A Four-Column Approach to Developing Stability-Indicating HPLC Methods

The authors describe a method development approach that incorporates four packing materials with unique selectivities. They found that using different packing materials and mobile-phase pH levels was the most powerful approach for optimizing the chromatographic separation of degradation products from active ingredients. They first used the four columns at low-pH conditions and then compared the resulting separations. If the authors obtained insufficient resolution, they used two of the columns with high-pH mobile phase as well.

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According to current good manufacturing practices, all drugs must be tested with a stability-indicating assay before release. The U.S. Food and Drug Administration (FDA) defines a stability-indicating assay as follows (1):

"A stability-indicating assay is a validated quantitative analytical procedure that can detect the changes with time in the pertinent properties of the drug substance and drug product. A stability-indicating assay accurately measures the active ingredients, without interference from degradation products, process impurities, excipients, or other potential impurities."

High performance liquid chromatography (HPLC) has become the method of choice for stability-indicating assays because of its inherent ability to separate degradation products, process impurities, and excipients from active ingredients. Typically, stability-indicating HPLC methods are developed in a sequential fashion. Analysts establish retention using a given column, perform degradation studies, and analyze the degraded sample to see if all potential degradation products are resolved from the active ingredient. If not, they choose a new column and repeat the process.

Different HPLC columns are well known to provide potentially different selectivities for given separations. In the case of a common ligand such as C18, these differences are attributable to variations in the ligand density or in properties of the base silica such as silanol or metal content. More recently, ligands such as those containing embedded polar groups have been shown to provide different selectivities than straight-chain ligands (2,3).

Recently introduced hybrid particle packings also offer unique selectivities compared with traditional silica-based packings, again because of differences in the silanol content of the base particles (4). Moreover, these materials enable separations at pH levels as high as pH 12 — conditions at which basic compounds are neutral and acidic compounds are fully ionized. This ability has led to the realization that pH is a very powerful selectivity tool for reversed-phase separations. Low-pH separations involve protonated acids and bases, but these acids and bases are deprotonated at high-pH levels. Because retention in reversed-phase chromatography is strongly dependent upon the analyte charge, pH can be used to make large changes in selectivity.

Given the powerful selectivity tools of column type and pH, we describe an approach to developing stability-indicating HPLC methods that uses four columns and two pH levels. Figure 1 shows the surface chemistries of the four columns used. The packing materials comprise two base particles —

silica and hybrid — and two ligands — straight-chain C18 and C18 with an embedded polar ligand. These four columns offer unique selectivity because of this combination of ligands and base particles. We performed separations at pH 3 on all four columns and then at pH 10 on the two hybrid particle columns. This approach had the advantage of testing the four columns in parallel, which saved time in the method development process. Through automation, the entire process could be completed in a few days.

We provide an example of how this approach can be implemented for the drug substance lansoprazole, which is an antiulcerative.

Experimental

Instrumentation: We performed separations using an Alliance HT 2790 separations module with a model 996 photodiode-array detector and a Millennium HPLC control system (all from Waters Corp., Milford, Massachusetts, USA).

Mobile phases: We performed all separations using acetonitrile as the organic modifier (mobile phase B). The low-pH buffer we used was 10 mM ammonium formate adjusted to pH 3.0 with formic acid (mobile phase A). The high-pH buffer we used was 10 mM ammonium bicarbonate adjusted to pH 10.0 with ammonium hydroxide. We purchased all chemicals from JT Baker (Phillipsburg, New Jersey, USA).

HPLC conditions: All columns had 100 mm × 4.6 mm dimensions with 3.5-µm d_P packings. The packings we used were Symmetry C_{18}, SymmetryShield RP_{18}, XTerra MS C_{18}, and XTerra RP_{18} (all from Waters). The flow rate was 1.4 mL/min, and the injection volume was 2–5 µL. The wavelength we monitored was 254 nm, and the columns were maintained at 30 °C.

Degradation studies: We obtained lansoprazole from Sigma-Aldrich (St. Louis, Missouri, USA). We prepared a solution of 0.14 mg/mL in 65:35 (v/v) water–acetonitrile to establish the isocratic conditions. For degradation studies, we prepared 10-mg/mL lan-

soprazole solutions in 0.4 N hydrochloric acid, in 0.4 N sodium hydroxide, and in 3% hydrogen peroxide. We allowed the solutions to degrade by stirring, and we then either diluted (hydrogen peroxide degradation) or neutralized (acid or base degradation) the solutions before HPLC analysis.

Results and Discussion

The first step in our method development process was to perform a pH 3 separation of the drug using all four columns. Our goal was to develop isocratic conditions that lead to a retention factor in the 4–6 range on all four columns. This approach was preferable to using the same conditions on all four columns because it enabled a better comparison of differences in selectivity.

Establishing isocratic conditions was accomplished two ways. We ran a gradient separation on each column, determined the percentage of organic solvent at which the compound was eluted, and optimized the isocratic conditions starting with that percentage of organic solvent to obtain a reten-
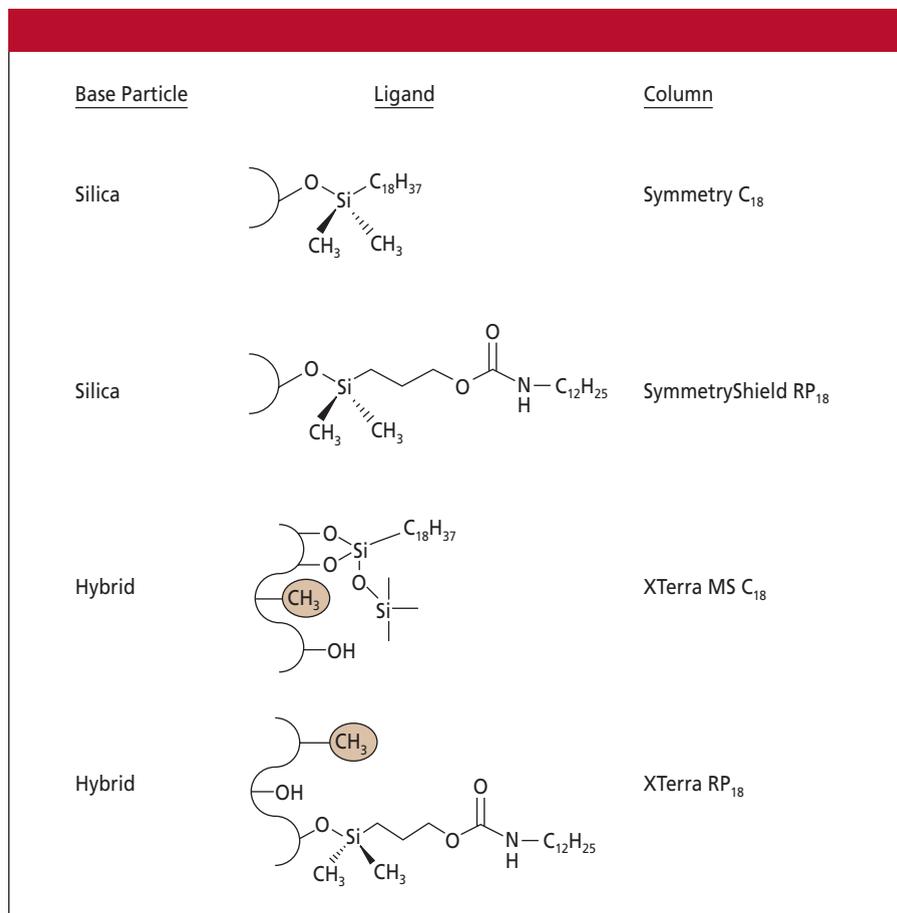

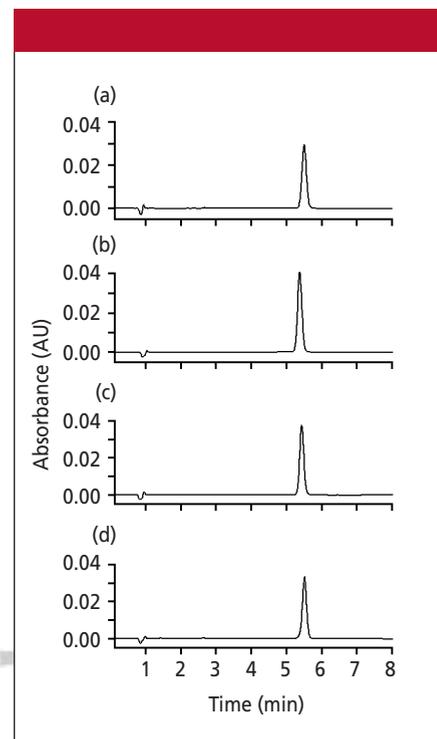**Figure 1:** Columns used for the method development approach.

Figure 2: Low-pH separations of lansoprazole using (a) XTerra MS C_{18}, (b) XTerra RP_{18}, (c) Symmetry C_{18}, and (d) SymmetryShield RP_{18} columns. Mobile phase: A = 10 mM ammonium formate (pH 3), B = acetonitrile; sample concentration: 137.5 µg/mL in 65:35 (v/v) water–acetonitrile. (a) 74:26 A–B, t_R = 5.5 min, N = 8365, k = 4.8; (b) 74:26 A–B, t_R = 5.4 min, N = 8679, k = 4.7; (c) 73:27 A–B, t_R = 5.5 min, N = 10,026, k = 4.8; (d) 73:27 A–B, t_R = 5.5 min, N = 9864, k = 4.8.
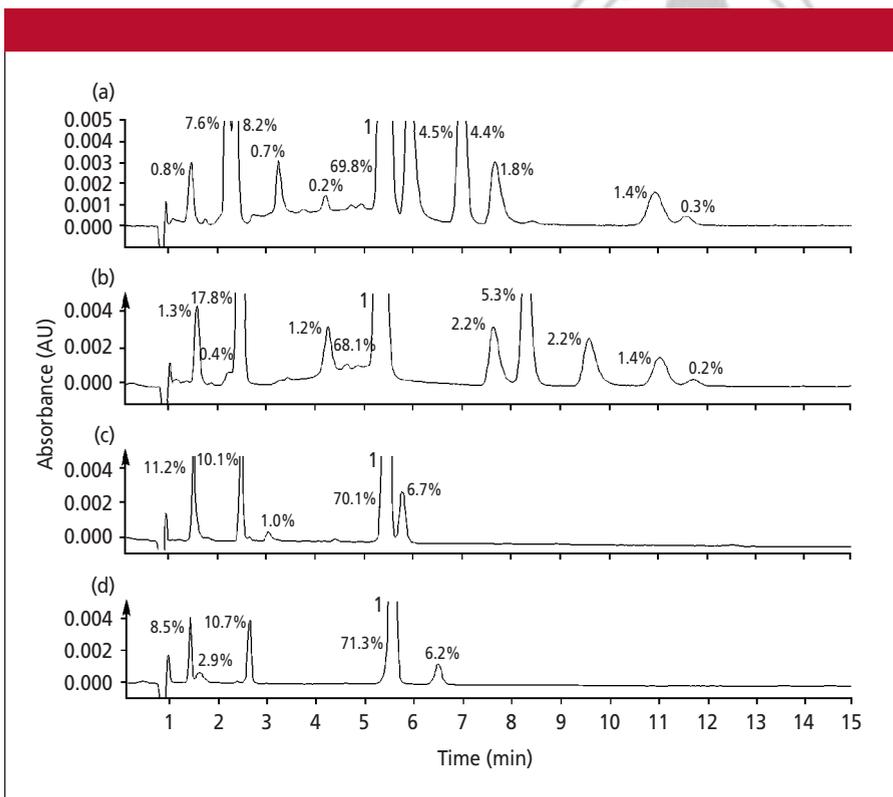
Figure 3: Low-pH separations of lansoprazole degraded with 0.4 N hydrochloric acid using (a) XTerra MS C$_{18}$, (b) XTerra RP$_{18}$, (c) Symmetry C$_{18}$, and (d) SymmetryShield RP$_{18}$ columns. Shown are enhanced-baseline chromatograms. Other conditions were the same as in Figure 2. Peak 1 is lansoprazole.

Figure 4: Low-pH separations of lansoprazole degraded with 0.4 N hydrochloric acid using (a) XTerra MS C$_{18}$, (b) XTerra RP$_{18}$, (c) Symmetry C$_{18}$, and (d) SymmetryShield RP$_{18}$ columns. Shown are full-scale chromatograms. Other conditions were the same as in Figure 2. Peak 1 is lansoprazole.

Figure 5: Plots showing the dependence of retention on pH.

tion factor of 4–6. Alternatively, we started with a high percentage of organic solvent, such as 90%, measured the retention factor, and then reduced the percentage of organic solvent until we reached the desired retention factor.

Figure 2 shows the isocratic separations of lansoprazole we obtained on all four columns at low-pH conditions. Note the excellent peak shape and efficiency obtained on all four columns. Because we had established the isocratic conditions on each column, our next step was to perform degradation studies on the drug. We investigated acid, base, and peroxide degradation. In this process, we dissolved the drug in concentrated acid, base, and peroxide. Then, we allowed the solutions to degrade approximately 10–30% (as measured by the reduction of parent peak area) and ran the degraded drug on all four columns. We compared the resulting separations to determine which column provided resolution of all potential degradants from the main drug peak. In addition to visual examination, we confirmed the purity of the main drug peak by photodiode-array detection as well.

Figure 3 shows the enhanced baseline sep-

arations of lansoprazole degraded by 0.4 N hydrochloric acid on all four columns. As the figure shows, the two hybrid particle columns' separations show several peaks, which are not present on the silica-based columns' separations, after the main peak. Moreover, several peaks in the region in front of the main peak in the hybrid particle columns' separations are not present in the silica-based columns' separations. Clearly, the hybrid particle columns provide superior resolution of the degradants in this separation. We would have needed to perform additional method development on the silica-based columns if quantification of all degradants was necessary. However, all four parent peaks in Figure 3 were free of any coelutions, according to photodiode-array detection peak-purity data. Therefore, if the quantitation of the main peak was the main focus, all four columns would be suitable.

We also observed differences when comparing the straight-chain hybrid column with the embedded polar ligand hybrid column. For example, we saw differences in the separation of the five peaks after the main peak and elution order changes within that
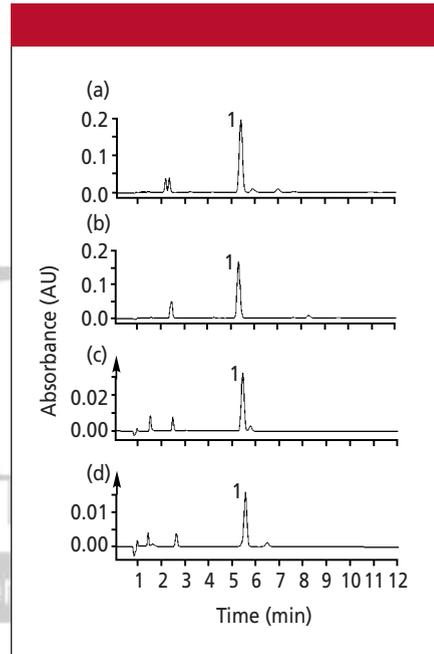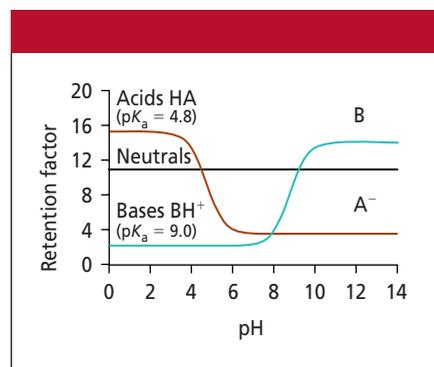
group of degradant peaks. To see an additional difference, we examined the full-scale chromatograms, which are shown in Figure 4. When comparing the two hybrid particle columns, the degradant peak before the main peak was resolved into two peaks on the straight-chain ligand versus a single peak on the embedded polar ligand.

If a column provided the desired separation at pH 3, our next step was to further optimize the separation on that column. We accomplished optimization by adjusting the percentage of organic solvent or by using methanol instead of acetonitrile. If we obtained no useful separation at low-pH
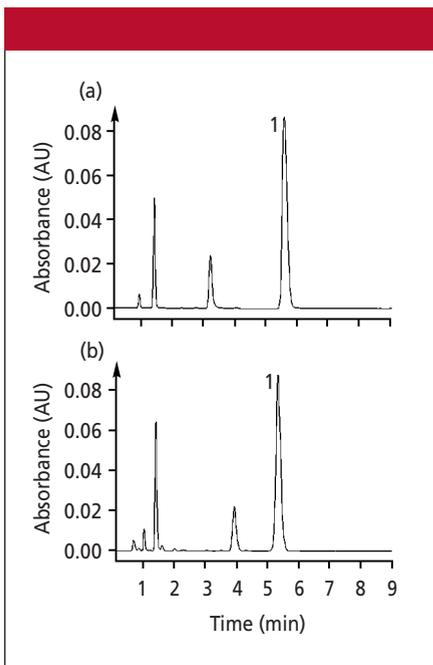
Figure 6: High-pH separations of lansoprazole degraded with 0.4 N hydrochloric acid using (a) XTerra MS C$_{18}$ and (b) XTerra RP$_{18}$ columns. Mobile phase: A = 10 mM ammonium carbonate (pH 10), B = acetonitrile; injection volume: 2 μL; sample concentration: 2.0 mg/mL in 40:60 water–acetonitrile. (a) 77:23 (v/v) A–B, (b) 78:22 (v/v) A–B. Peak 1 is lansoprazole.

Figure 7: Comparison of (a) pH 3 and (b) pH 10 separation of 0.4 N hydrochloric acid–degraded lansoprazole on the XTerra RP$_{18}$ embedded polar ligand column. Peak 1 is lansoprazole.

conditions, our next step was to run the two hybrid particle columns at pH 10. The change to pH 10 assumes that at least some of the analytes of interest are ionizable. Neutral compounds will be unaffected by changing the pH.

The pH levels chosen for this work — pH 3 and 10 — provide the greatest possible differences in selectivity, because complete protonization or deprotonization is achieved for most compounds. Figure 5 shows the dependence of acid, base, and neutral retention on pH. At pH 3, acids have their maximum retention because they are neutral, but bases have their minimum retention because they are fully charged. At pH 10, bases have their maximum retention because they are neutral, and acids are fully ionized and have their minimum retention. The retention of neutral compounds is unaffected by pH. The dependence of retention on pH for acids and bases at pH 3 and 10 is relatively insensitive to minor changes in pH. Therefore, methods developed at these pH levels will be more robust with respect to mobile-phase pH. Conversely, at more-neutral pH levels, the dependence is much more sensitive to slight changes.
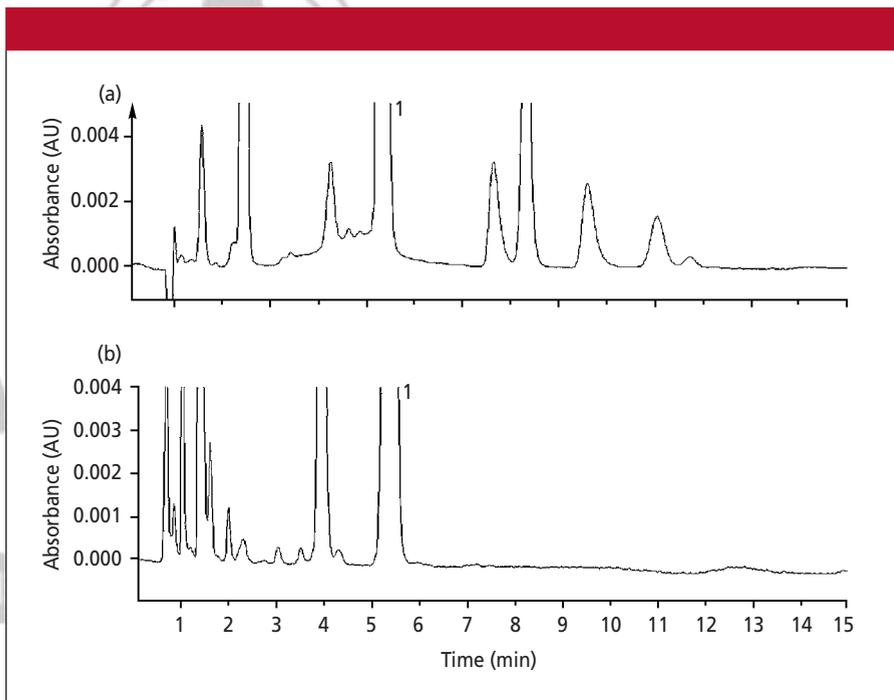
Figure 6 shows the separations of acid-degraded lansoprazole we obtained using the two hybrid particle columns at pH 10. Unlike the separations at pH 3, all the degradation products are eluted before the main peak. Photodiode-array detection peak-purity data also suggest that both peaks are free of coelutions. Separations performed at pH 10 would be preferable for quantitating degradation products, because the degradation products all are eluted before the main peak. As the concentration of the main peak increases to allow detector visualization of the smaller peaks, the main peak tends to tail, which can cause difficulties in quantitating compounds eluted after the main peak.

Figure 7 compares the separations of acid-degraded lansoprazole on the embedded polar ligand hybrid column at pH 3 and 10. The same sample exhibits large differences in selectivity, which demonstrate the power of pH adjustment in manipulating selectivity. The results indicate that the degradants are influenced more by the pH change than is the parent compound, which has less retention at high-pH than at low-pH conditions. Only columns that are stable at high-pH levels, such as the hybrid particle columns we used, can be used for these comparisons.

Conclusions

Using four columns and two pH levels, we showed how analysts can achieve substantial differences in separation selectivity. Although our example was for a degraded drug, our approach can be applied to any complex mixture. The main reasons for the differences in selectivity are that

- working at pH 3 versus pH 10 changes the charge state and, therefore, the retention of ionizable compounds;
- embedded polar ligands have different selectivities than straight-chain ligands; and
- hybrid particle–based packing materials have different surface characteristics and, therefore, different selectivity than silica-based packing materials.

Currently, we are investigating the use of ligands such as phenyl in this approach.

References

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