

Preparative Chromatography with Improved Loadability



In combinatorial chemistry and drug development, as well as in the full-scale production of pharmaceuticals, the most extensively used stationary-phase materials for preparative purposes are reversed-phased materials. Although silica-based materials are regarded as excellent stationary phases, their main drawback rests in their pH limitations. Hybrid packings are available today, and these packings are stable throughout a wide pH range and still can provide the retention characteristics of a silica material. The authors show that the loadability and throughput of ionizable compounds can be enhanced greatly by using the extended pH range of hybrid packings.

The intent of preparative chromatography is to purify materials and use them for additional testing or as final products (1–3). For example, preparative chromatography is used to purify compounds from combinatorial libraries, to obtain material for clinical trials, and in large-scale production of drugs and vaccines (4,5). Consequently, the scales of preparative chromatography vary substantially. Independent of the column size, the focus of preparative chromatography is the same: loadability of samples into the column, production rate of targeted compounds, and their purity and yield. Reversed-phase chromatography is one of the most frequently used modes of chromatography (6,7).

Silica-based materials are very popular, but they are used in a limited pH range because of silica's stability. On the other hand, hybrid particles maintain the retention characteristics of silica, and they are effective in the pH 2–12 range (8,9). Hybrid particles also provide good peak shape, independent of the chemistry of the solute. The retention factor of ionizable compounds varies with pH (10). In particular, the retention factor of bases increases with increasing pH, and the retention factor of acids increases as the pH of the mobile phase decreases. The study that we present here

shows that the loadability and throughput of ionizable compounds improves when working under suitable pH conditions. Also, we obtained the best loading capacity when the sample compounds were in nonionizable form. Furthermore, we used columns pulled randomly from production, and the scale-up applications show the consistency in retention times and baseline separation.

Experimental

Materials: We used 50 mm × 4.6 mm XTerra MS and 50 mm × 7.8 mm, 10 mm × 19 mm, 50 mm × 19 mm, and 50 mm × 30 mm XTerra Prep reversed-phase C18 columns randomly pulled from the Waters Corp. (Milford, Massachusetts) production line. The particle size of the stationary-phase material was 5 μm.

We purchased diphenhydramine, oxybutynin, terfenadine, buspirone, oxacillin, cloxacillin, and dicloxacillin from Sigma (St. Louis, Missouri). We obtained ammonium bicarbonate, ammonium formate, formic acid, and ammonium hydroxide from Sigma (St. Louis, Missouri). We purchased dimethyl sulfoxide from VWR (Westchester, Pennsylvania). We obtained high performance liquid chromatography (HPLC)–grade acetonitrile from J.T. Baker (Phillipsburg, New Jersey).

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Equipment: We performed analytical-scale experiments with basic compounds using an Alliance 2690 HPLC system and a model 996 photodiode-array detector (both from Waters). We used Waters Millennium³² data-management software, version 3.2.

We performed preparative chromatography with basic compounds using a model 4000 multisolvent pump system equipped with a model 2700 sample manager and a model 2487 UV-vis absorbance detector controlled by the Millennium chromatography software manager (all from Waters).

We performed analytical and preparative chromatography for acids using the Auto-purification system, which includes a model 2525 high-pressure dual-reservoir mixing pump, a model 2767 sample manager, and a column organizer (all from Waters). We used the photodiode-array detector for UV-directed monitoring of the column effluent. We used MassLynx software (Waters) for data-management purposes.

Procedures

Peak shape and retention times: We performed linear gradient experiments for the basic test mixture of diphenhydramine and terfenadine with the 50 mm × 19 mm column. Buffer A was deionized water, buffer B was acetonitrile, and buffer C was 100 mM ammonium bicarbonate (pH 10) or 100 mM ammonium formate (pH 3.8) (Figure

1). We equilibrated the column at 85:5:10 A–B–C and processed a 15-column-volume (volume of mobile phase in the column) linear gradient to 0:90:10 A–B–C. The flow rate was 30 mL/min, and the injection volumes were 1200 μL. We monitored the effluent at 254 nm.

Loadability studies: We performed the linear gradient experiments for the basic test mixture of diphenhydramine, oxybutynin, and terfenadine with the 50 mm × 19 mm column. Buffer A was deionized water, buffer B was acetonitrile, and buffer C was 100 mM ammonium bicarbonate (pH 10) or 100 mM ammonium formate (pH 3.8) (Figure 1). We equilibrated the column at 85:5:10 A–B–C and processed a 15-column-volume linear gradient to 0:90:10 A–B–C. The injection volumes ranged from 25 μL to 1200 μL. The flow rate was 30 mL/min. We monitored the effluent at 254 nm.

Reducing column length: We performed linear gradient experiments for the basic test mixture of diphenhydramine, oxybutynin, and terfenadine (200 mg/mL, 200 mg/mL, and 12 mg/mL in dimethyl sulfoxide, respectively) with the 10 mm × 19 mm column. Buffer A was deionized water, buffer B was acetonitrile, and buffer C was 100 mM ammonium bicarbonate (pH 10). We equilibrated the column at 85:5:10 A–B–C and processed a 10-column-volume linear gradient to 0:90:10 A–B–C. The flow rate was 30 mL/min, and the injection volumes were 200 μL. We monitored the effluent at 254 nm.

Plate-to-plate mapping: We performed linear gradient experiments with a 50 mm × 4.6 mm column. Buffer A was 95:5 (v/v) deionized water–100 mM ammonium bicarbonate (pH 10) and buffer B was 90:5:5 (v/v/v) acetonitrile–deionized water–

100 mM ammonium bicarbonate (pH 10). The sample mixture comprised 0.5 mg/mL of diphenhydramine and 10 mg/mL of oxybutynin in deionized water. The linear gradient slope was 10 column volumes from 60:40 to 10:90 A–B. The flow rate was 1.8 mL/min, and the injection volumes were 1000 μL. We monitored the effluent at 254 nm.

Scale-up: Buspirone: We performed linear gradient experiments for the buspirone scale-up. Buffer A was deionized water, buffer B was acetonitrile, and buffer C was 100 mM ammonium bicarbonate (pH 10). We equilibrated the column at 80:10:10 A–B–C and processed a 30-column-volume linear gradient to 30:60:10 A–B–C. Table I lists the column sizes, flow rates, and masses loaded. The sample concentration was 200 mg/mL in deionized water. We monitored the effluent at 254 nm.

Acids: We performed linear gradient experiments for the oxacillin, cloxacillin, and dicloxacillin mixture at a concentration of 20 mg/mL each. Buffer A was 90:10 (v/v) deionized water–100 mM ammonium formate (pH 3.8) and buffer B was 80:10:10 (v/v/v) acetonitrile–deionized water–ammonium formate (pH 3.8). We equilibrated the columns with 90:10 A–B and processed a 45-column-volume gradient to 40:60 A–B. Table II lists the column sizes, flow rates, and masses loaded in these experiments. We monitored the effluent at 273 nm.

Results and Discussion

The work presented in this article is aimed at investigating the loadability and purification of ionizable compounds under acidic and basic pH conditions. To do so, we used hybrid-type packings that have good stability in the alkaline pH range. In particular, our work focused on the loadability of acids

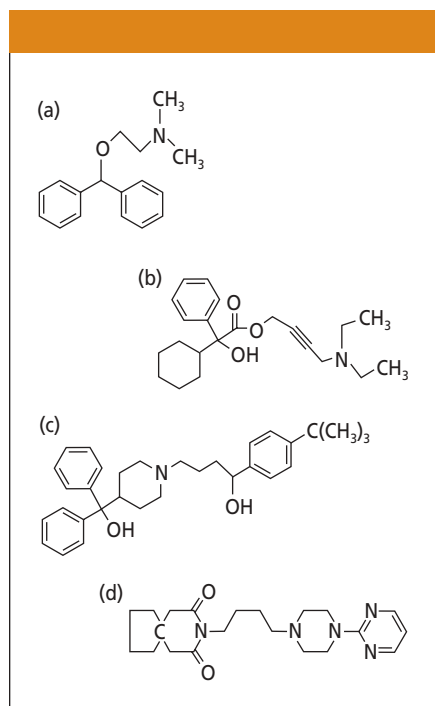


Figure 1: Structures of basic test compounds (a) diphenhydramine, (b) oxybutynin, (c) terfenadine, and (d) buspirone.

Table I: Buspirone scale-up data for XTerra MS C18 columns at high pH

Column Dimensions (mm)	Flow Rate (mL/min)	Total Mass Loaded onto the Column (mg)
50 × 4.6	1.8	5
50 × 7.8	5.0	14
50 × 19	30.0	85

Table II: Acids scale-up data for XTerra MS C18 columns at low pH

Column Dimensions (mm)	Flow Rate (mL/min)	Total Mass Loaded onto the Column (mg)
50 × 4.6	1.8	3
50 × 7.8	5.0	9
50 × 19	30.0	51
50 × 30	77.0	128

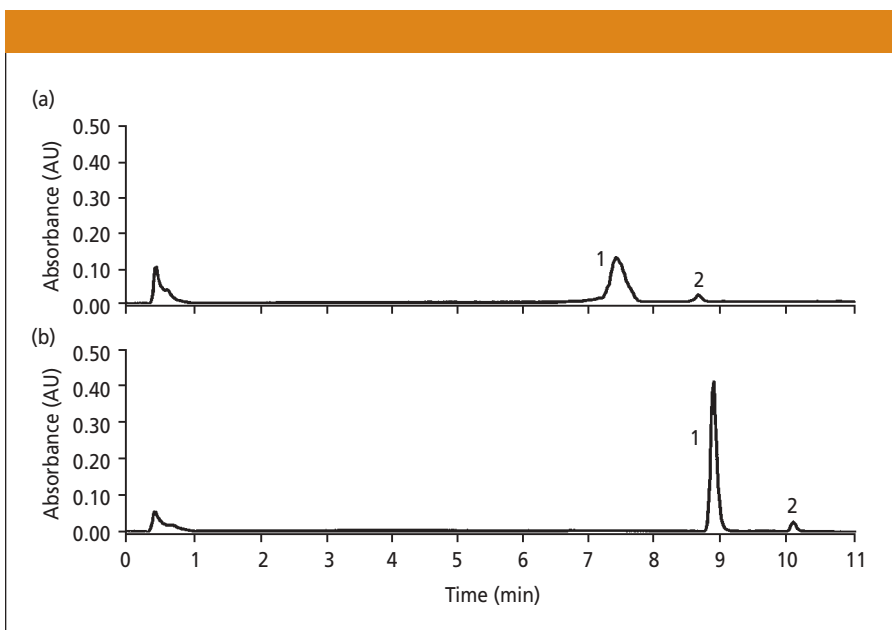


Figure 2: Peak shape and retention comparison of basic compounds at (a) pH 3.8 and (b) pH 10. Injection volume: 1.2 mL; detection: UV absorbance at 254 nm. Peaks: 1 = diphenhydramine (pH 3.8, 2.5 mg/mL; pH 10, 50 mg/mL), 2 = terfenadine (pH 3.8, 0.15 mg/mL; pH 10, 3 mg/mL).

and bases onto C18 columns using mass spectrometer-compatible buffers and on the purification of those compounds loaded onto various-size columns. In this investigation, we used model mixtures of ionizable drugs currently used by the pharmaceutical industry.

Peak shape and retention times: To compare the peak shapes and retention times of bases at low and high pH, we dissolved both diphenhydramine and terfenadine (Figure 1) in dimethyl sulfoxide and performed linear gradient experiments under acidic and basic pH conditions. We used dimethyl sulfoxide as the sample solvent in these experiments because it is a solvent widely used in combinatorial libraries. It also is a strong solvent for reversed-phase chromatography, which ensured a more challenging chromatographic application.

Figure 2 presents the chromatographic results. Although both chromatograms are the result of the same gradient slopes, injection volumes, and sample concentrations, the only variable introduced was the buffer to perform the experiments at pH 3.8 and 10. As expected, the retention of the bases increases with an increase in pH. In addition, the peak shapes improve dramatically when the diphenhydramine and terfenadine are run at high pH levels. For example, the width at 50% height for diphenhydramine at pH 3.8 is 0.2470 min, and this half-height width is reduced to 0.0997 min at pH 10.

Loadability studies: We performed loadability studies at low and high pH levels using the bases diphenhydramine, oxybutynin, and terfenadine dissolved in dimethyl sulfoxide. Figures 3 and 4 show our results.

As depicted in the figures, we performed the experiments for each pH condition by increasing the injection volume but maintaining the sample concentration. In particular, Figure 3 shows the chromatographic results for a 50 mm × 19 mm column when the injection volume increased from 25 μ L to 400 μ L with the total mass load ranging from 1 mg to 16.5 mg. The results shown in this figure indicate a loss of baseline separation between the 4- and 8-mg load.

Figure 4 shows the chromatographic results for a similar study at a high pH, and the injected samples are 10-fold more concentrated than those of the experiments performed at the low pH level. In fact, we were able to load 494 mg of a mixture of basic compounds into a column at pH 10 and still obtain a baseline separation. These results are compelling because they indicate that it is possible to load at least 60-fold more sample at high pH levels than at low pH levels. At the high pH, we loaded 118 mg/g packing material.

Although the pK_a s of diphenhydramine, oxybutynin, and terfenadine in water are 9.0, 7.0, and 9.5, respectively, these bases

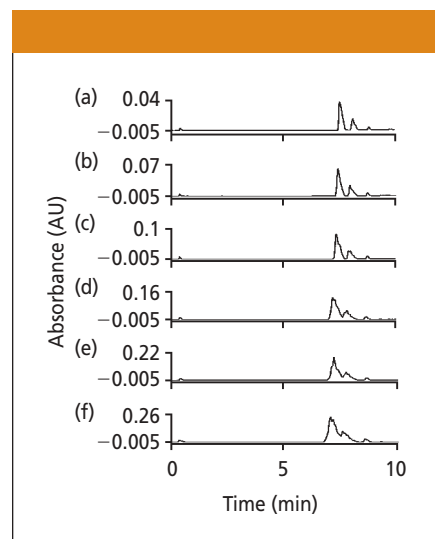


Figure 3: Loadability study at low pH using a 50 mm × 19 mm XTerra MS C18 column with total sample loads of (a) 1.0 mg (25 μ L), (b) 2.0 mg (50 μ L), (c) 4.0 mg (100 μ L), (d) 8.0 mg (200 μ L), (e) 10.0 mg (250 μ L), and (f) 16.5 mg (400 μ L). Buffer A: deionized water; B: acetonitrile; C: 100 mM ammonium formate (pH 3.8). Gradient: 85:5:10 to 0:90:10 A–B–C in 15 column volumes; flow rate: 30 mL/min; detection: UV absorbance at 254 nm; injection volume: 1.2 mL. Peaks: 1 = diphenhydramine (20 mg/mL in dimethyl sulfoxide), 2 = oxybutynin (20 mg/mL in dimethyl sulfoxide), 3 = terfenadine (1.2 mg/mL in dimethyl sulfoxide).

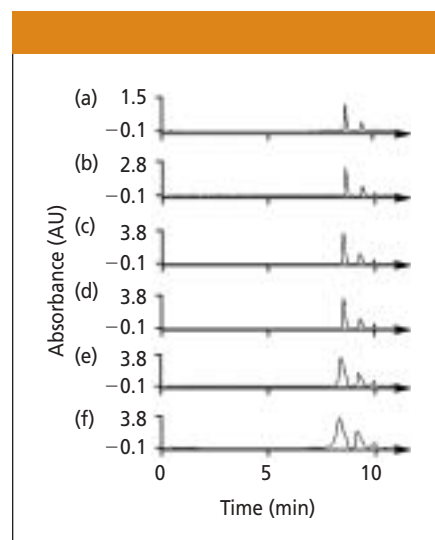


Figure 4: Loadability study at high pH using a 50 mm × 19 mm XTerra MS C18 column with total sample loads of (a) 41.2 mg (100 μ L), (b) 82.4 mg (200 μ L), (c) 164.8 mg (400 μ L), (d) 247.2 mg (600 μ L), (e) 329.6 mg (800 μ L), and (f) 494.4 mg (1200 μ L). Buffer A: deionized water; B: acetonitrile; C: 100 mM ammonium bicarbonate (pH 10). Gradient: 85:5:10 to 0:90:10 A–B–C in 15 column volumes; flow rate: 30 mL/min; detection: UV absorbance at 254 nm; injection volume: 1.2 mL. Peaks: 1 = diphenhydramine (200 mg/mL in dimethyl sulfoxide), 2 = oxybutynin (200 mg/mL in dimethyl sulfoxide), 3 = terfenadine (12 mg/mL in dimethyl sulfoxide).

are eluted in the presence of a certain percentage of organic solvent during the gradient run. Consequently, we expect the pK_a values to decrease; therefore, they are eluted as neutral compounds or close to their neutral state. These results demonstrate that it is possible to purify substantially more material when the buffer conditions are such that the compounds are in their neutral state. In addition, these results are possible due to the hybrid reversed-phase material used in this work, because traditional silica-based materials quickly would lose stability at pH 10.

Column length: Although the results so far are significant, we evaluated alternatives to generating faster purifications, especially to purify a large number of samples as for sample cleanup in combinatorial chemistry. One way to achieve faster separations is by using shorter columns. A shorter column reduces costs in run time, initial hardware, and solvent consumption and disposal.

Figure 5 illustrates a reduction in the run time from a 3-min fast separation using a 10 mm \times 19 mm cartridge. As the figure shows, we loaded a total of 82.4 mg of material onto the 10 mm \times 19 mm column. This load is impressive for both combinatorial chemistry cleanup of samples and process development. Because we used the same flow rate for the 10 mm \times 19 mm as for the 50 mm \times 19 mm columns, we achieved a significant solvent reduction per run.

Plate-to-plate mapping: In some cases samples produced by combinatorial synthesis that contain a few milligrams of the main compound must be purified with minimal handling to reduce time and potential errors. Often, combinatorial chemistry samples come in 96-well formats. One way of minimizing handling time and errors is to load samples from a 96-well plate and collect the pure fraction into a well of another 96-well plate. However, this procedure requires a small fraction volume for collection — less than 2 mL. Because of this need, we performed linear gradient chromatography at pH 10 with an analytical-scale column (50 mm \times 4.6 mm), injecting 1 mL of sample with a model mixture of diphenhydramine and oxybutynin in water (0.05 mg/mL and 10 mg/mL, respectively).

Figure 6 shows the baseline resolution obtained. Moreover, it is possible to collect the pure sample within 1.4 mL. When possible, running preparative chromatography in a smaller column results in reduced sample handling and lower column costs, flow

rates, pressures, fraction volumes, and solvent disposal costs.

Another advantage of performing preparative chromatography in an analytical column is that when a drug candidate is transferred to process development and then to production, the cost reductions also carry over to the large scale.

Scale-up: In this study, we performed scale-up experiments using the following equations:

$$F_{\text{prep}} = F_{\text{anal}} \left(\frac{V_{\text{prep}}}{V_{\text{anal}}} \right) \quad [1]$$

$$m_{\text{prep}} = m_{\text{anal}} \left(\frac{V_{\text{prep}}}{V_{\text{anal}}} \right) \quad [2]$$

$$t_{\text{prep}} = t_{\text{anal}} \left(\frac{V_{\text{prep}}}{V_{\text{anal}}} \right) \left(\frac{F_{\text{anal}}}{F_{\text{prep}}} \right) \quad [3]$$

where F_{prep} is the flow rate of the preparative column, F_{anal} is the flow rate of the analytical column, V_{prep} is the volume of the preparative column, V_{anal} is the volume of the analytical column, m_{prep} is the mass injected on the preparative column, m_{anal} is the mass injected on the analytical column, t_{prep} is the gradient time for the preparative column, and t_{anal} is the gradient time for the analytical column.

To maintain resolution, the change in mobile-phase conditions with respect to column volumes must be constant throughout the scale-up process. Maintaining consistent retention times requires an initial hold to mimic the system delay, expressed as number of column volumes, observed on the analytical-scale separation.

Using the formulas above, we performed scale-up experiments for the purification of the basic compound bupirone (Figure 1). Its utility as a pharmaceutical and the significant number of impurities makes bupirone a relevant and challenging purification. Table I shows the columns we examined and their flow rates and total masses loaded. For this study, we pulled columns at random from production lots, and the data indicated satisfactory lot-to-lot consistency.

Figure 7 shows detailed views of the overloaded experimental results. As the figure shows, the material loaded onto the column contained several impurities. It is possible to separate the main peak from the impurities at both the analytical and the preparative levels using the hybrid packing and to achieve a successful separation.

Figure 8 depicts the acidic compounds examined in this study. We selected oxacillin, cloxacillin, and dicloxacillin for the model mixture because they are pharmaceuticals with similar chemical structures and, therefore, they would present a challenging application.

Exploiting the concept that it is possible to load a larger amount of ionizable compounds when they are in their neutral state, we performed scale-up experiments for acids at low pH levels. Table II lists the col-

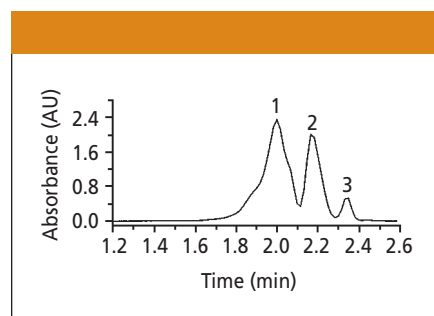


Figure 5: Scale-down to an XTerra Prep MS C18 10 mm \times 19 mm cartridge. Buffer A: deionized water; B: acetonitrile; C: 100 mM ammonium bicarbonate (pH 10). Gradient: 85:5:10 to 0:90:10 A–B–C in 10 column volumes; flow rate: 30 mL/min; detection: UV absorbance at 254 nm; injection volume: 0.2 mL; total load: 82 mg. Peaks: 1 = diphenhydramine (200 mg/mL in dimethyl sulfoxide), 2 = oxybutynin (200 mg/mL in dimethyl sulfoxide), 3 = terfenadine (12 mg/mL in dimethyl sulfoxide).

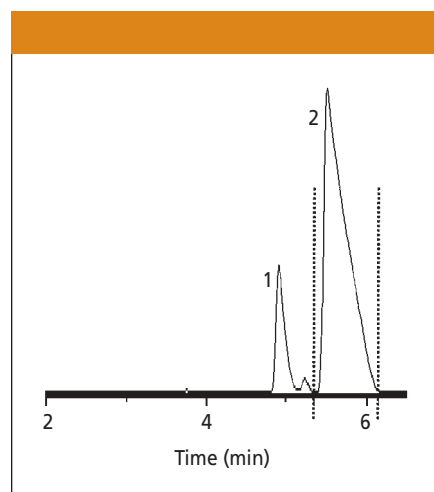


Figure 6: Complete recovery of pure material in one fraction. Column: 50 mm \times 4.6 mm XTerra MS C18 column. Buffer A: 60:40 deionized water–100 mM ammonium bicarbonate (pH 10); B: 90:5:5 acetonitrile–deionized water–100 mM ammonium bicarbonate (pH 10). Gradient: 60:40 to 10:90 A–B in 10 column volumes; flow rate: 1.8 mL/min; detection: UV absorbance at 254 nm; injection volume: 1 mL. Peaks: 1 = diphenhydramine (0.05 mg/mL in water), 2 = oxybutynin (10 mg/mL in water).

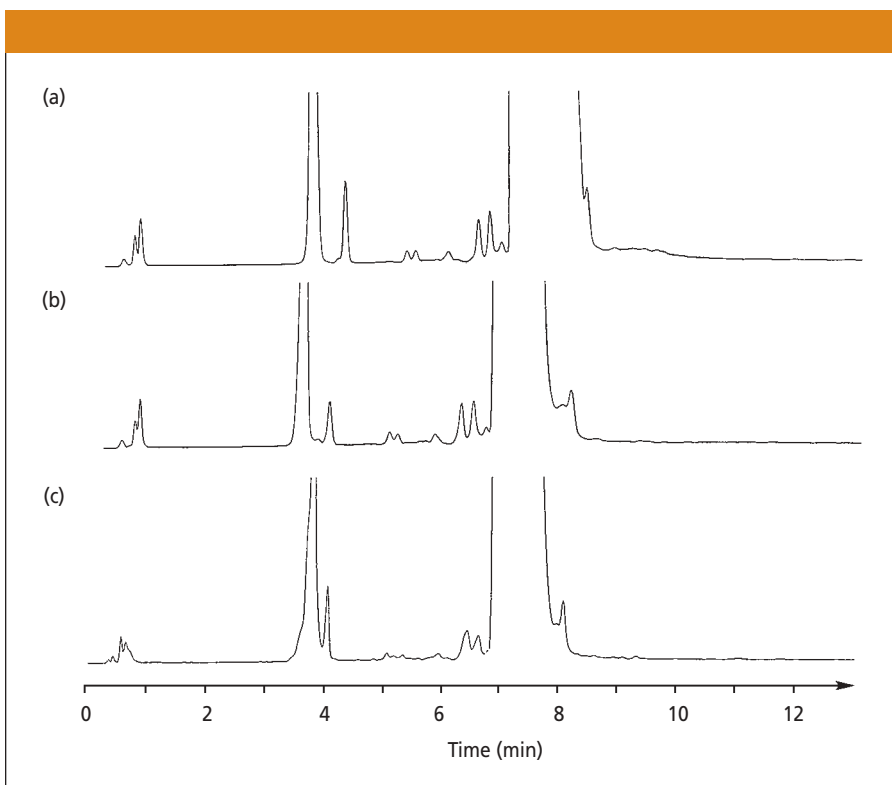


Figure 7: Impurity profile scalability study with buspirone. Column (a): 50 mm × 4.6 mm XTerra MS C18; mass load: 5 mg; flow rate: 1.8 mL/min. Column (b): 50 mm × 7.8 mm XTerra MS C18; mass load: 14 mg; flow rate: 5 mL/min. Column (c): 50 mm × 19 mm XTerra MS C18; mass load: 85 mg; flow rate: 30 mL/min. Buffer A: deionized water; B: acetonitrile; C: 10 mM ammonium bicarbonate (pH 10). Gradient: 80:10:10 to 30:60:10 A–B–C in 30 column volumes; detection: UV absorbance at 254 nm.

umn sizes, flow rates, and total masses loaded onto the columns. Figure 9 shows the chromatograms that correspond to the scale-up experiments. As the figure shows, we achieved baseline separations under overload conditions within 10 min, even with the 50 mm × 30 mm column on which the total load was 128 mg. The results show an increase in the production rate for a given compound from 0.2 mg/min (50 mm × 4.6 mm column) to 2.4 mg/min (50 mm × 30 mm column).

Although these findings are impressive, a decrease in pH from pH 3.8 to pH 3.0 permits an additional threefold increase in load while maintaining baseline resolution (Figure 10). The production rate is directly proportional to the volume injected into the column, and these findings indicate that the production rate in the 50 mm × 30 mm column could increase to 7.2 mg/min. In addition, the results illustrate the consistency in retention times, the resolution between different size columns, and the reproducibility among different lots of stationary-phase material and their packing procedures.

Conclusions

The results presented here show that hybrid packings provide fast separations without sacrificing resolution. The nature of the stationary-phase material enables chromatographers to use a wider range of pH values and to exceed the conventional limits of silica-based resins. In particular, our study indicates that it is possible to load at least 60 times more basic sample material onto the column at high pH levels compared with that at low pH levels and still obtain baseline separation.

At a high pH level, we loaded 118 mg/g of packing onto a column. These results show the dramatic improvement in performance when performing chromatography under conditions at which the compounds are in a neutral state. We were able to use an analytical-scale column to purify 10 mg of a main compound, still achieve plate-to-plate mapping, reduce the time required for the handling of samples, and minimize potential operator errors.

The two scale-up examples presented in this article illustrate the consistency in

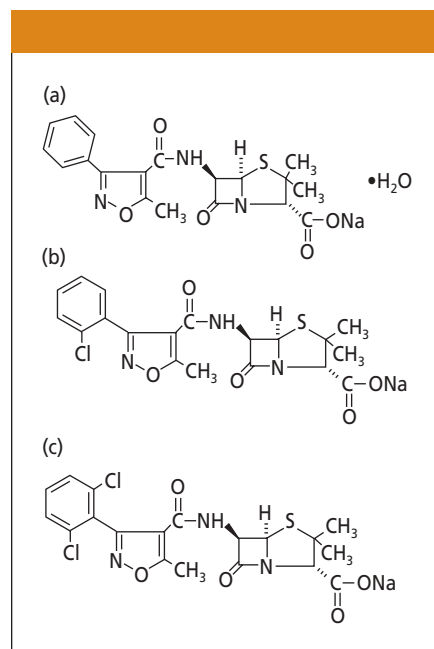


Figure 8: Acid test compounds (a) oxacillin, (b) cloxacillin, and (c) dicloxacillin.

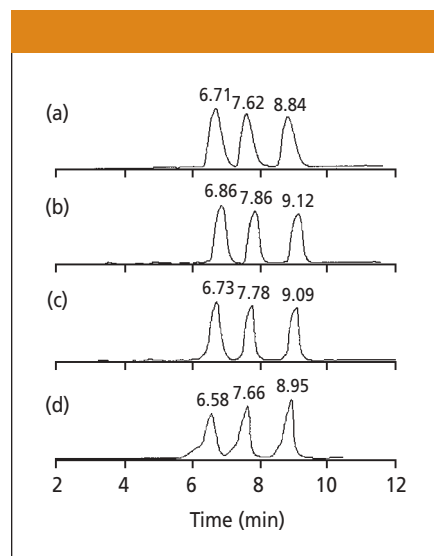


Figure 9: Scalability study of the XTerra Prep MS C18 packing material with acids at pH 3.8. Column (a): 50 mm × 4.6 mm; flow rate: 1.8 mL/min; load: 3 mg. Column (b) 50 mm × 7.8 mm; flow rate: 5.2 mL/min; load: 9 mg. Column (c): 50 mm × 19 mm; flow rate: 30 mL/min; load: 51 mg. Column (d): 50 mm × 30 mm; flow rate: 77 mL/min; load: 128 mg. Buffer A: 10 mM ammonium formate (pH 3.8) in water; B: 10 mM ammonium formate (pH 3.8) in 95:5 acetonitrile–water. Gradient: 90:10 to 40:60 A–B in 45 column volumes; detection: UV absorbance at 270 nm. Feed: oxacillin (20 mg/mL in water), cloxacillin (20 mg/mL in water), dicloxacillin (20 mg/mL in water).

retention times and resolution between different size columns and the reproducibility among lots of stationary-phase material and their packing procedures. Although loadings vary according to the nature of the compounds and the difficulty of the model mixtures, scale-up loadings ranged from 5 to 16 mg material/g packing.

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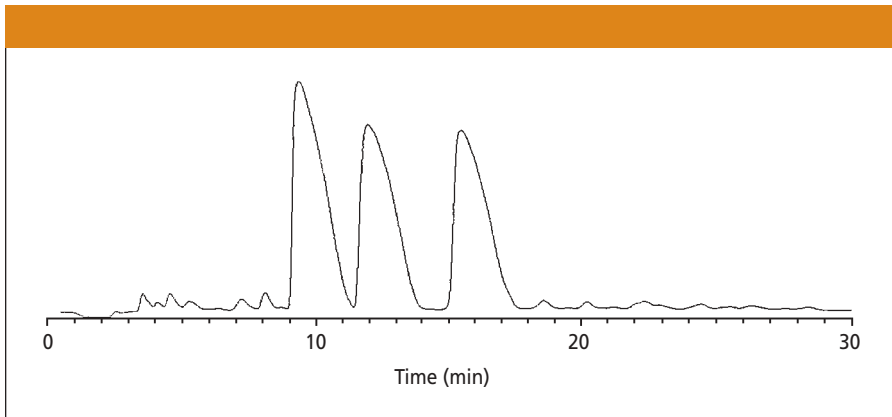


Figure 10: Scalability study of the XTerra Prep MS C18 packing material with acids at pH 3.0. Buffer A: 10 mM ammonium formate (pH 3.0) in water; B: 10 mM ammonium formate (pH 3.0) in 95:5 acetonitrile–water. Gradient: 90:10 to 40:60 A–B in 45 column volumes; detection: UV absorbance at 270 nm. Feed: oxacillin (20 mg/mL in water), cloxacillin (20 mg/mL in water), dicloxacillin (20 mg/mL in water).