated by measuring 6 successive injection of 20µl of standard solution. The peak response were measured from the following chromatogram and system precision data area shown in Table-15&16.

METHOD PRECISION:

Procedure

Twenty tablets were weighed and powdered. 512.6mg sample tablet DYCERIN-A(equivalent to 100.1mg of Aceclofenac and 50.1mg Diacerein) was taken into 100ml dried volumetric flask. The powder was first dissolved in 10ml of THF and sonicated and finally the volume was adjusted to 100ml with mobile phase. From this solution 5ml was transferred to 50ml volumetric flask

and volume was adjusted to 50ml with mobile phase to get a concentration of 100µg/ml of Aceclofenac and 50µg/ml of Diacerein. 20µl of the solution was injected and the chromatogram obtained is shown in following graph.

The amount of Aceclofenac and Diacerein present in the tablet formulation was calculated by comparing the peak area of the standard and reports are given in Table-17-19

Table-15

System Precision data

S.No.	Area of Diacerein	Area of Aceclofenac
1.	5481.978	4222.529
2.	5484.719	4194.068
3.	5491.212	4196.643
4.	5499.890	4200.154
5.	5500.224	4163.354
6.	5498.099	4125.231
MEAN	5492.687	4183.663
S.D	7.9790	34.3103
%RSD	0.1452	0.820

Table-16

Relative Standard Deviation	Diacerein	Aceclofenac	Acceptance Criteria
	0.1452	0.820	2%

Table-17

Method Precision 0f Diacerein

S.No.	Area Obtained	Assay value in(mg)	% Label claim w/v
1.	5481.929	49.82	99.64
2.	5469.929	49.68	99.37
3.	5491.016	49.82	99.64
4.	5510.121	49.91	99.82
5.	5488.954	49.72	99.74
6.	5517.952	50.00	100.00
	MEAN		5493.316
	STANDARD DEVIATION		17.834
	RELATIVE STANDARD DEVIAION		0.3246

Method Precision 0f Aceclofenac-18

S.No.	Area Obtained	Assay value in(mg)	% Label claim w/v
1.	4219.969	99.61	99.61
2.	4168.401	99.06	99.06
3.	4196.525	99.67	99.67
4.	4225.467	100.20	100.20
5.	4185.737	100.21	100.21
6.	4210.123	100.70	100.70
	MEAN		4201.037
	STANDARD DEVIATION		21.7133
	RELATIVE STANDARD DEVIAION		0.5168

Table-19

Relative Standard Deviation	Diacerein	Aceclofenac	Acceptance Criteria
	0.3246	0.5168	2%

Limit of detection (LOD)

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

It is given by

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

σ = standard deviation of the response

m= slope of the calibration curve

TABLE-20

LIMIT OF DETECTION

DRUG	STANDARD DEVIATION	SLOPE	L.O.Dµg/ml
DIACEREIN	7.9790	53.710	0.490
ACECLOFENAC	34.3103	22.075	5.129

Limit of Quantitation:

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

It is given by

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

σ = standard deviation of the response

m= slope of the calibration curve

TABLE-21

LIMIT OF QUANTITATION

DRUG	STANDARD DEVIATION	SLOPE	L.O.Q µg/ml
DIACEREIN	7.9790	53.710	1.4855
ACECLOFENAC	34.3103	22.075	15.542

RUGGEDNESS

The Ruggedness of an analytical method is degree of reproducibility of

test result obtained by the analysis of the same sample under a variety of normal test condition, such as different laboratories, different analyst, different instruments, different lots of reagents, different elapsed assay times, different assay temperature, different days, etc.

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

Determination:

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

The assay of DIACEREIN and ACECLOFENAC were performed in different conditions like different analyst on different days.

Method:

The standard and sample solutions were prepared by different analysts

on different days and the resulting solution were injected and chromatograms are recorded and shown in following graphs and ruggedness of the method and report of Diacerein and Aceclofenac are shown in Table 22.

Table-22

RUGGEDNESS

Analyst	Date	Amount Found		%purity	
		Diacerein mg	Aceclofenac mg	Diacerein	Aceclofenac
I	05.11.2008	50.17	100.05	100.34	100.05
II	06.11.2008	49.99	100.72	99.98	100.72

ROBUSTNESS

Robustness of an analytical method is measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides

an indication of its reliability during normal usage.

Determination:

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

Method:

Standard solution preparation:

100.2mg of Diacerin and 200.2mg Aceclofenac was transferred into a 100ml dried volumetric flask. The compounds were first dissolved in 10ml of THF.Then the volume was adjusted to 100ml with mobile phase. From the stock solution 5ml was transferred to a 50ml volumetric flask and the volume was adjusted to 50ml with mobile phase to get a concentration of 50µg/ml of Diacerin and 100µg/ml of Aceclofenac.

Sample preparation:

Twenty tablets were weighed and powdered. 512.6mg sample tablet

DYCERIN-A(equivalent to 100.1mg of Aceclofenac and 50.1mg Diacerein) was taken into 100ml dried volumetric flask. The powder was first dissolved in 10ml of THF and sonicated and finally the volume was adjusted to 100ml with mobile phase. From this solution 5ml was transferred to 50ml volumetric flask and volume was adjusted to 50ml with mobile phase to get a concentration of 100µg/ml of Aceclofenac and 50µg/ml of Diacerein. 20µl of the solution was injected and the chromatogram obtained is shown in following graphs.

The amount of Aceclofenac and Diacerein present in the tablet formulation was calculated by comparing the peak area of the standard and reports are given in Table-23-30

Table 23

Chromatographic condition:- change in flow rate (2 - 0.2ml/min)

Change in flow rate	1.8ml/min
Column	C_{18}
Wave length	265nm
Temperature	Ambient25^{o}c
Injection Volume	20µl

Table 24

Change in flow rate (1.8 ml/min)

S.No.	**Drug**	**Average Standard Area**	**Average Sample Area**	**% Purity w/ v**
1.	Diacerein	6542.189	6585.098	100.3
2.	Aceclofenac	4952.103	4974.121	100.12

Table 25

Chromatographic condition:- change in flow rate (2+0.2ml/min)

Change in flow rate	2.2 ml/min

Column	C_{18}
Wave length	265nm
Temperature	Ambient25°c
Injection Volume	20µl

Table 26

Change in flow rate (2.2 ml/min)

S.No.	Drug	Average Standard Area	Average Sample Area	% Purity w/ v
1.	Diacerein	4747.625	4770.182	100.12
2.	Aceclofenac	3838.522	3850.198	99.98

Table 27

Chromatographic condition:- change in Lambda max 263 nm

flow rate	2.0 ml/min
Column	C_{18}
Wave length	263nm
Temperature	Ambient25°c
Injection Volume	20µl

Table 28

Change in Lambda max 263 nm

S.No.	Drug	Average Standard Area	Average Sample Area	% Purity w/ v
1.	Diacerein	5581.541	5596.858	99.92
2.	Aceclofenac	4201.808	4258.008	101.01

Table 29

Chromatographic condition:- change in Lambda max 267 nm

flow rate	2.0 ml/min
Column	C_{18}
Wave length	267nm
Temperature	Ambient25^{o}c
Injection Volume	20µl

Table 30

Change in Lambda max 267 nm

S.No.	Drug	Average Standard Area	Average Sample Area	% Purity w/ v
1.	Diacerein	5317.509	5357.509	100.4
2.	Aceclofenac	4106.019	4152.287	100.8

SYSTEM SUITABILITY PARAMETERS

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

Method:

A solution of 100.1 µg/ml Diacerein and Aceclofenac 200.2 µg/ml were prepared by diluting with mobile phase and same was injected and a

chromatogram was recorded and they are shown in the following graph and system suitability report are shown in the following Table-31

Table 31

System suitability parameters

S.No.	Parameters	Diacerein	Aceclofenac
1.	Theoretical plates	3830	6722
2.	Tailing factor	1.263	0.917
3.	Resolution	9.948	

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

Aceclofenac and Diacerein

UV spectrophotometry by simultaneous equation method and reverse phase high performance liquid chromatography were developed for analysing Aceclofenac and Diacerein in combined tablet dosage form.

For UV spectrophotometry linearity was obtained in the concentration range of 5 to 50 µg/ml for Aceclofenac and 5 to 50 µg/ml for Diacerein. In quantitative determination the Percentage Drug content was found to be 100.11% and 100.10% for Aceclofenac and Diacerein respectively. Recovery experiments were performed and it was within 98 – 102% , the percentage relative standard deviation were found to be <2% which shows high precision and accuracy of the method.

In HPLC method, HPLC conditions were optimized to obtain an adequate separation of eluted compounds. Initially various mobile phase were tried, to separate drugs. Mobile phase and flow rate selection was based on peak parameters (height, tailing, theoretical plates, etc). The system with buffer (Potassium di hydrogen ortho phosphate pH 3): Acetonitrile : methanol (45:35:20 v/v) with 2 ml/min flow rate is quite robust. The optimum wavelength for detection was 265nm at which better detector response for drugs was obtained. The average retention times for 3.52 minutes for Diacerein ,6.2

minutes for Aceclofenac respectively.

According to USP system suitability test are an integral part of chromatographic method. They are used to verify the reproducibility of the chromatographic system. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared stock solution. Theoretical plates are found to be 3830 and 6722 for Diacerein and Aceclofenac respectively.

Tailing factors are 1.263 and 0.917 for Diacerein and Aceclofenac respectively.The resolution between these drugs is 9.948.

The calibration curve was found to be linear for both Diacerein & Aceclofenac.

The low values of Percentage RSD indicate the method is precise and accurate. The developed method was very specific without the interference of excipients.

The percentage purity was 99.78% and 100.29% for Diacerein and Aceclofenac respectively.

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

Limit of detection for Diacerein and Aceclofenac was found to be 0.490 µg/ml & 5.129 µg/ml respectively.

Limit of quantitation for Diacerein and Aceclofenac was found to be 1.4855 µg/ml & 15.542 µg/ml respectively.

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

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

Mosapride and Pantoprazole

UV spectrophotometry by simultaneous equation method and reverse phase high performance liquid chromatography were developed for analysing Mosapride and Pantoprazole in combined dosage form.

For UV spectrophotometry linearity was obtained in the concentration range of 5 to 50 µg/ml for Aceclofenac and 5 to 50 µg/ml for Diacerein. In quantitative determination the Percentage Drug content was found to be 101.35% and 99.20% for Mosapride and Pantoprazole respectively. Recovery experiments were performed and it was within 98 – 102% , the percentage relative standard deviation were found to be <2% which shows high precision and accuracy of the method.

In HPLC method, HPLC conditions were optimized to obtain an adequate separation of eluted compounds. Initially various mobile phase were tried, to separate drugs. Mobile phase and flow rate selection was based on peak parameters (height, tailing, theoretical plates, etc). The system with buffer

(Glacial acetic acid pH 6): Acetonitrile : methanol (60:30:10 v/v) with 2 ml/min flow rate is quite robust. The optimum wavelength for detection was 278nm at which better detector response for drugs was obtained. The average retention times for 6.13 minutes for Mosapride,2.65 minutes for Pantoprazole respectively.

According to USP system suitability test are an integral part of chromatographic method. They are used to verify the reproducibility of the chromatographic system. To ascertain its effectiveness, system suitability tests were carried out on freshly prepared stock solution.Theoretical plates for Mosapride and Pantoprazole are 9699,5602 respectively. Tailing Factors are found to be 1.789 and 1.184 for Mosapride,Pantoprazole respectively.

The Resolution between these two drurs was 17.841.

The calibration curve was found to be linear for both Mosapride & Pantoprazole.

The low values of Percentage RSD indicate the method is precise and accurate. The developed method was very specific without the interference of excipients.

The percentage purity was 100.53% and 100.50% for Mosapride and Pantoprazole respectively.

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

Limit of detection for Mosapride and Pantoprazole was found to be 0.00166 µg/ml & 0.0034 µg/ml respectively.

Limit of quantitation for Mosapride and Pantoprazole was found to be 0.00503 µg/ml & 0.0103 µg/ml respectively.

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

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

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

1. **Aceclofenac and Diacerein** available in combined dosage form were analysed by UV–spectrophotometric simultaneous equation method and reverse phase high performance liquid chromatography.

On comparing both the methods reverse phase high performance liquid chromatography was found to be more accurate, simple, and rapid than simultaneous equation method.

The values of standard deviation and relative standard deviation were found to be satisfactory.

2. **Mosapride and Pantoprazole** available in combined dosage form were analysed by UV–spectrophotometric simultaneous equation method and reverse phase high performance liquid chromatography.

On comparing both the methods reverse phase high performance liquid chromatography was found to be more accurate, simple, and rapid than simultaneous equation method.

The values of standard deviation and relative standard deviation were found to be satisfactory.

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