Critical Pairs in Column Chromatography: A Primer for Pharmaceutical Method Validation

A column chromatographer’s goal is to produce separation methods that provide baseline resolution of each analyte peak in a reasonably short analysis time. One way chromatographers have defined the effectiveness of their chromatographic separations is by monitoring the separation of a critical pair of components. A critical pair represents the two components of the chromatogram with the lowest calculated resolution between them. Although the concept of a critical pair of solutes in a chromatographic separation is seemingly straightforward, it is reinterpreted or used in several ways. Often, analysts mistakenly note several critical pairs in their separation, when in fact only one appears. This article reviews several common applications of the critical-pair approach to column chromatography as it is applied under pharmaceutical guidelines and validation strategies.

The Holy Grail for today's column chromatographers is the quest for separation methods that provide baseline resolution of each analyte response from the sample matrix in a reasonably short analysis time. This process entails the development and search for efficient methods that can be applied to samples and matrices of increasing complexity. As any seasoned chromatographer knows, this quest often concludes with lower expectations than those at the beginning of the project.

Since the pioneering works of Snyder and Kirkland (1,2) and Giddings (3), chromatographers have defined the effectiveness of their chromatographic separations by the separation of a critical pair of components. A critical pair represents the two components of a chromatogram with the lowest calculated resolution between them. Accordingly, the resolution of the critical pair is called the critical resolution, and the pair is considered baseline resolved if the resolution ($R_s$) between them is greater than or equal to 1.5. For simple applications, when the critical pair is separated, the remainder of the peaks in the chromatogram also will be separated. Whether this holds true under changing method conditions is specific to the robustness of the analytical method.

Although the concept of a critical pair of solutes in a chromatographic separation seemingly is straightforward, it has been our experience that the concept gets reinterpreted or used in several ways (4–9). Most separations have a single critical pair in their chromatographic profile, yet several critical pairs often are addressed in development. This error generally is the result of an analyst citing several close peaks as being critical. However, because these peak pairs behave correspondingly to changes in chromatographic conditions, the solutes with the minimum resolution are representative of the profile as a whole and should be the only assigned critical pair. Other resolution pairs in the chromatographic profile are important, but it is possible to profile a separation based on a single or the true critical pair — the solutes with the minimum resolution.
resolution necessary to sustain the integrity of the profile, it should be established as such.

Complex matrices can have more than one critical pair, if the solute pair with the critical resolution is nonindicative of the profile as a whole. As discussed below, this situation generally can be confirmed by robustness studies. As changes to the chromatographic conditions are applied, some solute peaks might not respond in the same manner. In this case, it is acceptable to assign additional pairs as critical. When more than one critical pair exists, it often is indicative of complex matrices that involve analytes of varying chemistries.

In the pharmaceutical industry, the critical-pair approach takes on added accountability as methods are validated using industry guidelines (10–16). Chromatographers routinely use a method’s critical pairs to conform to the policies of regulatory agencies. Critical pairs are either directly present or indirectly modeled in the resolution criteria for the analytical method and are an integral part of the establishment of system suitability. In addition, whether a method can serve as a stability-indicating method is established by the effectiveness of addressing the critical pair or pairs used with the active pharmaceutical ingredient and drug formulations.

The article reviews several common applications of the critical-pair approach to column chromatography as it is applied under pharmaceutical guidelines and validation strategies. The application of the critical-pair strategy is shown for a simple suitability standard and progresses to its application in robustness, degradation, and complex sample applications.

Critical Pairs and Pharmaceutical Method Guidelines
In its simplest case, the critical pair in a chromatographic separation is the two analytes with the minimum resolution between them. That minimum resolution is defined as the critical resolution. Obviously, a single-peak elution profile has no critical pair; for two components, analysts can find resolution only between the two responses. Interestingly, the current industry norm for single-component chromatographic investigations is to develop a method resolution standard by spiking a suitable standard into the standard matrix so that a resolution profile with a critical pair can be established and monitored. Although it is hard to say exactly how the term critical pair became part of the chromatographic vernacular, the extensive work of Snyder and Kirkland in this field certainly has been quite influential and should be credited.

Figure 1 is an example of a chromatogram produced by gradient liquid chromatography (LC). The three analytes (peaks 1–3) are fully resolved, and each of the peaks has a neighbor with a resolution greater than 1.5 (Table I). In this simple case, it is easy to discern that the critical pair for this separation comprises peaks 2 and 3. What does this designation tell us? At this point, the defining critical pair simply tells us that as long as the resolution is maintained at 2.1 between peak 2 and peak 3, the suitability of the separation for the profile is intact or as designed.

Current method validation guidelines such as the International Conference on Harmonization of Technical Requirements for the Registration of Drugs for Human Use (ICH), the U.S. Pharmacopeia, and the European Pharmacopoeia — do not refer to the term critical pair. The U.S. Food and Drug Administration (FDA) indirectly refers to this term in discussing selectivity for LC, gas chromatography, and capillary electrophoresis methods:

“If the analytical procedure is used to control their level of impurities, the minimum resolution between the active and the closest eluting impurity, or the two peaks eluting closest to each other, should be given” (17).

The referenced guidance also uses the term critical impurity, which is an impurity greater than the identification or qualification threshold. This impurity should not be confused as a component of the chromatographic critical pair, but it might be. Although the term critical pair is not directly referenced in the official guidance, critical pairs are used routinely in pharmaceutical laboratories to demonstrate that the intended separation method meets the criteria established in ICH and compendial guidelines. In addition, the use of representative critical pairs in the resolution standard often overcomes the necessity to inventory impurity and degradant standards on a case-by-case basis. The concept of a representative pair is discussed in more detail below.

Routine Case — Critical Pair in a Standard or Sample
For chromatographic separations under pharmaceutical industry guidelines, separation scientists must establish the robustness of their analytical procedures. Typically, they establish robustness by two components: robustness against the variability of the column packing materials and robustness against the variability of method conditions. Variability in the column packing material can be evaluated using the resolution standard and samples representative of each stress condition in the accelerated degradation study in which degradation was observed. The column packing material is acceptable if the specificity and the resolution criterion are maintained. Variability of method conditions can be evaluated by altering the method conditions, one condition at a time, as listed below:

• ± 2% relative change in the volume of the lesser component (organic or aqueous) of the mobile phase (the larger component volume remains unchanged)
• ± 2°C change in column temperature
• ± 5% relative change in the mobile phase flow rate
• ± 0.1 pH units in mobile-phase pH

Suppose the separation in Figure 2 is a profile for a method being validated as a pharmaceutical stability-indicating method. To establish robustness under ICH guidelines, a separation scientist would inject the solution used to create Figure 2 under each of the above conditions and examine the change, if any, in the chromatographic result (Table II). The analyst would inspect the data to verify whether peaks 3 and 4 are still resolved and remain the critical pair. Assuming the critical-pair peaks (peaks 3 and 4) remain the critical pair throughout the robustness study, the minimum resolution between these critical-pair peaks from this study becomes the foundation for the resolution specification for suitability of the method. The analytical method then would specify that as long as the resolution of this

<table>
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<th>Table I: Resolution values for Figure 1</th>
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<tr>
<td>Peak Pairs</td>
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<tr>
<td>--------------------------</td>
</tr>
<tr>
<td>Peaks 1 and 2</td>
</tr>
<tr>
<td>Peaks 2 and 3</td>
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</table>

Figure 1: Simple chromatogram example.
pair is maintained at or above this critical value, the method is suitable for use and the integrity of the chromatographic separation is maintained. This information is valuable to separation scientists because it is used for establishing system suitability and as the reference for establishing that integrity of the system is maintained throughout individual runs. In addition, it can be used as a diagnostic tool for the analytical method. In all cases in which critical resolution values are calculated, analysts should give appropriate consideration to repeating the analysis of this degradant simply because another degradation pathway produced it.

Typically, the resolution and tailing factor criteria established for the critical pair must be met for each robustness investigation condition. In doing so, separation scientists establish a great deal of control for the analytical method. In all cases in which critical resolution values are calculated, analysts should give appropriate consideration to repeating the study and reporting a confidence interval near the critical resolution.

**Critical-Pair Modeling**

In the previous example, separation scientists traced the effect of the robustness conditions upon the critical pair. How the critical pair responds to each stress also should be compared with the other responses in the profile. Why? Typically, the chromatographic critical pair is established with a degradant or process impurity, often at a time before a reference standard for the compound has been produced. Thus, system suitability commonly is established using retained samples from the validation study. After the degradant or impurity standard is produced, the method suitability is established by spiking this component into the method standard matrix. Not only is this process cumbersome to the routine assay, it often is unnecessary. If the critical pair is responding in a representative manner to other peaks in the profile during the robustness studies, these other peaks (the representative pair) can be used to model the actual critical-pair components. Thus, the actual critical-pair components need not be present in the method's suitability or resolution solution. By maintaining the representative pair at greater than an established minimum criterion, analysts can assure that the method will maintain the resolution of the critical pair, if it had been present. For example, a minimum criterion for resolution might be set at 10, which seems absurdly higher than 1.5. What the method author likely was conveying is that if the resolution of X and Y is greater than 10, the chromatographic profile is suitable to ensure the resolution of the components produced under stress and validation conditions, including the true critical pair.

Suppose in Figure 2 that peak 3 is a degradant produced from stressing the product sample, peaks 1 and 4 are preservatives, and peak 2 is the active pharmaceutical ingredient. The result is illustrated in Figure 3a (Table III), which leaves the remaining peaks as impurities. During the robustness investigations, the critical pair between the degradant and preservative 2 was assessed under various analytical conditions. Changing the gradient from the normal condition of 45–75% mobile phase B to 45.9–75% mobile phase B resulted in a minimum resolution of 1.9 for the critical pair. Because the resolution of the active pharmaceutical ingredient and preservative 2 changed in a manner that was parallel to the critical pair as the method conditions changed, this information can be used to determine minimum resolution of the critical pair indirectly. When the critical-pair resolution is 1.9, the resolution of the active pharmaceutical ingredient and preservative 2 peaks is 9.7. As long as the chromatographic system maintains the resolution of the active pharmaceutical ingredient and preservative 2 greater than 9.7, the validation data confirm that the critical pair of the separation also will be maintained. Thus, the method resolution standard does not need to be spiked with the degradant to validate separation of the critical pair; the resolution solution simply needs to reflect the critical pair criteria, which it does indirectly using the active pharmaceutical ingredi-
dient and preservative 2 standard (the representative pair), and reduces to Figure 3b (Table IV).

**Multiple Critical Pairs**

As pharmaceuticals become more complex, so do their associated chromatograms. Separation scientists in the pharmaceutical industry routinely are challenged by chromatographic profiles that contain more than one critical pair. Does this outcome mean that many band pairs all result in the same minimum value? No, multiple band pairs present in the chromatographic profile do not respond in the same representative manner as the traditional critical pair with the lowest calculated resolution. How can this result happen? Usually, it is due to small differences in chemistry between the chromatographic analytes and the target pharmaceutical ingredient. The sources of these components are typically synthesis impurities, fermentation by-products, or intended formulation components.

In the previous section, the critical pair was monitored to qualify the method’s robustness. Robustness testing produced a corresponding change in the separation of the chromatographic profile. In Figure 4a, the chromatographic profile of a fermentation product illustrates two critical pairs labeled CP1 and CP2. The pair designated CP1 has the minimum resolution, but the analytes are not considered important to the product and are not monitored for suitability. CP2 is an important band pair and is designated a critical pair for the method because it has the next lowest resolution value. The robustness of this method was challenged at another temperature. The separation profile, illustrated in Figure 4b, shows two additional band pairs that yield resolution at 1.5. The resolution between these additional pairs — CP3 and CP4 — was affected by these conditions (Table V) and required surveillance by an analyst to maintain the integrity of the separation. The band pairs at CP3 and CP4 also are labeled as critical pairs for the suitability of the method and need to be monitored during chromatographic analyses. Because the suitability of the separation must be established with two sets of peaks, the separation can be referred to as having multiple critical pairs.

**The Concept of Maximum Resolution**

Good manufacturing guidelines require that regulated processes establish and maintain control of the intended process. The establishment of a resolution range using a maximum-resolution parameter in the suitability section of a chromatographic method is an additional attempt to ensure the method maintains its performance integrity and control. Maximum resolution is defined as the greatest value that can exist between the critical pair and still maintain resolution between the remaining components in the chromatographic profile. The resolution range is the established suitability of the method between the minimum and maximum resolution of the critical pair. The need to establish a maximum resolution and corresponding resolution range occurs when a change in a method condition improves the resolution between the critical pair but at the same time decreases resolution between some other band pair in the chromatographic profile. Commonly in pharmaceutical separations, one analyte peak moves to longer retention times at a faster rate than the next peak, which causes potential coelution issues. If the maximum resolution factor was omitted, separation scientists might assume that the chromatography was quite good because the minimum resolution had increased by a large amount; however, in reality the overall separation of

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**Figure 4:** Complex chromatographic profiles with multiple critical pairs, including (a) the method profile and (b) the profile obtained at another temperature. Peaks labeled CP1–CP4 are critical peak pairs. Peak 1 is the active pharmaceutical ingredient.

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<thead>
<tr>
<th>Peak Pairs</th>
<th>$R_s$</th>
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<tbody>
<tr>
<td>Preservative 1 and the active pharmaceutical ingredient</td>
<td>9.62</td>
</tr>
<tr>
<td>Active pharmaceutical ingredient and preservative 2</td>
<td>9.90</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Peak Pairs</th>
<th>Original $R_s$</th>
<th>$R_s$ under Challenged Condition</th>
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<tbody>
<tr>
<td>CP1</td>
<td>0.8</td>
<td>0.5</td>
</tr>
<tr>
<td>CP2</td>
<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>CP3</td>
<td>2.69</td>
<td>1.5</td>
</tr>
<tr>
<td>CP4</td>
<td>2.0</td>
<td>1.5</td>
</tr>
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</table>
the resolution values. Figure 5b represents the routine method conditions, and the minimum resolution calculated between the critical pair (peaks 2 and 3) was 1.9. Under a new set of chromatographic conditions, such as a robustness challenge, peak 4 moved to a longer retention time at a faster rate than peak 5. Under these conditions, as Figure 5b shows, a new critical pair was established in peaks 4 and 5. The investigation was further challenged to a point at which the separation between peaks 4 and 5 decreased to baseline resolution ($R_s = 1.5$). In this profile, the resolution factor calculated for the original critical pair (peaks 2 and 3) was found to be 2.9. This resolution is the largest value the original critical pair could have, and it still yields acceptable resolution throughout the chromatographic profile — the maximum resolution. When the resolution of the critical pair is kept within the resolution range between the minimum and maximum values, it ensures that all the components of the chromatographic profile are separated.

The use of maximum resolution parameters has not yet become routine practice in the pharmaceutical industry. We believe the practice should be incorporated into methods in which the robustness of the method warrants such a designation.

**Conclusion**

Pharmaceutical guidelines, in accordance with current good manufacturing practice regulations, require analytical methods to exhibit and maintain control of the system used. Pharmaceutical scientists are challenged by the need to control each component that responds at 0.1% of the active pharmaceutical ingredient or higher, and this control in terms of specificity and robustness becomes increasingly difficult as the complexity of pharmaceutical matrices evolves. The application of critical-pair principles to a chromatographic separation allows separation scientists to effectively challenge analytical methods and also to maintain and safeguard the integrity of the separation profile.

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**References**