PEAK RESOLUTION IN HPLC

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Abstract:
HPLC is a dynamic adsorption process. Analyte molecules, while moving through the porous packing beads, tend to interact with the surface adsorption sites. Depending on the HPLC mode, the different types of the adsorption forces may be included in the retention process: Hydrophobic (non-specific) interactions are the main ones in reversed-phase (RP) separations. Dipole-dipole (polar) interactions are dominant in normal phase (NP) All these interactions are competitive. Analyte molecules are competing with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface. The weaker the eluent interaction, the longer the analyte will be retained on the surface. The resolution of peak in HPLC not only depends on chemical parameter of drug & mobile phase but also on instrumental parameter. Some extra or unexpected peaks are seen during the resolution which can be overcome by changing the condition or changing parameter.

KEY WORDS: HPLC, Retention time, buffer, pH etc

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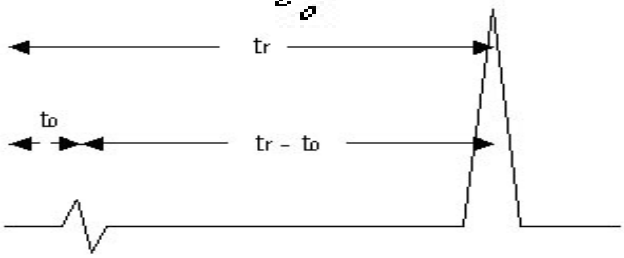
Introduction:

High Performance Liquid Chromatography (HPLC) is one mode of chromatography, one of the most used analytical techniques. Chromatographic process can be defined as separation technique involving mass-transfer between stationary and mobile phase. HPLC utilises a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. These components are first dissolved in a sol-vent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mix-ture separates into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both sol- vents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromato-graphic systems and it has the ability to easily separate a wide variety of chemical mixtures. "Time is money" is one of the slogans of modern economy. This is also true for Chromatography, the more separations per time unit that are achieved the better. In order to allow faster separations, columns are packed with smaller particles. In smaller particles the diffusion path into and out of the particle is shorter and peak broadening is reduced. This allows faster mobile phase flow rates which reduces analysis time. Unfortunately back pressure is massive, so that modern fast columns with 1.7 ìm particles make special UPLC systems nec essary which are expensive as regards purchase price and operation.

There are three basic parameters which we can mea-sure from a chromatogram, which affect peak resolution.

1) **Relative Retention** Time, k' Relative retention time is a measure of retention time which is independent of flow rate or column length or diameter. It is also known as the Capacity Factor.

$$k^1 = \frac{t_r - t_o}{t_o}$$



Here, tr is the retention time of the peak, and t0 is the retention time of the solvent front (unretained peak). In isocratic chromatography (elution with a constant eluent strength), elution occurs in a given volume of eluent. So if we double the flow rate, the elution volume passes through the column in half the time so we halve the retention time. But k' remains constant. In isocratic HPLC, we vary ' k' by using a stronger or weaker mobile phase. So for example in reversed phase HPLC, using a higher % methanol in the eluent reduces the retention time of all the peaks, but the retention time of the solvent front remains unchanged.

2) Selectivity, á

Selectivity is a measure of the extent to which two components are separated.

$$Selectivity\ \ \alpha = \frac{k_2^1}{k_1^1}$$

Because k' is a thermodynamic parameter, and hence constant for a given phase system, so is selectivity.

Selectivity is a function of the interaction between the stationary and mobile phases in equilibrium with the sample components. So to change selectivity, we need to change the stationary or mobile phases. By far the greatest changes can be achieved by changing the mo-bile phase. There are several parameters of the mobile phase which we can change to vary selectivity. It should be noted that selectivity can get worse as well as better!

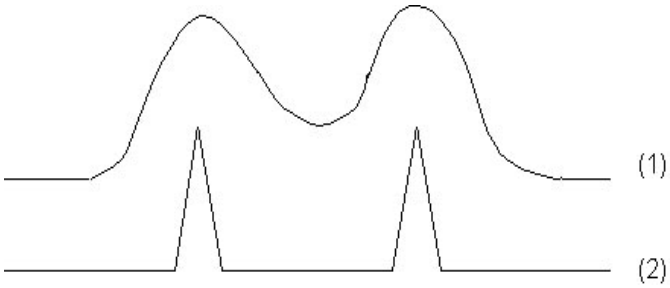
- **Temperature.** Increasing the temperature in-creases the energy of all the molecules, improving mass transfer in and out of the pores. As a consequence, and as a general rule, peaks are sharper and elute faster at higher temperatures. However this tends to make the peaks closer together, reducing resolution.
- **pH.** Components which are ionisable, such as acidic or basic compounds, give rise to elution times which are very pH dependent. The more charged the species is, the shorter the retention time. So for acidic compounds, retention times are longest at low pH; for basic compounds, retention times are longest at high pH (above 7); and for

zwitterionic species (containing both acidic and basic groups such as an amino acid) longest retention time come in the pH region 5-7. Having established the most suitable pH to use, accurate and precise control with a suitable buffer is essential.

- **Solvent type.** Normally we would use three solvents in reversed phase HPLC to achieve the great- est selectivity differences. Acetonitrile has a dipole influence, methanol a slight proton donor effect and tetrahydrofuran a slight proton acceptor effect. Each of these three solvents may be mixed with water to form a reversed phase eluent, and so changing from MeOH:H2O to MeCN: H2O would be expected to give rise to a change in selectivity.
- **Eluent composition.** Changing the eluent composi tion changes k', but the rate of change is different for different components. Hence the rate of migration of peaks in the chromatogram as eluent composition is varied is different, and this can cause peaks to over- lap, separate, and even change places.
- **Buffer type**. See the section on buffers for a guide as to which buffer to choose. But changing from one bufferto another can cause selectivity changes.
- **Buffer concentration**. Increasing buffer concentration causes peaks to elute more quickly. But as with %B changes, the effect on some components is greater than others. For a given separation there will be an ideal buffer concentration, and once found, it is important that this is controlled carefully.
- **Ion Pair Reagent**. An ion pair reagent can be used to pair up with polarised sample components, enablingthem to elute with less polar species in the chromatogram. It is not a recommended eluent system, because ion pair methods are often not robust. But it does affectselectivity. Changes to the stationary phase offer less possibilities, but may prove effective. This can be for example, a change from C18 to C8, or from one type of C18 to another. After each column change, the system must be re-equilibrated thoroughly before evaluating the success or otherwise of the change.

3) Efficiency, N

The above properties define where in the chromatogram the peak maximum occurs and the extent of the separation between them, but they cannot indicate theextent of resolution of the peaks.



Both the above chromatograms have the same thermodynamic properties but chromatogram (2) is clearly better resolved than chromatogram (1) because the peaks are sharper. Efficiency is calculated from the equations below.

$$N = 16\left(\frac{t_r}{w_b}\right)^2$$

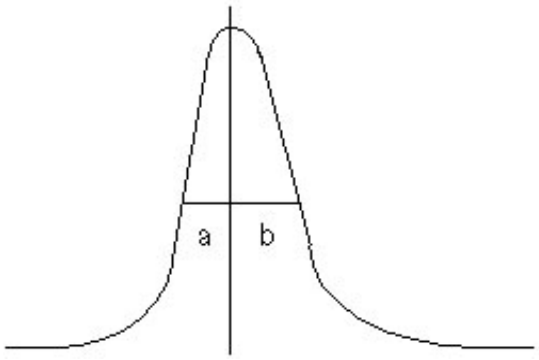
N = efficiency.
tr = Retention time of the peak wb = the peak width at its base

Since tr an w are both measured in either time or volume units, N is essentially dimensionless, and is ex- pressed as a number. It is normal to use to concept of theoretical plates (as in a distillation column), when de- scribing efficiency. Rather than the number of theoretical plates per column, efficiency is sometimes measured in plates per meter (N/m). This is then independent ofcolumn length.

Efficiency is affected by several parameters:

·Particle Size. The smaller the particle size of the column packing, the greater the surface area. Since the separation process of adsorption and desorption occurs on the surface of the stationary phase, increasing the surface area increases the efficiency of this process, giving rise to sharper peaks. Note that 90% of the surface area of a packing material is inside the pores, so it is essential that the molecular size of the sample components is not too great to enter the pores. For an 80A pore diameter silica, a maximum molecular weight of about 2000 is recommended, while for a 120A silica, the molecular weight limit is nearer 5000.

Eluent temperature. The higher the eluent temperature, the more efficient the mass transfer process, and hence the sharper the peaks.

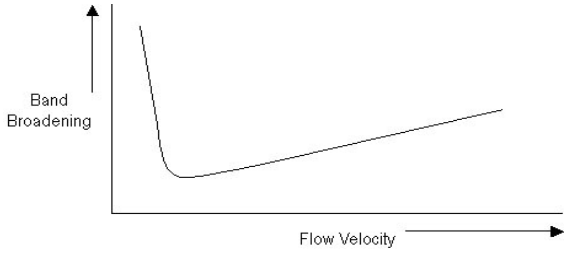


Column Length. A longer column gives shaper peaks, until the time in the column is so long that diffusion becomes an issue.

Eluent viscosity. The lower the viscosity of the eluent, the more efficient the transfer process in and out of the pores. This may be achieved either by selecting a non-viscous eluent component such as acetonitrile, or by raising the temperature, or both.

Linear velocity of the eluent. At very low flow rates the diffusion rate of the solute approaches the flow velocity and very poor efficiency is ob- served. At higher flow rates the contributions from the other components of band broadening become significant and they increase with flow velocity.

Hence it is anticipated that at high and low flow rates efficiency will be poor and in between an optimum flow corresponding to a maximum efficiency. This was first plotted by van Deemter



For a given column diameter, eluent linear velocity approximates to flow rate. For a 4.6mm id column, the optimum flow rate for efficiency is about 0.5ml/min, but as can be seen from the above diagram, at 1ml/min the efficiency is not a lot worse, but the analysis time is halved, so as a general rule, 1ml/min is the standard flow rate used in HPLC. Higher flow rates are also possible, with the limiting factor then being the system back pressure.

Some other parameters are also important which are

a) Peak Asymmetry, As

Not all peaks are symmetrical and asymmetry is defined as follows:

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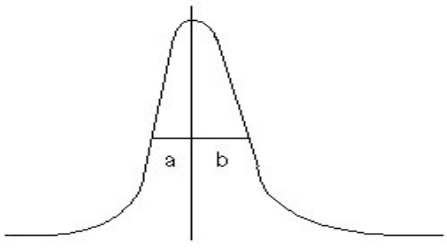


Fig: peak asymmetry

As = b / a

a and b are measured at 50% of peak height

The width of a statistical distribution is defined by its standard deviation and this can be applied to a chromatographic peak. Hence the standard deviation has the same dimensions as retention measurement (usually time, occasionally volume). The longer a peak is retained the more it is subject to band broadening and it becomes wider. Thus the standard deviation of the peak is de- pendent upon retention time.

Peaks should of course be symmetrical, and so

the Asymmetry should be as close to 1 as possible. Deviations from this suggest a problem with the method (per-haps the wrong pH has been specified) or a problemwith the column.

b) Resolution, Rs

Resolution between peaks is measured as the ratio of the difference in retention times to the average of their baseline peak widths. So it can be measured directly from the chromatogram using the equation below:

$$R_s = \frac{t_{R_2} - t_{R_1}}{(w_1 + w_2)/2}$$

tr2 =Retention time of peak 2
tr1 =Retention time of peak 1
w1=Peak width of peak 1
w2=Peak width of peak 2

However it is more usually expressed as shown below, in terms of N, k' and á:

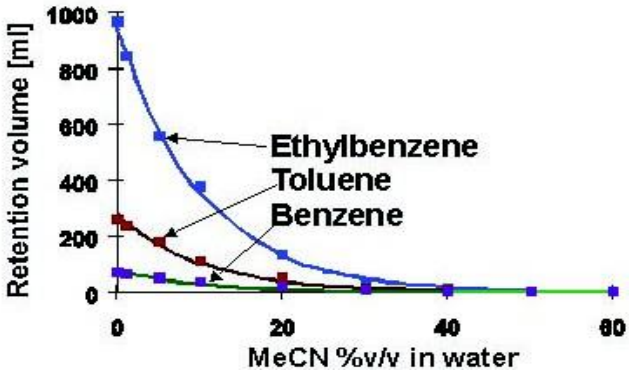
$$Rs = \frac{1}{4} N^{\frac{1}{2}} \left(\frac{k^1}{(1+k^1)} \right) (\alpha - 1)$$

Resolution is expressed as a number, and for baseline resolution of two peaks, a resolution of 1.5 or greater is required.

For a chromatogram containing more than two peaks, the concept of Critical Resolution is useful. This is a measure of the resolution of the least-well-resolved pair of peaks in the chromatogram. By definition therefore, all other peaks in the chromatogram are better resolved.

c) Mobile phase effect

Optimum pH control will usually result in mobile phase containing buffer and acid compositions that will resist change when the sample is introduced and force ionizable analytes into predominantly one form (ionized or neutral) as they enter the column. Good laboratory practice in preparing mobile phases should be followed to ensure that results can be reproduced within and between laboratories In most of the HPLC separations bi-nary eluents are employed. One of the solvents in the eluent is usually inert relative to the surface interactions. In Reversed-phase HPLC (RP HPLC) one of the eluent components is water, which does not interact with the hydrophobic adsorbent surface. And it does not compete with the analyte for the adsorption sutes. In nor- mal-phase HPLC (NP HPLC) one of the eluent components is usually hexane, which also does not interact with the very polar silica surface. Another component of any binary eluent is an active one. It usually called a "modifier" because it can interact with the adsorbent surface. and compete with analyte molecules for the adsorption sites. Increasing of the concentration of the "modifier" in the eluent leads to the decreasing of the analytes retention. For the binary eluent system only the "modifier" can interact with the surface. For RP HPLC it will be an organic component of the eluent, and water is assumed not to interact with the surface. Figure below is shows the experimentally measured dependencies of the retention of alkylbenzenes in reversed phase HPLC mode on C18 column with acetonitrile/water eluent at different compositions. Points are experimental values and a curves was calculated using the above equation. As we can see this simple approximation al- lows us to describe experimental dependencies pretty well.



Retention dependencies of alkylbenzenes vs. the eluent composition. Eluent: acetonitrile/water, column: Prodigy-C18 (150x4.6 mm)

This type of the influence of the mobile phase composition and its thermodynamic explanation are true for the chromatographic system with only hydrophobic interactions (dispersive forces). In case of the presence of any specific adsorption sites, the analyte behavior may significantly differ from that described above. Any specific interactions of the analyte molecules with the eluent mol- ecules also may introduce significant deviations to the analyte retention dependence. Ionizible components usually show a specific behaviour Organic acids are easily solvated with the water molecules, which block possible interaction of the hydrophobic part of the molecule

with the adsorbent surface, and lead to the very early elution of these compounds. For example, benzoic acid is eluted before the dead volume at any composition of acetonitrile/water eluent on the reversed-phase column. Organic basis usually shows a low retention also due to solvation, but in case of presence of strong acidic accessible adsorption sites on the adsorbent they show a strong retention.

d) Effect of pH

A general approach to the separation of the mixtures containing an ionisible components is to suppress their ionization. Suppression of the ionization decreases a power of the molecular solvation and exposes the hydrophobic (organic) part of the molecule to the surface interaction. Ionization suppression is usually made by the adding a buffer into the solvent, which shift a pH to the certain value. In the absence of buffer, easy ionizible components are eluted from the column as very broad peaks.

Table 1. Properties of Common Buffers

Common Buffers		pK_a	Useful pH Range
Phosphate	pK_1	2.1	1.1-3.1
	pK_2	7.2	6.2-8.2
	pK_3	12.3	11.3-13.3
Citrate	pK_1	3.1	2.1-4.1
	pK_2	4.7	3.7-5.7
	pK_3	5.4	4.4-6.4
Formate		3.8	2.8-4.8
Acetate		4.8	3.8-5.8
Tris		8.3	7.3-9.3
Ammonia		9.2	8.2-10.2
Borate		9.2	8.2-10.2
Diethylamine		10.5	9.5-11.5

So selection of right buffer is very important. While it is not always strictly necessary to operate under buffered conditions, one should recognize that poor peak shape and variable retention can result when the sample pH differs significantly from the pH of the non buffered mobile phase and when ionizable compounds are present in the sample.

f) PKa

One of the most important things to know about a buffer is its pKa. The pKa (or Dissociation Constant, or Ionisation Constant) is a measure of the strength of an acid or base, and allows us to determine the degree of ionisation at a given pH. Strong acids and strong bases are those which are fully ionised within the pH range 0-14, and weak acids and weak bases are those which are incompletely ionised within the pH range 0-14.

For acids, the Equilibrium Coefficient for the neutral and charged forms is:

Ka = { H+ } { A- } / { H }

The pKa is given by:

pKa = -log10 (Ka)

At a given temperature these are Thermodynamic Ionisation constants, which are independent of concentration. Since log 1 = 0, The pKa corresponds to the pH at which the concentration of ionised and neutral forms are equal. Note that pKa's are temperature dependent. It is normal to quote the pKa at 25oC.

Which buffer to use at which pH?

A pH Buffer should be used within +/- 1pH unit of its pKa. When used in this manner, the buffer will change its level of ionisation to counter any attempt to change the pH. Many HPLC methods are designed with inappropriate buffers, often a long way from the pKa. In this situation, the only help given by the buffer in resisting pH change arises because its concentration is very much higher than that of the sample. The table on the following page gives a list of buffers showing their pKa and the pH range within which they should be used.

Procedure for buffer selection

Set up the HPLC method and run trials at different pH's to establish the effect of pH change. Establish the optimum pH for the separation. Using the table, obtain a list of suitable buffers with their pKa within 1 pH unit of the desired pH. If using UV or fluorescence detection, check the UV cut-off of the buffer to ensure that it does not conflict with the detection wavelength being used.

Buffer	pKa	pH Range	UV Cutoff (A > 0.5)
1-methylpiperidine HCl/1-methylpiperidine	10.1	9.1 - 11.1	215 nm (10 mM)
Ammonium acetate	4.8	9.2 3.8 - 5.8 8.2 - 10.2	205 (10mM)
Ammonium formate	3.8 9.2	2.8 - 4.8 8.2 - 10.2	
Ammonium hydroxide./ammonia	9.2	8.2 - 10.2	200 nm (10mM)
Bis-tris propane HCl/Bis-tris propane	6.8	5.8 - 7.8	215 nm (10mM)
Bis-tris propane HCl/Bis-tris propane 9.0	8.0 - 10.0	225 nm (10mM)	
Borate (H3BO3/Na2B4O7 10 H2O)	9.2	8.2 - 10.2	
Diethylamine HCl/diethylamine	10.5	9.5 - 11.5	
Glycine HCl/glycine	9.8	8.8 - 10.8	
KH2PO4/ K2PO4/	7.2	6.2 - 8.2	<200 nm (0.1%)
KH2PO4/phosphoric acid	2.1	1.1 - 3.1	<200 nm (0.1%)
Potassium acetate/acetic acid	4.8	3.8 - 5.8	210 nm (10mM)
Potassium formate/formic acid	3.8	2.8 - 4.8	210 nm (10 mM)
Pyrollidine HCl/pyrollidine	11.3	10.3 - 12.3	
Triethylamine HCl/triethylamine	11.0	10.0 - 12.0	<200 nm (10 mM)
Trifluoroacetic acid	< 2	1.5 - 2.5	210 nm (0.1%)
Tri-K-Citrate/hydrochloric acid 1	3.1	2.1 - 4.1	230 nm (10mM)
Tri-K-Citrate/hydrochloric acid 2	4.7	3.7 - 5.7	230 nm (10mM)
Tri-K-Citrate/hydrochloric acid 3	5.4	4.4 - 6.4	230 nm (10mM)
Tris HCl/Tris	8.3	7.3 - 9.3	205 nm (10 mM)

Buffer Concentration

The buffer concentration is important for three reasons. Firstly, selectivity is affected by buffer concentration. As the concentration increases, the faster that polar species are eluted. However the rate at which their elution time decreases may be different for different sample components. Hence by changing the buffer concentration, peaks may be caused to co-elute. Conversely, it also follows that co-eluting peaks may be resolved at a different buffer concentration. If the buffer concentration is too low, it will not be able to act as a buffer. Hence the pH will no longer be held at the required level, and as a consequence, results may be different from one day to the next. Generally speaking a buffer should be present at at least 0.005M. If the buffer concentration is too high, the eluent solution becomes viscous (and hence the back-pressure becomes unacceptable), a silica based column packing will tend to dissolve, even below pH7, and solubility of the buffer becomes an issue when mixed with an organic solvent, making eluents hard to make up and gradients a risky

Conclusion:

We have looked at two of many possibilities for sources of unexpectedly peaks in chro- matograms. The resolution of peak in HPLC not only depends on chemical parameter of drug & mobile phase but also on instrumental parameter. The polarity, Pka, PH, ioniza- tion, particle size, viscosity, etc are very important for peak resolution. Also column length, tubing system, intensity of lamp detector, electrical fitting is alsotaken in to consideration.

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