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TITLE: Liquid Chromatography: LC Basics and Separation Techniques

PRESENTER: Y. Victoria Zhang, PhD, DABCC

Slide 1:

Hello, my name is Yan Victoria Zhang, an Associate Professor in the Department of Pathology and Laboratory Medicine at the University of Rochester Medical Center and Director of the Clinical Mass Spectrometry and Toxicology Laboratory at Strong Memorial Hospital. Welcome to this Pearl of Laboratory Medicine on "Liquid Chromatography – LC Basics and Separation Techniques."

Slide 2: Outline

Liquid Chromatography (or LC) is a very broad and complex topic and this Pearl will serve as a general introduction. I will discuss the basic components of LC, column chemistry, and the types of separations that can be used for clinical analysis.

Slide 3: What is Chromatography?

Chromatography, derived from Greek words, can be translated into "color writing" or separating the colors in a mixture. It is attributed to the Russian botanist, Mikhail Tsvet, who was able to separate the colored components in plant extracts using a column made of ground up chalk as the solid phase and a mixture of petroleum ether and ethanol as the liquid phase.

There are several types of chromatography. Most of the abbreviations end in "C" which sometimes gives you a clue that we are talking about a separation using chromatography. Specifically, besides LC, there are Gas Chromatography and Supercritical Fluid Chromatography (SFC), which are based on different types of mobile phases; and ion chromatography, gel permeation, and size exclusion chromatography, which are based on separation types.

What is Liquid Chromatography? The name tells you a lot. LC is a separation approach based on a liquid mobile phase.

Slide 4: LC Components

The main components of LC system are those required to handle liquids. The liquid in LC is also referred as Mobile Phase or Eluent. To work with liquids, you need reservoirs to hold it, one or more pumps to move it, and a bottle to collect it when you are done. While the pumps deliver defined flow of liquid, an Injector is used to insert a well-defined amount of the sample into the fluid path. Columns are where the separation occurs, and one or more detectors will let you know what you have accomplished with all of the complicated and expensive equipment.

Slide 5: Columns: Key Parameters

Columns are essential for the separation process. The key parameters of a column include the material that is used to provide the separation, that is, the packing material, the size of the packing material particles, and the dimensions of the column. Most columns are described by a string of numbers, as shown here. In this example, the column is 50 mm long and has an inner diameter of 2.1 mm. The packing material has an average particle size of 2.5 um, and has a C18, or long chain hydrocarbon bonded to it. Finally, the pore size of the particles is 100 Angstroms. Mechanically, columns are often made of stainless steel and stainless steel frits are used to keep the packing material inside.

Slide 6: Columns: Stationary Phase/Packing

Let's look more closely at the stationary phase. Since the column is where the separation happens, a lot of effort has been made to optimize the performance of the column. This has resulted in a wide variety of packing materials being available. Originally, liquid chromatography was performed using silica or alumina as the stationary phase. These materials, shown chemically in the right hand portion of the slide, feature a lot of hydroxyl groups on their surface. Those hydroxyl groups make the columns very polar. As interest in analyzing non-polar molecules grew, techniques were developed to modify the surface of silica, which resulted in a non-polar surface. Since this was the opposite polarity of the original packing materials, it became known as "Reverse Phase" and the original packings became known as "Normal Phase."

Reverse Phase packing materials can be thought of as grease-like or greasy. This comes from the modification of the silica surface by attaching hydrocarbons. Shown here are a variety of common phases such as C4, C18, Cyanopropyl, and Phenyl-Hexyl. There are two ways to draw the backbone structures, which are shown here. C4 has the carbons drawn explicitly, and C8 shown as a "stick figure." The names refer to the chemistry of the attached groups. Please note that these pictures are not to scale – the hydrocarbon chains are tiny compared to the support particles, and the surface is completely covered.

Slide 7: Columns: Dimensions

Columns are available in a variety of shapes and sizes, and the packing materials also come in a variety of sizes. It isn't important to remember all of the sizes, but it is important to know that substituting a column that differs in any dimension will result in a very different separation.

Slide 8: Columns: Particle Sizes

Columns are also available with a variety of particle sizes. This slide illustrates the effects of different particle sizes on separation efficiency and back pressure. In general, the larger the particle size is, the lower the number of theoretical plates and the lower the back pressure. The number of theoretical plates is a measure of the separation efficiency and the back pressure impacts the types of pumps and other tubing requirements for the HPLC system. Similarly, increasing the length of a column increases both the number of theoretical plates and the backpressure. The tendency in LC is to adapt shorter, narrower columns with smaller particle sizes.

Slide 9: Chromatography Pressure Regimes

This brings us to a discussion of the pressure regimens you may encounter while doing liquid chromatography. These regimens are defined by the equipment used to control the liquid flow, the history of the technique, and the column physical characteristics.

Low pressure chromatography does not use a pump to produce liquid flow. Instead, this chromatography uses gravity or a slight pressurization from compressed gas to produce flow. This type of chromatography is typically encountered on a laboratory bench and is often used for sample preparation and purification.

High Performance Liquid Chromatography (HPLC) has been the standard form of analytical chromatography for many years. In general, the upper working range of HPLC systems is around 400 bar or 6,000 psi. HPLC, in the past, also stood for High Pressure Liquid Chromatography, but that verbiage has gone out of favor.

Ultra High Performance Liquid Chromatography (UHPLC) came about in response to the need for faster, better separations and is made possible by improvements in the technology. UHPLC systems typically have an upper pressure limit of roughly 15,000 psi or 1,000 bar. Waters Corporation, an LC manufacturer, trademarked the term “UPLC,” but it is sometimes used and can stand for Ultra Performance or Ultra Pressure LC.

Chromatographers and instrument companies commonly use two sets of pressure units, Pounds per square inch (PSI) and bar. Neither are parts of the International System of Units (SI). Common usage is trending toward bar, but both units will be seen.

Slide 10: Progress in Column Technology

Columns are the heart and brain of chromatography separations. As such, there has been continuous research and development in column technologies. These examples illustrate the progress in column technology and its impact on chromatography separation. Column developments include the particle sizes and packing materials used, which impact column dimensions. Separations are continuously becoming faster and more sensitive, which brings new opportunities and new challenges to those of us using chromatography on a daily basis. Choice of columns will be determined by the purpose of the separation and priority in the separation process. The tradeoffs that we mentioned previously should be considered along with the hardware availability in the lab.

Slide 11: Types of Separation

There are several types of HPLC available, and generally, the different types are based on the separation mechanism or the analyte. The commonly used ones include Normal Phase, Reverse Phase, Hydrophilic Interaction Liquid Chromatography (HILIC), Size Exclusion Chromatography / Gel Permeation Chromatography (SEC/GPC), ion chromatography, and chiral chromatography. There are others, but they tend to be aimed at a more specific set of problems than we need to talk about here. We will discuss each of the common types of separation methods for the remaining of the presentation.

Slide 12: Normal Phase

Normal Phase chromatography has a polar stationary phase and is often comprised of alumina or silica particles. Its mobile phase is non-polar, typically hydrocarbons or chlorinated solvents. Tsvet, who is credited with the invention of chromatography, used calcium carbonate as a stationary phase. Paper chromatography is also a form of Normal Phase chromatography. Normal Phase chromatography was widely used until Reverse Phase chromatography became popular in the 1970s. The separations possible on Normal Phase chromatography are now mainly performed on HILIC or Ion Exchange Chromatography.

Slide 13: Reverse Phase

Reverse Phase (RP) chromatography reverses the polarity of the phases from Normal Phase chromatography. RP uses a non-polar stationary phase and a polar, usually aqueous-based, mobile phase. This results in retention of non-polar molecules on the column. A large variety of stationary phases have been developed, including C18, one of the most common stationary phases in use, C8, and C4 (the C-numbers refer to the hydrocarbon chain length attached to a particle). Other RP columns are available and are used to vary the separation of complex mixtures or to provide an orthogonal separation in method development.

Slide 14: Reverse Phase: Partition and Separation

Since Reverse Phase chromatography is one of the most commonly encountered separations, I would like to use it to illustrate how separation happens in LC through a process called partitioning. If we have a “typical” separation, we are interested in a non-polar analyte like a drug or vitamin D. These molecules will be retained because they are attracted to the stationary phase when the mobile phase is predominantly polar, or aqueous. This is shown in the top diagram, where the non-polar stationary phase, in this case a C8, is shown on the bottom of the box. Since polar molecules are not attracted to the stationary phase and are attracted to the mobile phase, they are not retained and are eluted.

As the solvent conditions are changed to become more non-polar, the analytes are attracted to both the stationary phase and the mobile phase. The analytes then partition between the mobile phase and the stationary phase, spending time in both the stationary phase and the mobile phase. Since different molecules will have different affinities for both the stationary phase and the mobile phase, a separation happens. Slight differences in polarity cause different molecules to group together as they move down the column, resulting in the desired separation.

I found it helpful to understand the process when "thinking like a molecule"– you can imagine the molecule not liking the polar mobile phase initially, and then being more attracted to the mobile phase as it becomes less polar.

As the solvent becomes even more non-polar, the analytes spend more time in the mobile phase and are eluted from the column.

Slide 15: HILIC (Hydrophilic Interaction Liquid Chromatography)

Hydrophilic Interaction Liquid Chromatography (HILIC) is the modern adaptation of Normal Phase Chromatography. Like Normal Phase Chromatography, HILIC uses a polar stationary phase and a non-polar yet water-miscible mobile phase. It uses designed stationary phases instead of bare alumina or silica particles. HILIC fills a need in analytical separations and is used for the separation of very polar molecules like acids, bases, and zwitterions. In clinical chemistry, HILIC is being used to separate compounds such as glycosylates and metabolites.

Slide 16: SEC/GPC/GFC

Size Exclusion Chromatography (SEC), Gel Permeation Chromatography (GPC), and Gel Filtration Chromatography (GFC) are a set of techniques developed to deal with macromolecules. There are multiple names for the technique based on the branch of analytical chemistry using the technique – SEC was the name given to the technique by polymer chemists, while the other names come from the biological chemistry arena. The separation is based on the size of the molecules being analyzed. To be technically correct, the separation is based on the molecule's Stokes radius, a descriptor of the shape of the molecule. The stationary phase contains pores of different sizes. As the analyte passes by, smaller molecules diffuse into the pores, while larger molecules pass by. As a result, the larger molecules in a sample are eluted sooner than smaller molecules. SEC is used for large molecule separations, such as protein and polymer molecular weight determinations, and peptide purification.

Slide 17: Ion Exchange Chromatography

Ion Chromatography (or Ion-Exchange Chromatography), as the name implies, is used to separate ionic species. The separation takes place on a column that has bound charge sites – positive charges are bound to the column for the analysis of anions, and negative charges are bound to the column for the analysis of cations. The analytes are eluted from the column by increasing the salt content of the solution, changing the pH, etc. Like SEC/GPC, the technique is often tailored to a specific set of analytes.

Slide 18: Chiral Chromatography

Chiral Chromatography is a fairly specialized variant of LC, but it is important for particular biochemical analytes. Many active pharmaceutical ingredients are Chiral, which means that the molecule is not superimposable on its mirror image – like our hands. That means that the two or more enantiomers of a molecule may have different biological effects, so we need to be able to measure them. Typically, this is done by using a chiral stationary phase, often based on β-cyclodextrin. This method is not commonly used in clinical chemistry. Recent research on detecting antifungal drugs has demonstrated its applications.

Slide 19: Summary
Liquid chromatography is a complex technology. This presentation provides a very high level overview of the LC basics and separation techniques. Other Pearls in the future will discuss separation mechanisms and method development. I hope you will join me on those presentations as well.

Slide 20: References

Slide 21: Disclosures

Slide 22: Thank You from www.TraineeCouncil.org
Thank you for joining me on this Pearl of Laboratory Medicine on “Liquid Chromatography: LC Basics and Separation Techniques.” I am Yan Victoria Zhang.