Adjusting Conditions for a Routine Reversed-Phase HPLC Assay, Part I: Changing the Column

Routine high performance liquid chromatography (HPLC) assays often are used over a period of years or even decades. During the use of such procedures, it often is necessary to replace the column several times with an equivalent column, usually a column of the same designation (part number) from the same manufacturer. Such changes are made routinely with little or no thought to changes in method performance. Certainly no regulatory red flags would be raised by such routine column replacement, so long as system suitability requirements are met. Because of possible variations over time in the selectivity of columns from different production batches (1,2), a new column of the same designation and source might not be able to reproduce the original separation or meet system suitability requirements. In some cases, manufacturers might discontinue a particular column and the user can no longer purchase a replacement column. At such times, it is necessary to find either a different column that is equivalent to the original column, or other separation conditions must be changed (method adjustment [3]). A similar situation arises for older methods, when one wishes to replace an older column with a newer column that has better separation characteristics.

A change in the column or separation conditions might not lead to an exact duplication of the original separation. Even when the original separation appears to have been replicated, consideration must be given to all possible consequences of such method changes. For methods used in a regulated environment, as in the pharmaceutical industry, the revised procedure must also conform to pertinent rulings by groups within FDA, United States Pharmacopeia (USP), or other agencies. In this article, recent advances in our ability to select equivalent columns will be reviewed, and in a future installment of “Column Watch” (4), we will turn to method adjustment; that is, a change in the mobile phase or temperature. In both of these articles, we will try to address both technical and related regulatory issues. The present two articles represent reviews rather than new round, and no new regulatory policy is implied. However, work toward official regulations on these topics has been underway for several years, and will be reviewed here.

Characterization of Column Performance: Equivalent Columns
A change of column in a routine assay can lead to differences in run time, column efficiency (plate number N), peak shape (tailing), selectivity (peak spacing or values of α) and the resolution R of one or more pairs of peaks. The efficiency of columns sold today usually is guaranteed by the manufacturer, and run time can be adjusted by changes in flow rate, usually with little other affect on the separation. Peak tailing is less of a problem today, with the increasing use of type B columns and separation conditions that favor good peak shape (5). Comparable selectivity and resolution therefore represent the main challenges in the selection of an equivalent column.

A previous installment of “Column Watch” (6) described a new approach for the selection of columns of equivalent selectivity based on the hydrophobic-subtraction model. As reviewed recently (7), this characterization of column selectivity has been expanded and further evaluated during the past two years. On the basis of more than 7000 measurements of retention for 150 compounds of widely varied structure and more than 300 different columns of varied functionality, it was found that equation 1 can predict values of retention factor k with an accuracy of 1−3% (thus assuring that all contributions to selectivity have been measured):

$$\log \left( \frac{k}{k_{EB}} \right) = \eta' H - \sigma' S^* + \beta' A + \alpha' B + \kappa' C$$

[1]
Here, $k_{EB}$ is the value of $k$ for a reference compound (ethylbenzene), the quantities $\eta^s, \sigma^s, \beta^s, \alpha^s$, and $\kappa^s$ refer to properties of the compound being separated, and the remaining terms characterize column selectivity: $H$ (hydrophobicity); $S^*$ (steric resistance of the stationary phase to insertion of a sample compound); $A$ (silanol hydrogen-bond acidity); $B$ (column hydrogen-bond basicity); and $C$ (negative charge on the column due to ionization of silanols). Values of the column-selectivity parameters $H$, $S^*$, $A$, $B$, and $C$ therefore characterize column selectivity. If two columns have equal values of each parameter, they are expected to be equivalent in terms of selectivity for any sample and any separation conditions.

Values of $H$, $S^*$, $A$, $B$, and $C$ do not change with separation conditions, whereas $C$ varies with mobile phase pH because of varying silanol ionization. Therefore, values of $H$, $S^*$, $A$, $B$, and $C$ can be measured consistently using a single set of isocratic conditions (50% acetonitrile–buffer, 35 °C), with pH varied in order to determine $C$ as a function of pH. A collaborative effort by four pharmaceutical laboratories has shown that these measurements of $H$, $S^*$, $A$, $B$, and $C$ can be replicated in different laboratories with suitable precision.

Values of $H$, $S^*$, $A$, $B$, and $C$ are reported in reference 7 for more than 300 columns (C$_1$–C$_30$, phenyl, cyan, fluorosubstituted, with embedded or endcapped polar groups, and so forth), including approximately 200 C8 and C18 columns.

**Comparison of Column Selectivity**

Exact matches of values of $H$, $S^*$, $A$, $B$, and $C$ for two different columns are not expected. However, a sufficiently close match still can lead to equivalent separations. Acceptable matches of column selectivity can be evaluated in terms of a function of values of $H$, $S^*$, $A$, $B$, and $C$.

$$F_S^* = \frac{[12.5 (H_2 - H_1)^2 + 100 (S^*_{-2} - S^*_{-1})]^2 + [30 (A_2 - A_1)]^2 + [143 (B_2 - B_1)]^2 + [8.3 (C_2 - C_1)]^2]^{1/2}}{2}$$

Here, $H_1$ and $H_2$ refer to values of $H$ for columns 1 and 2, respectively, (and similarly for values of $S^*_{-1}$ and $S^*_{-2}$ and so forth). If $F_S^* < 3$ for two columns, then equivalent separations on those two columns are expected; that is, changes in $R_s < 0.5$ units. For larger values of $F_S^*$, the two separations might or might not be acceptably similar, but the likelihood of equivalent separation decreases as $F_S^*$ becomes larger. If a sample does not contain acids and/or ionized bases, $F_S^*$ can be replaced by a quantity $F_S^*$ (where the last two terms of equation 2 are dropped). Because $F_S^* < F_S^*$, this often leads to a larger number of possible “equivalent” columns or a better chance of matching columns.

Recently, five pharmaceutical laboratories evaluated the replacement of one column by another for 12 routine assay procedures. In every case, equation 2 provided a reliable basis for choosing one or more equivalent columns (9). An example from the latter study is shown in Figure 1. The original separation (Figure 1a) provides baseline separation of 10 impurities and the drug product (marked with an asterisk). Two different columns (Figure 1b and 1c) have values of $F_S^* < 3$ and are expected to provide equivalent separation; this is seen to be the case. A fourth column (Figure 1d) has $F_S^* = 10.1$, and the last two peaks overlap (this column is not equivalent).

A second, similar example is shown in Figure 2. Note the similar separations in Figures 2a and 2b for $F_S^* = 1.3$, and the very different separation in Figure 2c for $F_S^* = 248$. Although equation 2 with simi-
lar values of $H$, $S^*$, $A$, $B$, and $C$ for two different columns is not guaranteed to result in a successful prediction of column equiva-
lency, values of $F_i$ need to be selected with great care to screen columns for a subsequent experimental verification of equiva-
lency. In each of the examples of Figures 1 and 2, the selection of equivalent columns was made using commercial col-
umn-comparison software (Column Match, Rheodyne LLC, Ro h n et Pa rk, California). An evaluation copy of this program can be downloaded from www.rheodyne.com.

Most workers will agree that because the regulatory requirements are sufficiently vague and the risk of problems due to non-
compliance are sufficiently large, one generally should “play it safe” and perform some revalidation of the method when an equiva-
lent column is substituted for a previously used column. Of course, one way to avoid this situation is to include one or more equivalent columns in the original valida-
tion of the method. At a minimum, one should demonstrate that the least-resolved peak pairs are still adequately separated and system suitability is achieved.

Development of Orthogonal Separations

Orthogonal separations are complementary to an original HPLC method, being designed to achieve an adequate separation of a given sample (as provided by the original method), while at the same time achieving a very different separation selectivity. An example of an orthogonal separation is shown in Figure 2, where only the column is changed. The separations in Figures 2a and 2b were performed on a C8 column while the separation in Figure 2c was achieved on a polar-embedded column containing an amide linkage. Note the major changes in selectivity for the separations of Figure 2a versus Figure 2c, with elution order reversals and major changes in the resolution of other peak pairs. In the event that a sample component was unsepa-
rated and therefore overlooked in the original method (Figure 2a), it is likely that the “hidden” peak will no longer overlap the same peak in the orthogonal procedure (Figure 2c). Orthogonal methods can be based upon a completely different chromatographic principle (for example, gas chromatography or thin-layer chromatography), but it is more convenient to use a dif-
ferent reversed-phase HPLC method. A change in selectivity can be achieved by a change in any of several separation condi-
tions, but we favor a large change in column selectivity combined with a change in the organic solvent (such as methanol replacing acetonitrile). Together, these two changes can be shown to lead to changes in selectivity that are likely to result in the separation of any peaks that were overlapped in the original method.

A change in column and organic solvent generally provides an adequate change in selectivity for the separation of two peaks that are overlapped in the original method, but samples that contain more than a few peaks can exhibit an overlap of other peak pairs. Therefore, after changing column and solvent, further adjustments in conditions might be required. It has been shown else-
where (10) that further changes in temperature and either organic solvent percentage (isocratic) or gradient time usually are able to achieve the separation of samples with less than 15 components, using computer simulation (11) for the convenient selection of final conditions.

The foregoing procedure currently is being evaluated by several different pharma-
caceutical laboratories. Figure 3 shows the results of one orthogonal method devel-
opled in this way. The original method (Figure 3a) consists of the drug product (peak 3) and four known impurities or degradation products (peaks 1, 2, 4, and 5). When the orthogonal procedure (Figure 3b) was applied to the same sample, a new (previously unrecognized) peak (peak 6) was separated from peak 3. The results of this collabo-
ration are described in the conclusion of this investigation.

Most workers would agree that a change in a method by use of an orthogonal pro-
cedure represents sufficient change in the method that revalidation is required. How-
ever, this generally should not be a problem, because orthogonal procedures are used primarily during the prevalidation phase of method development to ensure that no hidden peaks are present, so the additional investment in validation at this point would be minimal.

Alternative Means for Characterizing Column Selectivity

During the past 30 years, dozens of chromatographic tests have been proposed for the purpose of measuring the selectivity of reversed-phase columns. Apart from the present procedure based on equations 1 and 2, none of these past column characteriza-
tion procedures appears to meet all of the practical requirements for establishing column equiva-
lence.

- A test procedure that is based upon a rea-
sonable physico-chemical model that allows a logical interpretation of column selectivity as a function of column prop-
ties (ligand length and concentration, pore diameter, endcapped or not, and so fo th);
- A test procedure that has been shown to measure all significant sample–column interactions that contribute to selectivity for most commercially available column types (for example, C18–C30 based upon either type A or B silica; columns with embedded or endcapped polar groups; phenyl, cyano or fluoro columns; and so fo th);
- Column selectivity results for example, values of $H$, $S^*$, $A$, $B$, and $C$ for a large number of commercial columns, espe-
cially the more popular columns;
- Column selectivity results that are applicable for any sample and any separation conditions;
- A convenient, reproducible test pro-
cedure, with test results for a significant fraction of all reversed-phase columns;
- Availability of verified means (for exam-
ple, equation 2) for the quantitative comparison of selectivity for two columns (including commercial software for convenient implementation); and
- Experimental proof that equivalent columns can be identified by the column selectivity results.
dozen technical publications based upon this work have either appeared, are in various stages of publication, or are in preparation. During the last half of this program, organizations such as National Institutes of Health (NIH), USP, and the Product Quality Research Institute (PQRI; a consortium of industry, academic, and regulatory entities concerned with pharmaceutical analysis) have become involved in both support and evaluation roles. To our knowledge, this combined effort represents the most intensive investigation of a column test procedure that has yet been attempted. Values of $H$, $S^*$, $A$, $B$, and $C$ now can be used to select replacement columns that are likely to give equivalent separations for any sample or experimental conditions. Values of $H$, $S^*$, $A$, $B$, and $C$ also can be used in the design of orthogonal separations for use in detecting hidden peaks from an original HPLC method. Other possible applications of this approach should be apparent to the experienced reader.

Because new reversed-phase columns are being introduced continually, it is important that the present column database be updated on an ongoing basis. Column

manufacturers with newly developed columns are invited to contact one of the authors for inclusion of additional columns into this database.

References
(2) R.E. Majors, LCGC 15, 1008 (1997).

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