Chapter: 1

General introduction to analytical methodology
CHAPTER-1

Assay of drugs in dosage forms

Drugs are typically developed and formulated into dosage forms prior to their administration. Dosage forms require a variety of tests and standards and to assure therapeutic benefit. The intricacies of drug delivery system complicate efforts to develop control assays and tests. The drug delivery matrix may complicate procedures for extraction or recovery of the drug from its excipients prior to qualitative analysis.

Novel dosage forms, such as controlled release capsules and inserts are often designed to deliver drugs in vivo for 12 hours or more, designing laboratory tests and procedures to control such performance present challenges.

Pharmaceutical chemistry is a science that makes use of the general laws of chemistry to study drugs i.e., their physical and chemical nature, composition, structure, influence on an organism and also studies the methods of quality control and conditions of their storage. The enormous number of drugs available for the treatment of various diseases has made it necessary to classify them. Hence, a distinct division into the following two classes has been made.

Pharmacodynamic agents

These drugs have certain effects on animal organs but are not specific remedies for particular diseases. They may be further subdivided into different classes like central nervous system modifiers (depress or stimulate), adrenergic stimulants and blocking agents, cholinergic and anticholinergic agents, cardiovascular agents, diuretics, anti-inflammatory agents, antispasmodics, antihistaminic, anticoagulants and hormones (steroidal and non steroidal).
Chemotherapeutic or anti-infective agents

Anti-infective agents treat infection by suppressing or destroying the causative microorganisms—bacteria, mycobacteria, fungi, protozoa or viruses. Anti-infective agents derived from natural origin are said to be antibiotics, and those produced from synthetic substances are said to be antimicrobials. However, these two terms are now used interchangeably. An anti-infective agent should be chosen on the basis of its pharmacological properties and spectrum of activity as well as on various dependent factors.

Types of Instrumental Techniques

The methods of estimation of drugs are divided into physical, chemical, physicochemical and biological. Among them, physical and physicochemical methods are widely used. Physical methods of analysis involve the study of the physical properties of substance. They include the determination of solubility, transparency or degree of turbidity, colour intensity or specific gravity (for liquids), moisture content, melting, freezing and boiling points. Physicochemical methods are used to study physical phenomenon that occurs as a result of chemical reactions. Among the physicochemical methods, optical (refractometry, polarimetry, emission and fluorescent methods of analysis, photometry including photocolorimetry and spectrophotometry, nephelometry or turbidimetry), electrochemical (potentiometry, amperometry, coulometry, polarography) and chromatography (column, paper, thin layer, gas-liquid, high performance liquid) methods are generally preferable. Methods involving nuclear reactions such as nuclear magnetic resonance (NMR) and paramagnetic resonance (PMR) are becoming more and more popular. The combination of mass spectroscopy with
gas chromatography is one of the most powerful tools available. The chemical methods include gravimetric and volumetric procedures, which are based on complex formation, acid-base, precipitation and redox reactions. Titrations in nonaqueous media and complexometry have been widely used in pharmaceutical analysis whenever the drug levels are in milligram level and the interference is negligible. These methods (HPLC, GLC, NMR and Mass Spectroscopy) of choice for assay involve sophisticated equipment that are very costly and pose problems of maintenance. Hence they are not in the reach of most laboratories and small scale industries, which produce bulk drugs and pharmaceutical formulations. However the use of sophisticated equipment eliminates the difficulties encountered in the determination of minute amounts of degradation products or the analysis of metabolites of drugs in body fluids.

**Selection of Analytical Techniques for the Present Work**

Qualitative analysis yields information about the identity of atomic or molecular species or the functional groups in the sample whereas quantitative analysis provides numerical information as to the relative amounts of one or more of these components.

**Classification of analytical methods**

Analytical methods are often classified as being either classical or instrumental.
**Classical methods**

In the early years, most analysis were carried out by separating the components of interest (the analyte) in a sample by precipitation, extraction or distillation. For qualitative analysis, the separated components were then treated with reagents that yielded products that could be recognized by their colors, their optical activities or their refractive indexes.

**Instrumental methods**

Early in the twentieth century, chemists began to exploit phenomenon other than those used for classical methods for solving analytical problems. Thus, measurements of physical properties of analytes—such as conductivity, electrode potential, light absorption or emission, mass-to-charge ratio, and fluorescence began to be used for quantitative analysis. Furthermore, highly efficient chromatographic and electrophoretic techniques began to replace distillation, extraction and precipitation for the separation of components of complex mixture prior to their quantitative or qualitative determination. These newer methods for separating and determining chemical species are collectively known as instrumental methods of analysis.

**Types of instrumental methods preferred in the present investigation**

The spectrophotometric methods involve interactions of the analyte with electromagnetic radiation. The atoms present in the analyte are excited by electromagnetic radiation from a selected region of the spectrum and the excited state atoms then emit characteristic electromagnetic radiation, which are then quantitatively measured by the instrument by means of the electrical quantities.
with voltage or current. In spectrophotometric methods, absorption of radiation is the characteristic property of the analyte. The analyte is able to absorb some fraction of characteristic electromagnetic radiation, which is the quantity measured by the instrument.

The name high performance liquid chromatography (HPLC) is employed to the column chromatography in which the mobile phase is a liquid. Here the separation and elution of analytes can be done very quickly based on any property of the analyte such as partition between stationary and mobile phase liquids or adsorption on solid stationary phase or ion exchange property of the analyte or size exclusion, because increase in flow rate increases plate heights.

Every country has legislation on bulk drugs and their pharmaceutical formulation that sets standards and obligatory quality indices for them. These regulations are present in separate articles – general and specific – relating to individual drugs, and are published in the form of book called a Pharmacopoeia (eg. India- IP; USA-USP; Britain- BP). Pharmaceutical analysis deals not only with medicaments (drugs and their formulations) but also with their precursors i.e., with the raw material on whose degree of purity, the quality of the medicament depends. The quality of a drug is determined, after establishing its authenticity, by testing its purity and the quality of the pure substance in the drug and its formulations. The methods of estimation of drug can also be divided into physical, chemical, physiochemical and biological ones. Physiochemical and physical methods are used the most. Physical methods of analysis involve the studying of the physical properties of a substance. They include determination of solubility transparency or degree of turbidity, color, density or specific gravity (for liquids), moisture content and melting, freezing and boiling points.
Physiochemical methods are used to study the physical phenomenon that occurs as a result of chemical reactions. Among the physicochemical methods, the most important are optical, (refractometry, polarimetry, emission and fluorescence methods of analysis, photometry including photocolorimetry and spectrophotometry covers UV, visible and IR regions, nephelometry or turbidimetry), electro-chemical (potentiometry, amperometry, coulometry and polarography) and chromatography(column, paper, thin layer, gas liquid ,high performance liquid chromatography) methods. Methods involving nuclear reactions such as nuclear magnetic resonance (NMR) and paramagnetic resonance (PMR) are becoming more and more popular. The combination of mass spectroscopy with gas chromatography is one of the most powerful tools available. The chemical methods include the gravimetric and volumetric procedures, which are based on complex formation, acid-base, precipitation and redox reactions. Titrations in nonaqueous media and complexometry have been used in pharmaceutical analysis.

**Importance of HPLC in pharmaceutical analysis**

HPLC provides reliable quantitative precision and accuracy, along with a linear dynamic range sufficient to allow for the determination of the active pharmaceutical ingredients and related substances in the same run using a variety of detectors, and can be performed on fully automated instrumentation. HPLC provides excellent reproducibility and is applicable to a wide array of compound types by judicious choice of HPLC column chemistry. Separation of chiral molecules into their respective enantiomers is possible by HPLC. This involves pre-column derivatisation to form diasteriomers or addition of the derivatization
reagents to the chromatographic mobile phase to form dynamic diastereomers during the separation process.

Alternatively, special columns prepared with cyclo dextrins or specific chiral moieties as stationary phase may be used.

1.3.2.1 Advantages of HPLC

- For accurate quantitative measurements.
- Repetitive and reproducible analysis using the same column.
- Automation of the analytical procedure and data handling.
- Adsorption, partition, ion-exchange and exclusion column separations are excellently made.
- Both aqueous and non-aqueous samples can be analyzed with little or no sample pretreatment.
- A variety of solvents and column packing are available, providing a high degree of sensitivity for specific.
- Provides a means for determination of multiple components in a single analysis.

Chromatography (from Greek χρώμα:chroma, color and γραφεῖν:graphein to write) is the collective term for a set of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which
separates the analyte to be measured from other molecules in the mixture and allows it to be isolated.

- Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive.

- The **analyte** is the substance that is to be separated during chromatography.

- **Analytical chromatography** is used to determine the existence and possibly also the concentration of analyte(s) in a sample.

- A **bonded phase** is a stationary phase that is covalently bonded to the support particles or to the inside wall of the column tubing.

- A **chromatogram** is the visual output of the chromatograph. In the case of an optimal separation, different peaks or patterns on the chromatogram correspond to different components of the separated mixture.

Plotted on the x-axis is the retention time and plotted on the y-axis a signal (for example obtained by a spectrophotometer, mass spectrometer or a variety of other detectors) corresponding to the response created by the analytes exiting the system. In the case of an optimal system the signal is proportional to the concentration of the specific analyte separated.
• A **chromatograph** is equipment that enables a sophisticated separation e.g. gas chromatographic or liquid chromatographic separation.

• **Chromatography** is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction.

• The **effluent** is the mobile phase leaving the column.

• An **immobilized phase** is a stationary phase which is immobilized on the support particles, or on the inner wall of the column tubing.

• The **mobile phase** is the phase which moves in a definite direction. It may be a liquid (LC and CEC), a gas (GC), or a supercritical fluid (supercritical-fluid chromatography, SFC). A better definition: The mobile phase consists of the sample being separated/analyzed and the solvent that moves the sample through the column. In one case of HPLC the solvent consists of a carbonate/bicarbonate solution and the sample is the anions being separated. The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated.

• **Preparative chromatography** is used to purify sufficient quantities of a substance for further use, rather than analysis.

• The **retention time** is the characteristic time it takes for a particular analyte to pass through the system (from the column inlet to the detector) under set of conditions.
• The **sample** is the matter analysed in chromatography. It may consist of a single component or it may be a mixture of components. When the sample is treated in the course of an analysis, the phase or the phases containing the analytes of interest is/are referred to as the sample whereas everything out of interest separated from the sample before or in the course of the analysis is referred to as waste.

• The **solute** refers to the sample components in partition chromatography.

• The **solvent** refers to any substance capable of solubilizing other substance, and especially the liquid mobile phase in LC.

• The **stationary phase** is the substance which is fixed in place for the chromatography procedure.

**Techniques by chromatographic bed shape**

**Column chromatography**

Column chromatography is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) or be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column). Differences in rates of movement through the medium are calculated to different retention times of the sample.
In 1978, W. C. Still introduced a modified version of column chromatography called **flash column chromatography** (flash). The technique is very similar to the traditional column chromatography, except for that the solvent is driven through the column by applying positive pressure. This allowed most separations to be performed in less than 20 minutes, with improved separations compared to the old method. Modern flash chromatography systems are sold as pre-packed plastic cartridges, and the solvent is pumped through the cartridge. Systems may also be linked with detectors and fraction collectors providing automation. The introduction of gradient pumps resulted in quicker separations and less solvent usage.

A spreadsheet that assists in the successful development of flash columns has been developed. The spreadsheet estimates the retention volume and band volume of analytes, the fraction numbers expected to contain each analyte, and the resolution between adjacent peaks. This information allows users to select optimal parameters for preparative-scale separations before the flash column itself is attempted.

In expanded bed adsorption, a fluidized bed is used, rather than a solid phase made by a packed bed. This allows omission of initial clearing steps such as centrifugation and filtration, for culture broths or slurries of broken cells.

**Planar Chromatography**

Thin layer chromatography is used to separate components of chlorophyll

**Planar chromatography** is a separation technique in which the stationary phase is present as or on a plane. The plane can be a paper, serving as such or
impregnated by a substance as the stationary bed (paper chromatography) or a layer of solid particles spread on a support such as a glass plate (thin layer chromatography). Different compounds in the sample mixture travel different distances according to how strongly they interact with the stationary phase as compared to the mobile phase. The specific Retardation factor (Rf) of each chemical can be used to aid in the identification of an unknown substance.

**Paper Chromatography**

Paper chromatography is a technique that involves placing a small dot or line of sample solution onto a strip of chromatography paper. The paper is placed in a jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture which starts to travel up the paper with the solvent. This paper is made of cellulose, a polar substance, and the compounds within the mixture travel farther if they are non-polar. More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far.

**Thin layer chromatography**

Thin layer chromatography (TLC) is a widely-employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate. Compared to paper, it has the advantage of faster runs, better separations, and the choice between different adsorbents. For even better resolution and to allow for quantitation, high-performance TLC can be used.
Displacement Chromatography

The basic principle of displacement chromatography is a molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser affinities.\(^4\) There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds, and there be resolved, there must be substantial differences in some interaction between the biomolecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with gradient elution and low column loadings. Thus, two drawbacks to elution mode chromatography, especially at the preparative scale, are operational complexity, due to gradient solvent pumping, and low throughput, due to low column loadings. Displacement chromatography has advantages over elution chromatography in that components are resolved into consecutive zones of pure substances rather than “peaks”. Because the process takes advantage of the nonlinearity of the isotherms, a larger column feed can be separated on a given column with the purified components recovered at significantly higher concentrations.
Techniques by physical state of mobile phase

Gas chromatography

Gas chromatography (GC), also sometimes known as Gas-Liquid chromatography, (GLC), is a separation technique in which the mobile phase is a gas. Gas chromatography is always carried out in a column, which is typically "packed" or "capillary" (see below).

Gas chromatography (GC) is based on a partition equilibrium of analyte between a solid stationary phase (often a liquid silicone-based material) and a mobile gas (most often Helium). The stationary phase is adhered to the inside of a small-diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column). It is widely used in analytical chemistry; though the high temperatures used in GC make it unsuitable for high molecular weight biopolymers or proteins (heat will denature them), frequently encountered in biochemistry, it is well suited for use in the petrochemical, environmental monitoring, and industrial chemical fields. It is also used extensively in chemistry research.

Liquid chromatography

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Present day liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred to as high performance liquid chromatography (HPLC).
In the HPLC technique, the sample is forced through a column that is packed with irregularly or spherically shaped particles or a porous monolithic layer (stationary phase) by a liquid (mobile phase) at high pressure. HPLC is historically divided into two different sub-classes based on the polarity of the mobile and stationary phases. Technique in which the stationary phase is more polar than the mobile phase (e.g. toluene as the mobile phase, silica as the stationary phase) is called normal phase liquid chromatography (NPLC) and the opposite (e.g. water-methanol mixture as the mobile phase and C18 = octadecylsilyl as the stationary phase) is called reversed phase liquid chromatography (RPLC). Ironically the "normal phase" has fewer applications and RPLC is therefore used considerably more.

Specific techniques which come under this broad heading are listed below. It should also be noted that the following techniques can also be considered fast protein liquid chromatography if no pressure is used to drive the mobile phase through the stationary phase. See also Aqueous Normal Phase Chromatography.

**Affinity chromatography**

Affinity chromatography\textsuperscript{15} is based on selective non-covalent interaction between an analyte and specific molecules. It is very specific, but not very robust. It is often used in biochemistry in the purification of proteins bound to tags. These fusion proteins are labelled with compounds such as His-tags, biotin or antigens, which bind to the stationary phase specifically. After purification, some of these tags are usually removed and the pure protein is obtained.
Supercritical fluid chromatography

Supercritical fluid chromatography is a separation technique in which the mobile phase is a fluid above and relatively close to its critical temperature and pressure.

Techniques by separation mechanism

Ion exchange chromatography

Ion exchange chromatography uses ion exchange mechanism to separate analytes. It is usually performed in columns but can also be useful in planar mode. Ion exchange chromatography uses a charged stationary phase to separate charged compounds including amino acids, peptides, and proteins. In conventional methods the stationary phase is an ion exchange resin that carries charged functional groups which interact with oppositely charged groups of the compound to be retained. Ion exchange chromatography is commonly used to purify proteins using FPLC.

Size exclusion chromatography

Size exclusion chromatography (SEC) is also known as gel permeation chromatography (GPC) or gel filtration chromatography and separates molecules according to their size (or more accurately according to their hydrodynamic diameter or hydrodynamic volume). Smaller molecules are able to enter the pores of the media and, therefore, take longer to elute, whereas larger molecules are excluded from the pores and elute faster. It is generally a low resolution chromatography technique and thus it is often reserved for the final,
"polishing" step of a purification. It is also useful for determining the tertiary structure and quaternary structure of purified proteins, especially since it can be carried out under native solution conditions.

SPECIAL TECHNIQUES

Reversed-phase chromatography

Reversed-phase chromatography is an elution procedure used in liquid chromatography in which the mobile phase is significantly more polar than the stationary phase.

Two-dimensional chromatography

In some cases, the chemistry within a given column can be insufficient to separate some analytes. It is possible to direct a series of unresolved peaks onto a second column with different physico-chemical (Chemical classification) properties. Since the mechanism of retention on this new solid support is different from the first dimensional separation, it can be possible to separate compounds that are indistinguishable by one-dimensional chromatography.

Chiral chromatography

Chiral chromatography involves the separation of stereoisomers. In the case of enantiomers, these have no chemical or physical differences apart from being three dimensional mirror images. Conventional chromatography or other separation processes are incapable of separating them. To enable chiral separations to take place, either the mobile phase or the stationary phase must themselves be made chiral, giving differing affinities between the analytes. Chiral
chromatography HPLC columns (with a chiral stationary phase) in both normal and reversed phase are commercially available.

**Paper chromatography:**

Paper chromatography is an analytical chemistry technique for separating and identifying mixtures that are or can be coloured, especially pigments. This can also be used in secondary or primary colours in ink experiments. This method has been largely replaced by thin layer chromatography, however it is still a powerful teaching tool. Two-way paper chromatography, also called two-dimensional chromatography, involves using two solvents and rotating the paper 90° in between. This is useful for separating complex mixtures of similar compounds, for example, amino acids.

**Technique**

A small concentrated spot of solution that contains the sample of the solute is applied to a strip of chromatography paper about two centimetres away from the base of the plate, usually using a capillary tube for maximum precision. This sample is absorbed onto the paper and may form interactions with it. Any substance that reacts or bonds with the paper cannot be measured using this technique. The paper is then dipped in to a suitable solvent, such as ethanol or water, taking care that the spot is above the surface of the solvent, and placed in a sealed container. The solvent moves up the paper by capillary action, which occurs as a result of the attraction of the solvent molecules to the paper, also this can be explained as differential adsorption of the solute components into the solvent. As the solvent rises through the paper it meets and dissolves the sample.
mixture, which will then travel up the paper with the solvent solute sample. Different compounds in the sample mixture travel at different rates due to differences in solubility in the solvent, and due to differences in their attraction to the fibres in the paper. The more soluble the component the further it goes. Paper chromatography takes anywhere from several minutes to several hours. In some cases, paper chromatography does not separate pigments completely; this occurs when two substances appear to have the same values in a particular solvent. In these cases, two-way chromatography is used to separate the multiple-pigment spots.

**Ascending Chromatography**

In this method, the solvent is in pool at the bottom of the vessel in which the paper is supported. It rises up the paper by capillary action against the force of gravity.

**Descending Chromatography**

In this method, the solvent is kept in a trough at the top of the chamber and is allowed to flow down the paper. The liquid moves down by capillary action as well as by the gravitational force. In this case, the flow is more rapid as compared to the ascending method. Because of this rapid speed, the chromatography is completed in a comparatively shorter time. The apparatus needed for this case is more sophisticated. The developing solvent is placed in a trough at the top which is usually made up of an inert material. The paper is then suspended in the solvent. Substances that cannot be separated by ascending method, can sometimes be separated by the above descending method.
**R\textsubscript{f} value**

R\textsubscript{f} value may be defined as the ratio of the distance travelled by the substance to the distance travelled by the solvent. R\textsubscript{f} values are usually expressed as a fraction of two decimal places but it was suggested by Smith that a percentage figure should be used instead. If R\textsubscript{f} value of a solution is zero, the solute remains in the stationary phase and thus it is immobile. If R\textsubscript{f} value = 1 then the solute has no affinity for the stationary phase and travels with the solvent front.

**HPLC Instrumentation**

**HPLC EQUIPMENT BASICS**

The main components of HPLC are:

- **Solvent Reservoir**
- **Pump**
- **Injection Port**
- **Column**
- **Detector**
- **Data Acquisition System**

**Solvent Reservoir**

Solvent Reservoirs are used to store Mobile-Phase. Scott Duran bottles are commonly used as solvent reservoirs. The solvent reservoir must be made of inert material such as glass and must be smooth so as to avoid growth of microorganisms on its walls. It can be transparent or can be amber colored. A
graduated bottle gives a rough estimate of mobile-phase volume in the bottle. Solvent reservoirs are placed above HPLC system (at higher level) in a tray. They should never be kept directly above the system as any spillage of solvent on the system may damage electronic parts of HPLC.

**HPLC Pump**

The HPLC pump is very important component of the system. The Pump delivers the constant flow of the Mobile Phase or phases so that the separation of the components of the mixture occur in a reasonable time. There are two types of pumping systems Isocratic and Gradient.

**Modes of HPLC**

**Isocratic:** In the system the things are kept constant throughout the run. In case of pumping of Mobile phase, the mobile phase composition is kept constant throughout the run. The nominal flow rate accuracy required is $+ or - 1\%$ of the set flow.

**Gradient:** There is some change purposely incorporated during the particular sample run to achieve a better or/and faster separation. In case of pumping mobile phase, the composition of the mobile phase is continuously varied during the particular run for one of the above mentioned purpose. $+ or - 1\%$ of the step gradient composition is typical.

There are two types of gradient systems:

- Low pressure mixing
- High pressure mixing
Each system has its advantage. Low pressure mixing gives you a choice of three or four mobile phases. In High pressure mixing, generally two pumps are used and hence the choice is of two different mobile phases only. High pressure mixing is advantageous as even if one pump fails one can work in isocratic mode.

**Injection Port**

The sample introduction device such as injector to introduce the sample in a flow of mobile phase at high pressure. It is not possible to use direct syringe injection on column like GC as the inlet pressure in LC is too high. The valve injection through fixed or variable loop is a common way of introducing the sample. The Rheodyne valve is the mostly used devise. The loop can be partially or fully filled. There are both the types of injectors available. The advantage of partial filling is the possibility of using small amount of sample, when there is scarcity of sample. The precision of the injection is 1% RSD and carryover < 1%.

**HPLC Column**

Columns and related stuff

The HPLC Column holds the stationary phase for separating the components of the sample. The columns are usually made up of SS-316 grade steel. Apart from columns, the material of construction of tubing and fittings, plumbing and connections are also very critical. Apart from resistively to corrosion, connections and plumbing should have a very low dead volume. You can read more details about column from following links.
HPLC Column Basics

HPLC Column Hardware

Stationary Phase

Column Efficiency

Columns and Packing

HPLC Detectors

Detect various compounds as they elute out from column. The detector gives response in terms of a milivolt signal that is then processed by the computer (integrator) to give you a chromatogram. Basically detector consists of a flow-cell through which the mobile phase and resolved sample moves. Optics shine through the detector cell and variation in optical properties are detected. A Ultra violet or UV detector detects absorbance of UV light by chromophores in the analyte compound. A refractive index detector will sense variation in refractive index of mobile phase stream passing through flow-cell as the sample/analyte mixed M.P enters the detector. Similarly Fluorescence Detectors checks for Florescence.

The Photo Diode Array Detector: DAD is the most used detector in LC today. The DAD gives a three dimensional view of chromatogram (Intensity Vs Time) and Spectra (Intensity Vs Wavelength) simultaneously. It can be called as Spectro-chromatogram. The detailed analysis of the data reveals more information on the complexity of co elution and helps in identifying the merged peaks and gives information on peak purity.
Various types of HPLC Detectors

There are several detectors available in the market. However UV-VIS Detector, Photo-Diode Array Detector, fluorescence Detector, Conduct metric and coulometric detector are more commonly used. The new ELSD detector is proving to be important detector; while the MS as detector is outstanding.

HPLC Detectors

Universal detector - Refractive Index Detector

UV-Visible Detector

Fluorescence Detector

Electrochemical Detector

Conductivity detector

Mass Detector (MS)

Evaporative Light Scattering Detector (ELSD)

Various Detectors and their comparison

<table>
<thead>
<tr>
<th>HPLC Detector</th>
<th>RI Response</th>
<th>UV/VIS Sensitivity</th>
<th>Fluor Sensitivity</th>
<th>MS Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RI</td>
<td>Universal</td>
<td>Selective</td>
<td>Selective</td>
<td>Selective</td>
</tr>
<tr>
<td>UV/VIS</td>
<td>4 microgram</td>
<td>5 nanogram</td>
<td>3 picogram</td>
<td>1 picogram</td>
</tr>
<tr>
<td>Fluor</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>MS</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>
Data Acquisition System

Data Systems process the detector output and integrate it to form a meaningful chromatogram. Modern Integration systems do more than just that. They do processing of the chromatogram, calculations, statistical analysis, data back-up and storage. Data Systems also control various parameters of the system.

Thanks to these advanced systems that we no longer have to cut chromatogram peaks and weigh them on analytical balances for interpretation!

Agilent 1200 HPLC System
Shimadzu VP HPLC System
PROCEDURE FOR ANALYTICAL METHOD DEVELOPMENT

- HPLC method development is not very difficult when a literature reference for a same or similar compounds to be analyzed can be found but what happens when reference to the compounds interest do not exist

- The first consideration when developing a hplc method is to determine the solubility of the components knowing the nature of the analytes will allow the most appropriate mode of hplc top be selected.

- First step in method development is literature search, whether the compound is official is United states of pharmacopoeia, European pharmacopoeia and other journals, active pharmaceutical reports from

- Knowledge about the nature of sample

- The next step involved in method development is

- Selection of mobile phase

- Selection of Diluent

- Selection of column

- Selection of chromatography method

- Selection of buffer

- Selection of detector

- Selection of detector wavelength
SELECTION OF MOBILE PHASE:

- Since the mobile phase governs solute-stationary phase interactions, its choice is critical. Practical considerations dictate that it should not degrade the equipment for the column packing. For this reason, strong acids, bases and halide solutions should be avoided.

- Chemical purity of solvents is an important factor. Since large volumes of solvent are pumped through the column and eventually be detrimental to the results. Spectro or HPLC grade solvents are recommended.

- Volatility should be considered if sample recovery is required.

- Viscosity should be less than 0.5 Centipoise, otherwise higher pump pressures are required and mass transfer between solvent and stationary phase will be reduced.
CHOOSING HPLC MOBILE PHASE BUFFERS

- Buffers are used in HPLC mobile phase preparations in order to achieve reproducible chromatography. They are needed when an analyst is dealing with an ionizable sample species. In reversed phrase chromatography samples are separated based on their hydrophobicity. The less polar a sample is the longer it is retained on the column. When an analyte is ionized it becomes more polar and subsequently less retained on the column. Acids become ionized as the pH increases, conversely bases become ionized as the pH decreases.

- To develop a rugged method buffers should be employed that are at least 2 pH units away from the analyte pKa. This is drawn from the Henderson-Hasselback Equation: \( \text{pH} = \text{pKa} + \log([A^-]/[HA]) \)

- Essentially, operating at a pH near to the pKa of the sample analyte means that it will be in a partially dissociated state, the analyte will partially in its weak acid or base form and partially in its conjugate form. This will cause peak distortion in the chromatography and poor peak reproducibility. Operating with a mobile phase at least 2 pH units away from the analyte pKa ensures that in excess of 99% of the sample will be in a single state.

- Once the proper pH range for the mobile phase is determined choosing the correct buffer can begin. Buffer capacity is optimized at or near a pH equal to the pKa of the buffer. As a rule of thumb, most buffers work suitably well within ±1 pH unit of their pKa.
**BUFFERS FOR REVERSED PHASE HPLC**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pKa</th>
<th>Buffer Range</th>
<th>UV Cut off (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pK1</td>
<td>2.1</td>
<td>1.1-3.1</td>
<td>210</td>
</tr>
<tr>
<td>pK2</td>
<td>7.2</td>
<td>6.2-8.2</td>
<td></td>
</tr>
<tr>
<td>pK3</td>
<td>12.3</td>
<td>11.3-13.3</td>
<td></td>
</tr>
<tr>
<td>Citrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pK1</td>
<td>3.1</td>
<td>2.1-4.1</td>
<td>230</td>
</tr>
<tr>
<td>pK2</td>
<td>4.7</td>
<td>3.7-5.7</td>
<td></td>
</tr>
<tr>
<td>pK3</td>
<td>5.4</td>
<td>4.4-6.4</td>
<td></td>
</tr>
<tr>
<td>Formate</td>
<td>3.8</td>
<td>2.8-4.8</td>
<td>210</td>
</tr>
<tr>
<td>Acetate</td>
<td>4.8</td>
<td>3.8-5.8</td>
<td>230</td>
</tr>
<tr>
<td>Tris(hydroxymethyl) aminomethane</td>
<td>8.3</td>
<td>7.3-9.3</td>
<td>220</td>
</tr>
<tr>
<td>Borate</td>
<td>9.2</td>
<td>8.2-10.2</td>
<td>210</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>10.8</td>
<td>9.8-11.8</td>
<td>200</td>
</tr>
</tbody>
</table>

Another consideration when choosing a buffer is the type of detector being used. Citrate may not be suitable for some UV/Vis applications due to its high UV cutoff limit. Likewise, if mass spec detection is used, a volatile buffer such as TEA or acetate should be employed while non-volatile buffers such as phosphate or citrate should be avoided.
Once a buffer range and type are identified the proper concentration must be used. Ideally, unless using the buffer as ion-pair reagent, the buffer should have negligible effect on the overall separation and retention of your sample analytes. The concentration should be set just high enough to control the mobile phase pH. But still low enough to avoid possible precipitation of the buffer salts in the presence of organic solvents. Typically a buffer concentration in the range of 20-50mM is suitable.

It is advisable that you check the miscibility of your aqueous buffer solution in the highest concentration of organic mobile phase that will be present during the course of a gradient HPLC run before putting it on your system. This can easily be checked by mixing the aqueous portion with the correct amount of organic in a beaker and observe the presence of any salting out. Also, recall that pH is only defined in an aqueous system. So, when adjusting the pH of the mobile phase, it must be done prior to the addition of the organic solvent.

Proper selection and preparation of your mobile phase will help ensure good peak shape in your chromatography. An understanding of your analyte pKa and applying the recommendations outlined above will help in choosing and preparing the right buffer for your application.

Typically ionogenic analytes are analyzed at a mobile phase pH either two units greater or less than the analyte pKa to avoid any secondary equilibria effects that might compromise the chromatography. Accounting for the pH shift of the mobile phase will lead to faster method development, rugged methods and an accurate description of the analyte retention as a function of pH at varying organic compositions.
The pH of the mobile phase affects also the analyte UV response. Understanding the effects of charge delocalization and conjugation on the UV response will allow the chromatographer to choose the proper pH and wavelength of detection to obtain a method with high sensitivity.

Reversed-phase HPLC has become the dominant chromatography technique for separations and analysis. This is due to the subtle ways in which molecules interact with the reversed-phase chromatographic surface, offering the chromatographer remarkable control over the separation process through manipulation of the separation conditions. Optimizing separations may consist of enhancing resolution in order to better quantitate key components or reducing analysis time in order to increase analytical throughput. In order to optimize a separation, the chromatographer needs to understand the underlying factors that affect resolution and analysis time. This class will focus on the key components of resolution and will show the relative affects of each on reversed-phase separations. It will describe in detail the important attributes of columns currently in use that affect resolution and analysis time and how these may be optimized by selection of the optimum column chemistry. The class goes on to describe the important characteristics of the mobile phase that affect resolution and analysis time, including solvent type and strength, gradient conditions and mobile phase pH. The affect of temperature on optimizing reversed-phase separations is also discussed. The class finally suggests approaches to using this understanding to most effectively and efficiently develop robust HPLC separations. The class is intended for those who are currently using reversed-phase HPLC and have an interest in further optimizing separation methods to meet laboratory needs. The class will provide those new to HPLC with a sound understanding of the
separation process and will provide those more experienced in HPLC with a broad sense of how to optimize separations and develop methods.

**ADDITION OF MOBILE PHASE ADDITIVES:**

When a peak tailing is observed, increasing the salt concentration can reduce peak tailing for both bases and acids. For ionizable compounds an increase in ionic strength can suppress solute and silica ionization, as well as secondary interactions between them. Increase beyond 50mM is not recommended due to possible solubility problems of the salt in the organic portion of the mobile phase. Triethylamine (TEA) or diethylamine can be added to the mobile phase to control peak tailing for bases. TEA, DEA acts as a competing base and minimizes solute-silanol interactions.

- This is usually a final step to try because TEA/DEA will reduce retention, modify the column and complicate the mobile phase.

- For reducing the peak tailing for acids, 1% acetic acid is added to the mobile phase to minimize solute silanol interactions as it acts as competing acid.
ADDIITION OF DIFFERENT ORGANIC MODIFIERS OR ION PAIR REAGENTS IN MOBILE PHASE:

- Acetonitrile is the first best organic solvent, second is methanol and third is THF. These three solvents are widely used to control selectivity and separations.

- Select Acetonitrile as the default organic modifier because of favourable organic modifier and low viscosity. To avoid bumping problems associated with 100% acetonitrile use acetonitrile always with about 10% aqueous portion.

- Order of polarity: Methanol Acetonitrile > Ethanol > THF > propanol

- Order of solvents strengths: propanol > THF > ETHANOL > acetonitrile > methanol

- THF has some advantages, higher UV absorbance reactivity with oxygen and slower column equilibration.

- Sodium perchlorate can be used as an ion pair reagent to get the required selectivity for acidic compounds.

SELECTION OF DILUENTS:

- Select a diluents in which impurities, starting materials, byproducts, intermediates and degradation products and the analyte is soluble. Diluent should be compatible with mobile phase to obtain good peak shape.
• Select a diluent for finished dosage forms in which the analyte should be extracted at least 95%. Calculate the % of extraction against pure compound. The peak shapes of all compounds should be good in the selected diluents.

FOR API:

• It can be achieved by selecting a diluent in which API is completely soluble. Optimising the Extractions:
  
• Diluent selection is based on Extraction Efficiency:

FOR FORMULATIONS:

• Choose a diluent in which the drug is extract out of Excipient matrix.
  
• Optimising the Extractions:
  
• Understanding of the sample Matrix is key.
  
• Avoid Entrapments of Drug in Matrix of Excipients.

WHAT TO EXTRACT (RECOVER) ?.

• Excipients
  
• Active Drug
  
• Preservative

METHODS OF EXTRACTION

• Extraction by
  
• Complete dissolution.
• Swelling of sample Matrix.

• Breaking the matrix.

• Leaching out from the matrix.

• HOW TO RECOVER

• TOOLS EXTRACTION

EXTRACTION BY

• Sonication.

• Rotary shaking.

• Cyclomixing.

• Depending upon the solubility of the drug suitable diluent is selected. All compounds in mixtures must be soluble in the solvent selected at the desired concentration.

• For polar compounds use acetonitrile, methanol or water individually or in combination.

• For more non polar compounds use Dimethyl sulphoxide or Dimethyl formamide.

• Select a solvent which matches the initial mobile phase as closely as possible. If the sample is dissolved in a too strong solvent, significant disturbances can occur in the chromatogram.
SELECTION OF COLUMN:

- Knowledge of the sample influences the choice of column bonded phase characteristics

Column Chemistry:

60 Å PORE SIZE PROVIDES MAXIMUM RETENTION

100 Å PORE SIZE PROVIDES MODERATE RETENTION

3 μM PARTICLE SIZE PROVIDES FASTER SEPARATIONS
5 μM IS IDEAL FOR GENERAL SCREENING INITIAL METHOD DEVELOPMENT

SELECTION OF COLUMN:

Column Properties – terms and definitions

- Polarity
- Polar Selectivity
- Size (hydrophobic) selectivity
- Concentration of high energy sites –
  - (The sites normally should be suppressed for good peak shapes)
- Solvatic Retention Model in RP LC to describe column characteristics

POLAR SELECTIVITY

- Polar selectivity is a capability of a column under specified conditions to separate solutes that differ in their polarity and have no differences in the size.
SIZE SELECTIVITY

Size selectivity is a capability of a column under specified conditions to separate solutes that differ in their size and have no differences in the polarity.

COLUMNS BY PHASE

- C18 Columns
- C8 Columns
- Biphenyl Columns
- Cyano Columns
- Phenyl Columns
- Silica Columns
- Other Phase Columns

COLUMNS BY USE

- pH Stable Columns
- Columns for Acidic Analytes
- Columns for Basic Analytes
- Columns for Hydrophobic Neutral Analytes
- Columns for Hydrophilic Neutral Analytes
- Columns for Aromatic or Unsaturated Analytes
- Columns for Mixed Analytes
- Columns for LC/MS
- Columns for Large Molecules
- Columns for Small Particles
- Columns: Unique Phases
- Columns: Other Phases
- Choosing the Appropriate HPLC Column Should Be Based Both Upon Knowledge of the Sample and Goals for the Separation
Benefits of this Approach Include:

- Small initial time investment
- Big time savings in the HPLC laboratory
- More “informed” approach to column selection
- More efficient than “trial and error” approach
Knowledge of the Sample Influences the Choice of Column Bonded Phase Characteristics

Knowledge of the Sample
- Structure of sample components?
- Number of compounds present?
- Sample matrix?
- $pK_a$ values of sample components?
- Concentration range?
- Molecular weight range?
- Solubility?
- Other pertinent data?

Column Chemistry
(bonded phase, bonding type, endcapping, carbon load)

Goals for the Separation Influence the Choice of Particle Physical Characteristics

Goals for the Separation
- Max. resolution of all components?
- Partial resolution?
- Fast analysis?
- Economy (low solvent usage)?
- Column stability and lifetime?
- Preparative method?
- High sensitivity?
- Other goals?

Column Physics
(particle bed dimensions, particle shape, particle size, surface area, pore size)
Choosing the Particle Physical Characteristics

Example:
Sample Type: hydrophobic compounds
Method Goal: highest resolution

<table>
<thead>
<tr>
<th>Column Selection Chart</th>
<th>Default Column</th>
<th>Optimum Column†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column Bed Dimensions</td>
<td>150 x 4.6mm</td>
<td>250 x 4.6mm</td>
</tr>
<tr>
<td>Particle Size</td>
<td>5µm</td>
<td>3* or 5µm</td>
</tr>
<tr>
<td>Surface Area</td>
<td>200m²/g</td>
<td>&gt;200m²/g</td>
</tr>
<tr>
<td>Pore Size</td>
<td>100Å</td>
<td>100Å</td>
</tr>
<tr>
<td>Carbon Load</td>
<td>10%</td>
<td>16 - 20%</td>
</tr>
<tr>
<td>Bonding Type</td>
<td>Monomeric</td>
<td>Mono- or Polymeric</td>
</tr>
<tr>
<td>Base Material</td>
<td>Silica</td>
<td>Silica</td>
</tr>
<tr>
<td>Particle Shape</td>
<td>Spherical</td>
<td>Spherical</td>
</tr>
</tbody>
</table>

* mobile phase backpressure may be excessive
† Optimum Column: Alltima C18**, 5µm, 250 x 4.6mm (Part No. 88056)
*Note that the choice may represent a compromise. Here, the "optimum" column for resolution sacrifices speed.

Choosing the Particle Physical Characteristics

Column Dimensions
• Length and internal diameter of packing bed

Particle Shape
• Spherical or irregular

Particle Size
• The average particle diameter, typically 3-20µm

Surface Area
• Sum of particle outer surface and interior pore surface, in m²/gram
Column Dimensions

- This refers to the length and internal diameter of the packing media bed within the column tube.

Particle Shape

- Most modern chromatographic packings have spherical particles, but some are irregular in shape.

Particle Size

- This refers to the average diameter of the packing media particles. Standard particle sizes range from 3μm (high efficiency) to 15-20μm (preparative). Recently, Alltech introduced 1.5μm particles in Platinum™ C18 and Platinum™ EPS C18 phases for use in short, high efficiency columns. A 5μm particle size offers a good compromise between efficiency and back pressure.

Surface Area

- Expressed in m²/gram, the total surface area of a particle is the sum of the outer particle surface and the interior pore surface.
Choosing the Particle Physical Characteristics

**Pore Size**
- Average size of pores or cavities in particles, ranging from 60-10,000Å

**Bonding Type**
- Monomeric - single-point attachment of bonded phase molecule
- Polymeric - multi-point attachment of bonded phase molecule

**Carbon Load**
- Amount of bonded phase attached to base material, expressed as %C

**Endcapping**
- "Capping" of exposed silanols with short hydrocarbon chains after the primary bonding step

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**Pore Size**
- This refers to the average size of the pores or cavities present in porous packing particles. Pore sizes range from 60Å on the low end to greater than 10,000Å on the high end.

**Bonding Type**
- This refers to how the bonded phase is attached to the base material. Monomeric bonding uses single-point attachment of each bonded phase molecule to the base material. Polymeric bonding uses multi-point attachment of each bonded phase molecule to the base material.
Carbon Load

- Carbon load refers to the amount of bonded phase attached to the base material. For C18, C8 and phenyl packings, the carbon load is a good indicator of hydrophobic retention.

Endcapping

- Endcapping applies only to reversed phase chromatography and is the process of bonding short hydrocarbon chains to free silanols remaining after the primary bonded phase has been added to the silica base.

Column Dimensions

**Effect on chromatography**

<table>
<thead>
<tr>
<th>Column Dimension</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Short</strong> (30-50mm)</td>
<td>short run times, low backpressure</td>
</tr>
<tr>
<td><strong>Long</strong> (250-300mm)</td>
<td>higher resolution, long run times</td>
</tr>
<tr>
<td><strong>Narrow</strong> (&lt; 2.1mm)</td>
<td>higher detector sensitivity</td>
</tr>
<tr>
<td><strong>Wide</strong> (10-22mm)</td>
<td>high sample loading</td>
</tr>
</tbody>
</table>

Short columns (30-50mm) offer short run times, fast equilibration, low backpressure and high sensitivity. Long columns (250-300mm) provide higher resolving power, but create more backpressure, lengthen analysis times and use more solvent. Narrow column (2.1mm and smaller) beds inhibit sample diffusion and produce narrower, taller peaks and a lower limit of detection. They may require instrument modification to minimize distortion of
the chromatography. Wider columns (10-22mm) offer the ability to load more sample.

- Spherical particles offer reduced back pressures and longer column life when using viscous mobile phases like 50:50 MeOH:H₂O.

**Particle Size**

**Effect on chromatography**
Smaller particles offer higher efficiency, but also cause higher backpressure. Choose 3µm particles for resolving complex, multi-component samples. Otherwise, choose 5 or 10µm packings.

![Particle Sizes](image)

- Smaller particles pack into columns with a higher density, allowing less diffusion of sample bands between particles and causing narrower, sharper peaks. However, smaller particles also cause higher solvent back pressures. As a rule of thumb, choose 1.5 or 3µm particle sizes for resolving complex, multi-component samples. Otherwise, choose 5 or 10µm packings.
Surface Area

Effect on chromatography
High surface area generally provides greater retention, capacity and resolution for separating complex, multi-component samples. Low surface area packings generally equilibrate quickly, especially important in gradient analyses.

High surface area silicas are used in Alltech's Alltima™, Adsorbosphere® HS, and Adsorbosphere® UHS packings. Low surface area silicas are used in Alltech's Platinum™, Econosphere™, and Brava™ packings.

- Solute retention is greater on packings that have a high surface area. High surface areas generally provide longer retention, greater capacity and higher resolution. As a rule of thumb, choose a base material with maximum surface area for resolving complex, multi-component samples. High surface area silicas are used in Alltech's Alltima™, Adsorbosil®, Adsorbosphere® HS, and Adsorbosphere® UHS packings.
Pore Size

Effect on chromatography
Larger pores allow larger solute molecules to be retained longer through maximum exposure to the surface area of the particles. Choose a pore size of 150Å or less for sample MW \( \leq 2000 \). Choose a pore size of 300Å or greater for sample MW > 2000.

- Larger pores allow larger solute molecules to be retained longer through maximum exposure to the surface area of the particles. Choose a pore size of 150Å or less for sample MW \( \leq 2000 \). Choose a pore size of 300Å or greater for sample MW > 2000.
Bonding Type

Effect on chromatography

Monomeric bonding offers increased mass transfer rates, higher column efficiency, and faster column equilibration.

Polymeric bonding offers increased column stability, particularly when highly aqueous mobile phases are used. Polymeric bonding also enables the column to accept higher sample loading.

- Polymeric bonding offers increased column stability, particularly when highly aqueous mobile phases are used. Polymeric bonding also enables the column to accept higher sample loading.
Carbon Load

Effect on chromatography
Higher carbon loads generally offer greater resolution and longer run times. Low carbon loads shorten run times and may show a different selectivity, as in Alltech's Platinum line of packings.

- Higher carbon loads generally give higher column capacities, greater resolution and longer run times. Conversely, low carbon loads shorten run times and may show different selectivity because of greater exposure of the base material. Alltech's Platinum™ line of packings are specifically designed to offer unique selectivity through increased exposure of the base material, while still preserving peak shape. Choose high carbon loads for complex samples which require the maximum degree of separation. Choose low carbon loads to give shorter analysis times for simpler sample mixtures and for samples which require a high water content for solubility or stability.
Endcapping

Effect on chromatography
Endcapping reduces peak-tailing of polar solutes that interact excessively with the otherwise exposed, mostly acidic silanols. Non-endcapped packings provide a different selectivity than do endcapped packings, especially for such polar samples. Alltech's Platinum™ EPS packings are non-endcapped to offer enhanced polar selectivity.

Conclusion
In this approach to HPLC column selection, the bonded phase chemistry of the column is chosen on the basis of an analysis of the sample component structures. The physics of the column is chosen according to an analysis of the goals for the separation method. This approach succeeds in predicting unique, optimum bonded phase chemistries and particle bed physical characteristics that are likely to meet the goals for the separation method.
CHANGING THE PH:

- In reversed phase HPLC retention of analytes is based on hydrophobicity. The more hydrophobic the analyte, the longer it is retained. With increasing the pH acids loses a proton and ionized and become more hydrophobic and less hydrophilic. Therefore its retention decreases. With decreasing pH bases gain a proton and becomes ionized. When bases are ionized, it becomes less hydrophobic and more hydrophilic and hence retention decreases.