

An Executive
Summary

Reversed-Phase Liquid Chromatography Using Surrogate (Additional) Stationary Phases



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Introduction

Liquid chromatography (LC) has several uses in the pharmaceutical world: to identify an analyte or active pharmaceutical ingredient (API), to quantitate the amount of an analyte or API (assay), and as a preparative method for separating the analyte or API from their related substances. Techniques that can increase the resolution between related substances and the API are vitally needed when establishing the identification, assay, or preparative output. This manuscript discusses the use of quaternary ammonium salts that are bound to C8 or C18 reversed-phase media as surrogate or additional stationary phases, and their effect on the resolution parameters such as retention factor (k'), efficiency factor (i.e., the number of theoretical plates [N]), tailing factor (i.e., peak asymmetry), and the selectivity factor (α).

Chromatographic Method Objectives and Resolution Basics

In general, chromatography objectives can be separated into four categories: identification (ID), purity, assay, and preparative isolation. Almost all API specifications have an ID test that correlates the analyte's retention time with that of its reference standard. A purity or high-performance liquid chromatography (HPLC) method that separates the API from its impurities and quantitates each impurity by area percentage is also often required. Over the years, in fact, HPLC methods for the quantitative analysis of an API have largely replaced less-specific methods like titration or colorimetric assays. HPLC is also often used for preparative purposes by separating a compound from its impurities on the gram to kilogram scale.

Often, the first separation technique chromatographers are exposed to is thin-layer chromatography (TLC). In TLC, the mobile-phase composition is adjusted to get the sample of interest to move in the "retardation factor" (R_f) range of 0.3–0.7 for optimal component separation. Likewise, the mobile-phase composition in HPLC is adjusted to a k' of three to 10 for an optimal separation. Similar to a non-absorbed compound that moves to the solvent front in TLC, a non-absorbed compound in HPLC elutes in the void volume. In that way, column chromatography can be thought of as TLC (or planar chromatography) in three dimensions.

The "resolution equation" expresses the separation between any two components in a mixture, as illustrated in **Figure 1**. Resolution is affected by three things: retention, efficiency, and selectivity. The **retention factor** (k or k') is a means of measuring the retention of an analyte on the chromatographic column. It can be defined in terms of an analyte's elution time relative to that of an unretained compound (see **Figure 2**). A high k or k' indicates that the compound is highly retained and has spent a significant amount of time interacting with the stationary phase.

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Intuitively, one would think that higher k or k' values translates to longer periods of interaction and, therefore, leads to better separations. Especially in an analytical laboratory, however, one must carefully evaluate the tradeoff between analyte retention and the time and cost of components used in the separation. The growing prominence of ultra-performance liquid chromatography (UPLC), which uses very short columns packed with very small particles, and leads to very short retention times for analytes, attests to the way this trade-off is usually made.

Efficiency, or theoretical plates, is a measure of a column's performance defined in terms of the narrowness of an analytical peak, as shown in **Figure 3**. The narrower the peak's width, the higher the column's plate number. The theoretical plate number (N) is inversely proportional to the diameter of the stationary phase's particle size and directly proportional to the length; therefore, smaller particle sizes and longer columns result in better separations, within practical limits as discussed directly above.

The **selectivity factor** (α) is the ability of the chromatographic system to distinguish between two sample components. It is measured as the ratio of the two peaks' retention factors. When two peaks co-elute, alpha is one. The separation's selectivity is dependent upon interactions among the analytes, the mobile phase, and the stationary phase. Therefore, all of these factors can be altered to optimize HPLC separation selectivity.

Some factors that are usually modified include the nature of the organic solvent in the mobile phase, the solvent strength or ratio of that organic solvent to water, the presence of additives, the mobile phase pH (if any of the compounds of interest are ionic), and the stationary phase. Even temperature changes can affect the separation in reversed-phase HPLC. The addition of ion-pairing reagents such as an alkyl sulfonic acid can also have a marked effect on selectivity when chromatographing ionic substances. Using chiral stationary phases provides an opportunity to determine the enantiomeric purity of chiral molecules on both an analytical scale and to

Figure 1: The impact of selectivity on resolution.

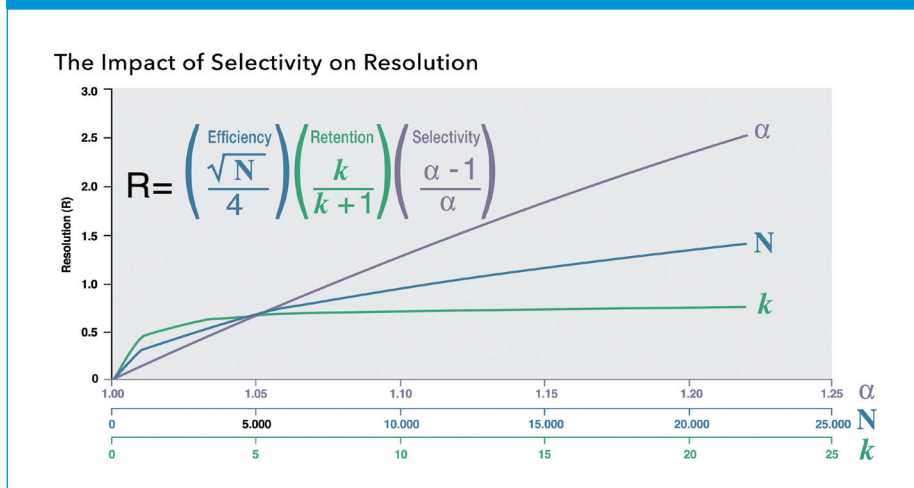
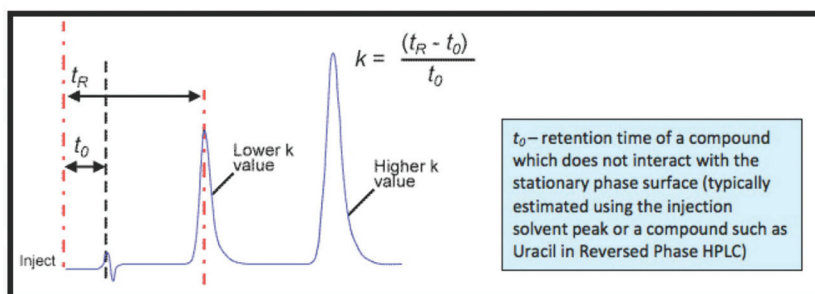


Figure 2: Capacity factor (k or k') or retention factor (residence time on column).



(Taken from www.chromacademy.com)

$$k' = \frac{V_R - V_0}{V_0}$$

V_R = Elution volume of Analyte

V_0 = Elution volume of a un-retained solute

perform chiral separations on a preparative scale.

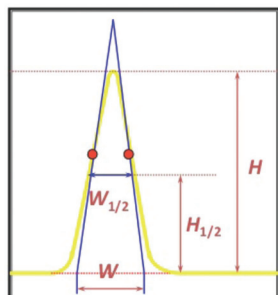
Another important parameter in any separation is the tailing factor, which is a measure of chromatographic peak symmetry. Ideally, peaks would be Gaussian and the tailing factor would be one. Realistically, a tailing factor of one is very rare and tailing or fronting is often observed. Tailing factors that are significantly less than or more than one can lead to doubt about what small peaks might be hiding under a large peak. With preparative separations, tailing can make it difficult or impossible to effectively remove impurities that have slightly larger retention factors than the desired component.

Surrogate or Additional Stationary Phases

The resolution equation is the heart and soul of chromatography. As we have discussed, one can increase resolution in

Figure 3: Efficiency term.

Theoretical Plate Number, N



$$N = 16 \left(\frac{t_R}{W} \right)^2$$

$$= 5.54 \left(\frac{t_R}{W_{1/2}} \right)^2$$

$$= 2\pi \left(\frac{t_R \cdot H}{\text{Area}} \right)^2$$

A mathematical concept that is calculated using the equations below.

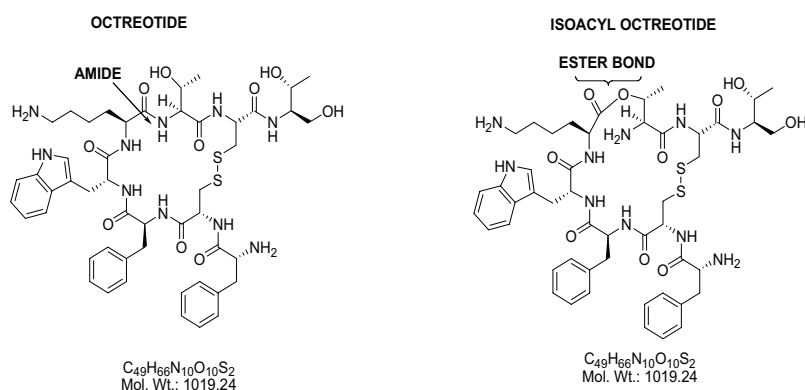
$$N \propto \frac{1}{dp} \quad N \propto \frac{L}{dp}$$

Where,

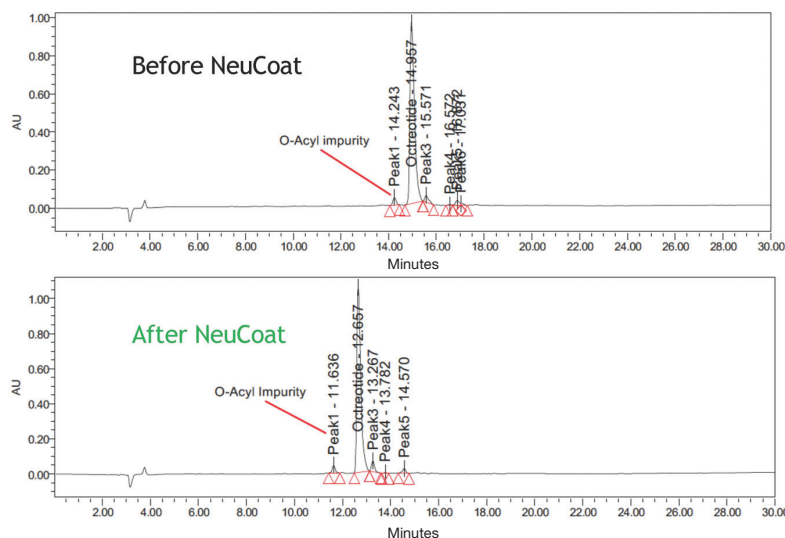
dp = diameter of particles

L = Length of column

W = Peak width

Figure 4: Structure of Octreotide and Isoacyl (O-acyl) Octreotide impurity.


H-D-Phe-Cyclo[Cys-Phe-D-Trp-Lys-Thr-Cys]-Threoninol

Figure 5: O-acyl impurity, and Octreotide: RRT:0.92 (after NeuCoat), 0.95 (Before NeuCoat), and API (RRT:1.00).


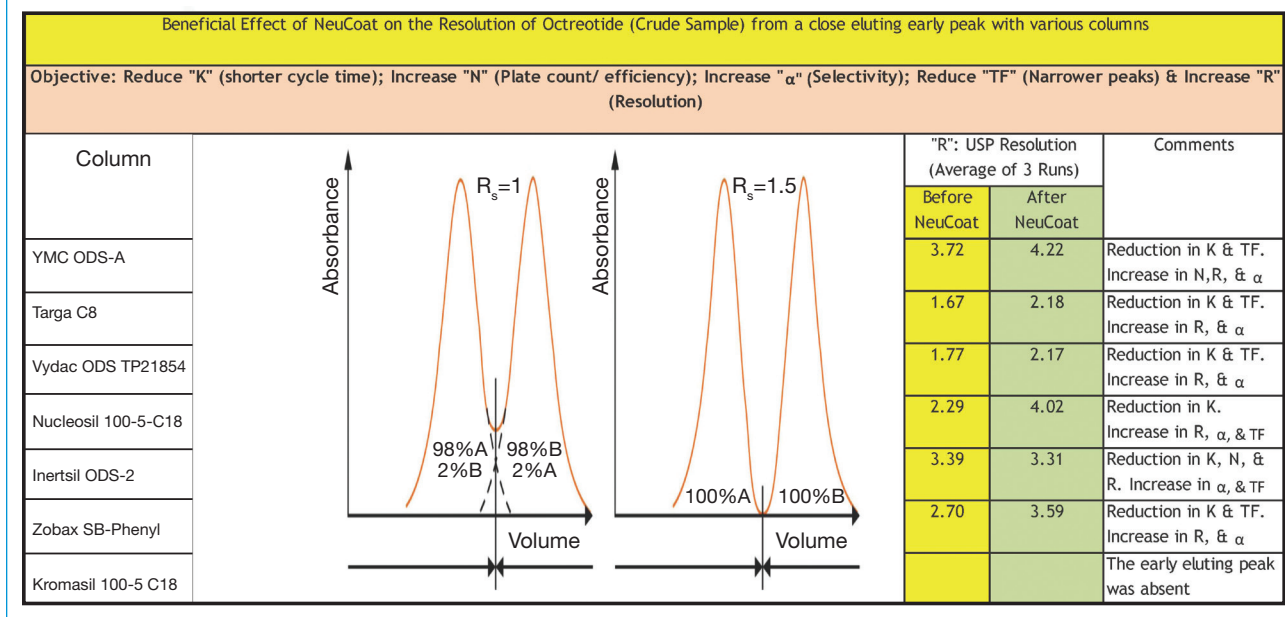
three ways: by increasing retention, efficiency, or selectivity. Increasing retention requires patience, due to longer separation times; increasing efficiency requires smaller-particle columns; and increasing selectivity requires an understanding of solute interactions with both the stationary and mobile phases.

Another way to affect resolution, however, is through the use of surrogate or additional stationary phases. An additional stationary phase is a hydrophobic compound like quaternary ammonium ions that binds with the C18 of a reversed-phase column, making it function as if it were a C8 column (a technique patented by Neuland Laboratories).

An example of the use of an additional stationary phase on the analytical scale is for the separation of octreotide and its impurity isoacyl-octreotide (O-acyl), shown in **Figure 4**. Octreotide is used to treat severe watery diarrhea and sudden reddening of the face and neck caused by certain pancreatic and intestinal tumors. The HPLC profiles of octreotide and its impurity before and after coating a typical Nucleosil C18 column with the additional stationary-phase NeuCoat is presented in **Figure 5**.

In the top chromatogram in Figure 5, the O-acyl impurity elutes close to octreotide. After coating, better separation is observed (see the bottom chromatogram of Figure 5). By examining several other C18 columns from multiple vendors, it was seen that, in general, efficiency was mainly unaffected. A trend toward slightly lower k' values and decreased tailing values was observed when an additional stationary phase was used. The latter is advantageous for increasing resolution.

The additional stationary phase had the biggest effect on α in that there was an increase that varied from 0.1 units to 0.5 units, which is substantial on a scale of 1–1.25. In general, a resolution value of ≥ 1.5 between two peaks of interest is needed for “baseline resolution”; **Figure 6** illustrates the cumulative effect on resolution of additional stationary phases for a variety of different C18 columns.

Figure 6: Effect of SSP on "R" (Resolution). Sample: Octreotide. Column dimensions: 0.46 cm (ID) x 25 cm (L).


Additional Stationary Phases and Preparative Chromatography

Preparative HPLC is used for the cGMP production of peptide APIs and other complex APIs that cannot be purified by crystallization. The purification output of a column depends on the amount of stationary phase, the efficiency dictated by the particle size, and selectivity. For normal-phase silica small-molecule separations, %loading (i.e., weight divided by the volume) is usually about 8% of the column's volume. For reversed-phase purification, it is typically around 1%. However, by using additional stationary phases, due to increased resolution, reports of %loading on reversed-phase columns as high as 10% can be achieved, rivaling those of normal-phase silica columns. Many quaternary ammonium salts are useful as surrogate stationary phases for preparative chromatographic applications such as for the purification of leuprolide, a gonadotropin-releasing hormone (GnRH) agonist used to treat prostate cancer (summarized in **Figure 7**).

Conclusion

Novel surrogate or additional stationary phases using quaternary ammonium salts as surrogates can increase resolution in analytical HPLC and increase the purification output in

Figure 7: Performance of quaternary ammonium and phosphonium as surrogate (additional) stationary phase (SSP/ASP). Experimental parameters: Reveleris C-18, 12 g, CV 18 mL, 40 μ , 60 \AA ; flow rate was 8 mL/min. The analyte was crude leuprolide, 800 mg net weight by Edelhoc method¹; 84.6% pre by modified *United States Pharmacopeia* method.

Entry	Additional stationary phase	HPLC Assay: Weight of >95% pure product in mgs (% yield)	Individual fraction with highest purity (%)
1	n-Tetrabutylammonium bromide (TBA-Br)	534 mgs (66.4%)	97.9%
2	n-Tetrabutylammonium hydrogen sulphate (TBA-HS)	515 mgs (64.4%)	96.4%
3	Cetyltrimethylammonium bromide (CTA-Br)	491 mgs (61.4%)	97.7%
4	n-Tetrabutylphosphonium chloride (TBP-Cl)	482 mgs (60.3%)	97.8%
5	n-Tetrabutylammonium chloride (TBA-Cl)	428 mgs (53.5%)	98.3%

¹There was "NO LEUPROLIDE" in the flow through or in the 100% B Wash in all the cases shown above.

preparative HPLC. Increased loading capacities of seven to 12 times versus conventional reversed-phase HPLC can be obtained, in some cases allowing the purification of 100 mg of a pure peptide on a simple analytical column using common HPLC instruments. This approach (patented by Neuland Laboratories) has many applications for use in any laboratory faced with improving efficiency and throughput using existing technology.