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TITLE: Liquid Chromatography – Separation Mechanisms

PRESENTER: Y. Victoria Zhang, PhD, DABCC

Slide 1:

Hello, my name is Yan Victoria Zhang. I am 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 – Separation Mechanisms."

Slide 2: Outline

Liquid chromatography, or LC for short, is a broad and complex field. A previous Pearl was focused on the basics of LC. In this Pearl, I will discuss the separation mechanisms, and factors that impact separation efficiency. I've found that thinking like a molecule helps me understand what happens in an LC separation, so I'm going to try to show you that analogy as we go through the talk.

Slide 3: Mechanism Overview

This slide demonstrates how LC works. This is where we start thinking like a molecule or the analytes. Let's assume that we inject a sample containing a mixture of red, green, and yellow molecules. The LC Column shown in our example has dimensions of 4.6mm by 300mm. Recall from previous Pearls that 4.6mm is the diameter of the column and 300mm is the overall length of the column.

The diagrams show what happens at time 0, 2, and 5 minutes. Time zero is defined as when the sample is injected at the front end or head of the column. From there, our mobile phase flows through the column and the analytes partition between phases. If an analyte, like the red one in this example, really likes to be in the mobile phase, it will spend a lot of time in the mobile phase and thus move faster. On the other hand, if the analyte, like the yellow one in this case, likes to interact with the packing material inside the column, then it spends more time with the packing material and moves more slowly through the column.

If your analyte doesn't interact at all with the column packing material or the stationary phase, it is unretained.

This process of moving between stationary and mobile phase is referred to as partitioning. So as time passes, we generate three distinct bands from our mixture. They are separated in space on the column, and, because the liquid is flowing continuously, they become separated in time and reach the detector separately. I will explain partitioning later in this Pearl.

Slide 4: Reverse Phase Overview

Here is a quick review of column chemistry. The packing material is based on silica gel, a network polymer that has a lot of polar hydroxyl groups on the surface. To make the surface less polar, hydrocarbon chains or other moieties are bonded to the hydroxyls. Different lengths of hydrocarbon chain give different retention properties – For a given non-polar molecule, a C_{18} stationary phase will be more retentive than a C_4. The column on the bottom left is packed with microscopic silica particles that have atomic scale hydrocarbon chains attached. The particles can be as small as 2um.

Slide 5: Mechanism of Reverse Phase

We discussed the features of columns and column selection based on property of compounds in a previous Pearl. I will use reverse phase as an example to further explain how chromatography works.

The different color dots indicate compounds with different hydrophobicity.

The reverse phase column illustrated in this slide is packed with silica that has octadecyl carbon chains bound to it. This creates a hydrophobic stationary phase typically referred to as a C_{18} phase. C_{18} attracts hydrophobic molecules. When the samples are loaded onto the column under a hydrophilic condition, hydrophilic molecules (red molecules) will not stick to the column, and hydrophobic compounds will be retained on the column. The gradient elution step increases the concentration of hydrophobic mobile phase B which typically has higher concentrations of acetonitrile or methanol.

As the hydrophobicity increases in the mobile phase, the hydrophobic molecules that like this environment will "partition" into the mobile phase and eventually will be eluted off the column (green and purple molecules). The more hydrophobic the molecule is, the higher the concentration of organic solution required for its elution off the column, such as the purple molecule here. The column will be "washed" at a very high concentration of organic compounds to wash off strongly hydrophobic molecules (such as orange molecules). The column must be re-equilibrated at initial conditions before starting another injection.

Slide 6: Partitioning

Partitioning is the most important concept in chromatography separation. I discussed this in a previous Pearl and it is worth some time to recall the concept. I use reverse phase as an example to illustrate the concept. This is where thinking like a molecule will help again. Like us, molecules want to spend more time with the environment that they like. That is, polar molecules

like to spend more portion in polar environment and non-polar molecules like to be surrounded by non-polar environment.

The three diagrams provide the snapshots of three separation conditions, which are retention, partitioning, and elution. 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 on the stationary phase because they are attracted to the non-polar stationary phase when the mobile phase is predominantly polar. This is shown in the top diagram, where the non-polar stationary phase, in this case a C_8, 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 off right away.

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 which results in the desired separation.

As the solvent becomes even more non-polar, the analytes move to the mobile phase and are eluted from the column.

Slide 7: Isocratic vs. Gradient Separation

There are two commonly used elution approaches in an LC system - Isocratic and Gradient Separations. The mobile phases are typically referred as “mobile phase A” and “mobile phase B” with mobile phase B having the higher elution strength. In a reverse phase separation, the “A” solvent is typically low concentration of organic solvent such as 0 - 5% acetonitrile or methanol and the balance water, and the “B” solvent is typically a high concentration organic solvent, such as 95% acetonitrile or methanol with the balance water. Other reagents, such as formic acid or an ammonium acetate / acetic acid buffer, may be added to obtain the correct pH.

As indicated here on the slide, during an isocratic elution (left graph), the mobile phase composition doesn’t change over the course of the separation. It stays constant at a level such as 40% mobile phase A + 60% mobile phase B. The ratio of aqueous to organic solvent stays constant. However, during a gradient elution, the mobile phase composition, that is, the ratio of Aqueous to Organic, is changed from, for example, 10% mobile phase B to 80% mobile phase B, and is done so in a well-controlled manner over a defined time period.

Each approach has its pros and cons. Isocratic is simple, and does not require additional time for re-equilibration. One concern with isocratic elution is that later eluting compounds come out as broader peaks, and some analytes may not ever come off the column. It is important to wash the column after a certain number of injections to remove compounds that are retained on the column to keep the column clean.

Gradient elution provides higher resolution and both separation and elution of a wider range of compounds; however, it needs “smarter” pumps and programming. In addition, the column must

be re-equilibrated between injections – essentially, the column needs to re-stabilize to the initial conditions of the analysis before starting the next analysis.

Slide 8: Isocratic vs. Gradient Separation

This slide shows the impact of three possible elution patterns on the separation efficiency based on a four-compound mixture on a reverse phase column. At the top, the separation uses an isocratic approach where mobile phase consists of 30% acetonitrile in water. As you can see, the fourth component is not off the column in the desired time. To rectify this, we increase the concentration of acetonitrile to 60% in the water. That elutes the fourth component off the column, but the first two components are no longer separated. One could try some modification: 45 percent acetonitrile might effect a decent separation, but a better solution would be to use a gradient. With the correct gradient from 20% to 70% acetonitrile, all four components are eluted and resolved.

As indicated here, the retention times for compounds are different in isocratic and gradient elution. Overall, compounds are eluted at their designated % organic B and higher concentrations. The required % B concentration for elution is determined by its physical properties; in the case of reverse phase, it is the compound's hydrophobicity. In the example, compounds 1 and 2 require about 30% and 35% of acetonitrile to be eluted off the column. With 60% acetonitrile isocratic elution, compounds 1 and 2 elute almost right away from the column at about 0.9 minute. This is what we previously mentioned as no retention on the column. With gradient elution, compounds 1 and 2 are not eluted until the percentage of acetonitrile reaches 30% and 35%, respectively. It is indicated as 1.5 minute and 2.3 minute in this example.

On the other hand, compounds that are more hydrophobic, such as compound 4, requires 70% acetonitrile to be eluted off the column. Compound 4 was not eluted at 30% acetonitrile within 5 minute time frame, started to be eluted at 3.5 minutes at 60% isocratic elution, but not complete within the LC run, and eluted at 4 minute with the gradient elution when the % B hit 70% acetonitrile.

Slide 9: Chromatographic Parameters

Several factors can affect the LC separations. I will now spend some time talking about those factors and the basic definitions that need to be understood to grasp a more mathematical description of LC.

Slide 10: Retention Time

First is retention time, t_R. Retention time is easy to understand. It is defined as the amount of time from injection, or when the injection valve closes, to the apex of the peak signal measured by the detector. As indicated here, these two peaks have retention times of 0.75 min and 1.25 min.

Slide 11: Resolution

Resolution (R_s) indicates if two peaks are separated from each other.

As shown on the left side of the slide, if two components are detected as distinct, separate peaks, they are resolved. Otherwise, they aren't resolved, as indicated in the diagram at the lower left corner.

Mathematically, resolution can be quantified as the difference in retention times between the two peaks, divided by the average of the combined widths of these peaks. W_b is used as the symbol for the width of a peak at the baseline. Using that definition, $R_s \geq 1.5$ means that the two peaks are baseline resolved. Higher resolution means peaks are well separated from each other. The resolution of the two peaks in the example is calculated as shown.

Slide 12: Three Major Chromatogram Factors Impacting Resolution

The goal of HPLC is to obtain optimum resolution in minimum time. Controlling and improving resolution are key considerations for clinical chemists during method development. There are three basic components that influence resolution: Efficiency, Selectivity, and Retention, which are reflected in the equation shown here, where N is the efficiency, kappa is the retention factor, and alpha is the selectivity.

Graphically, you can think of the parameters as indicated in the figure below. I will discuss each of these three factors in the next few slides.

Slide 13: Efficiency

Chromatographic efficiency is a measure of the dispersion or broadening of the analyte band as it travels through the column and entire HPLC system. Efficiency is a measure of how well the column keeps a band together and it is directly related to the ratio of the retention time and the peak width. A column has a better efficiency if the peaks that are eluted at a certain retention time t_R have narrower peak width W_b, where W_b is the peak width at the baseline.

Slide 14: Determination of Retention Factor

Retention factor is also known as capacity and retained the symbol kappa (κ). Retention is a measure of how well or how long an analyte is on the column – how much the analyte interacts with the packing material. If t_0 indicates the dwell time, retention factor is the ratio of the difference between the peak retention time and dwell time divided by dwell time t_0. Analytes that are eluted more slowly have higher retention. This is shown in the diagram: the upper figure shows an analyte being eluted early, with a lower kappa, due to a much steeper gradient than that used in the lower figure. The lower figure shows the same analyte with a higher retention factor due to a shallower gradient.

Slide 15: Selectivity (Separation) Factor

The next term is "selectivity," or the separation factor, alpha. It is defined as the ratio of two retention factors. Two components can be separated only if they have different retention factors. The selectivity factor reflects the ability of the chromatographic system to chemically distinguish between any two sample components. In other words, the difference between Retention and Selectivity is that Retention describes how any one component moves through the column while Selectivity describes the separation of two components on the column.

Slide 16: Parameter Summary

This slide summarizes the common parameters that you can change in a separation and the effect those changes have on the separation. For example, changing the column length will have a large impact on efficiency, but will make little or no change on capacity and selectivity. Selectivity has a number of parameters that are often changed, while capacity has few. Selecting and balancing those factors are very important for method development, which will be discussed in future Pearls.

Slide 17: Summary

To summarize, there are several factors influencing separation power or resolution of a column and they include efficiency, retention, and selectivity. Understanding those factors will help appreciate the complexity of the liquid chromatography technology and help troubleshoot the system when needed. More importantly, these factors are critical for method development in your practice.

Slide 18: References

Slide 19: Disclosures

Slide 20: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on “Liquid Chromatography – Part Two: Separation Mechanisms.”