Optimizing Sample Load Capacity and Separation Through a Series of Short Prep Columns

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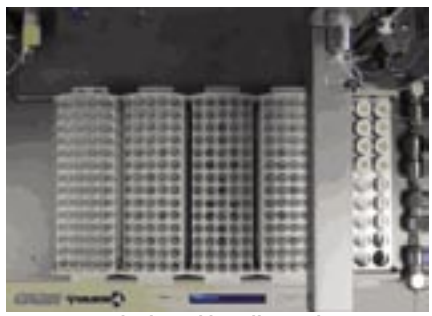
Preparative HPLC is still the dominate chromatography mode for separating compounds in large quantities. Purifying gram quantities requires using large preparative columns with 5-cm or larger internal diameters.

Once the diameter of an HPLC column approaches 5 cm, additional difficulties occur in operation. Frequently the bed settles after operating for only a short time, and the top of the column needs to be repacked. (The rate of settling is based on column diameter.) Sometimes bed channeling leads to peak broadening or splitting, negating use of the column altogether. When wall support decreases significantly as column diameter increases, a bed can become unstable. In analytical columns the wall is close to the center of the column, and "bridges" of packing particles can form. They enable longitudinal forces acting on the packing material to be dissipated to the walls of the column (**1**).

Additionally, the cost of the columns may be prohibitive to smaller companies, or purchasing multiple preparative columns to maintain consistency within lot batches may not be reasonable. Other problems to address include the practical lifetime of a column (which is uncertain) and reestablishing a method after column failure.

Changes in performance of preparative HPLC columns depend on the stability of the packed bed. One way to overcome these effects is to place smaller fast-prep columns (21.2 mm i.d. × 50 mm) in series. Serially connected, short prep columns can increase loading capacity and separations and shorten run times at semipreparative flow rates without sacrificing bed stability. Using a series of semiprep columns allows pressures to be maintained at lower levels. Bed stability improves when the pressure difference is reduced across the series of columns, preventing column channeling (**2**).

Implementing this technique of column stacking minimizes the replacement cost for a larger preparative column. Because the short semiprep columns are in series, a column can be replaced at a fraction of the cost of a large preparative column. Reestablishing the method after column failure requires little effort. Column conditioning is held to a minimum by the smaller size of the semiprep columns, and those maintained in use within the system facilitate system stability. Lot-to-lot variation is minimal, eliminating the need to reexamine fraction-collection parameters.



The liquid handler with four stacked columns
(WWW.GILSON.COM)

A chromatography application released by David Trail, Veronica Thomason, and John Urh examines column stacking in flash chromatography (**3**). Using this technique, they produced results that increased peak resolution. Our method uses the increase in resolution and plate number to demonstrate stacked column efficiency. Evaluating reversed-phase liquid chromatography stacked semiprep columns and prep columns with identical diameters and packing material demonstrates the stacked columns' comparable efficiency and chromatography.

Experimental

Equipment is detailed in the "Instruments and Materials" box. All solutions are prepared with filtered water. The buffer is produced with 6 g of sodium acetate in 4,000 mL water. Glacial acetic acid (2 mL) and triethylamine (2 mL) are added to the buffer. The standards (caffeine,

Product Focus: Proteins, antibodies, and other parenteral products

Process Focus: Analytical chromatography, QA/QC

Who Should Read: Process development and manufacturing, chromatographers, QA/QC

Keywords: HPLC, column stacking, reversed-phase liquid chromatography, semiprep and prep columns

Level: Intermediate

nicotinamide, 4-acetamidophenol) are prepared as follows.

Caffeine: 5 g of benzoic acid is placed in a 100-mL volumetric flask, 50 mL of filtered water is added, and 10 g of caffeine is added to the solution. The flask is filled with methanol to 100 mL The solution is sonicated until all solute is dissolved.

Nicotinamide: 20 g of nicotinamide is added to a volumetric flask, and water added to the 100-mL demarcation.

Acetamidophenol: 20 g of acetamidophenol is added to a 100-mL volumetric flask, and methanol added to the 100 mL demarcation. The solution is sonicated until the acetamidophenol is dissolved.

All sample dilutions are mixed in a 300-mL brown vial at a 1:1:1 ratio and covered. The GX-281 is used to inject the standard solution and collect the fractions. The component mixtures of 3 mL each are injected as a set of five into the system individually, placing 500 mg of sample on column. The UV detector is set at 254 nm. The flow rate is 15 mL/min. The gradient is 5% methanol for one minute, after which it is increased for 8.5 minutes to 95% methanol. The 95% column flush is one minute followed by a decrease in the gradient to 5% over 30 seconds. The mobile phase equilibrates for one minute at 5%. The first set of five standards is injected onto a single semiprep column. The second set is injected onto two columns, the third set onto three columns, and the fourth set onto four columns. The fifth set is injected into the 150-mm prep column. All instruments are controlled and data evaluated by Trilution software, LC version 1.4 (Gilson, Inc.).

Instruments and Materials

Instruments

Gilson Inc. (www.gilson.com): GX-281 liquid handler, UV–vis 155 detector, and 333- and 334-series pumps

Phenomenex (www.phenomenex.com): four Luna 5-µm C18 100Å 50 × 21.20 mm columns; one Luna 5 µm C18 100Å 150 × 21.20 mm column; 45 mm sections of 0.030" ID PEEK (polyetheretherketone) tubing joined each column in series

Materials

Burdick & Jackson (www.honeywell.com): methanol HPLC grade

Barnstead International (www.barnsteadthermolyne.com): filtered water

Fisher Scientific (www.thermofisher.com): sodium acetate

Mallinkrodt Baker(www.mallbaker.com): triethylamine

Sigma-Aldrich (www.sigmaaldrich.com): glacial acetic acid, 4-acetamidophenol, caffeine, and nicotinamide. Once you get to the website, just type in the chemical in the search engine entry box.

Figure 1: 21.2 i.d. × 50-mm stacked columns

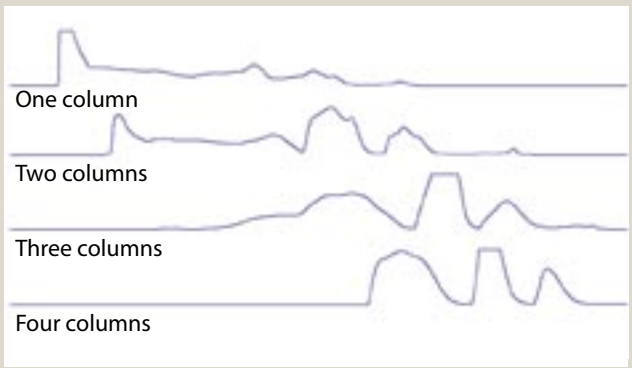
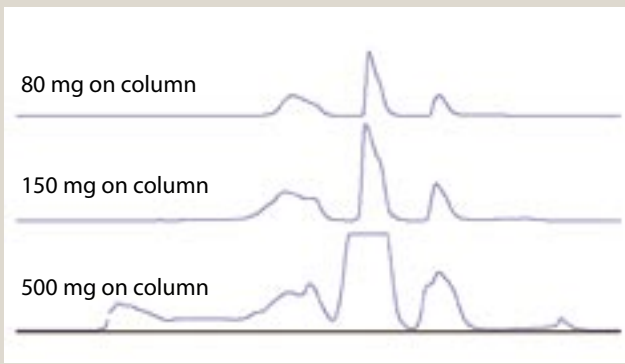


Figure 2: 21.2 mm i.d. × 150-mm column



Results

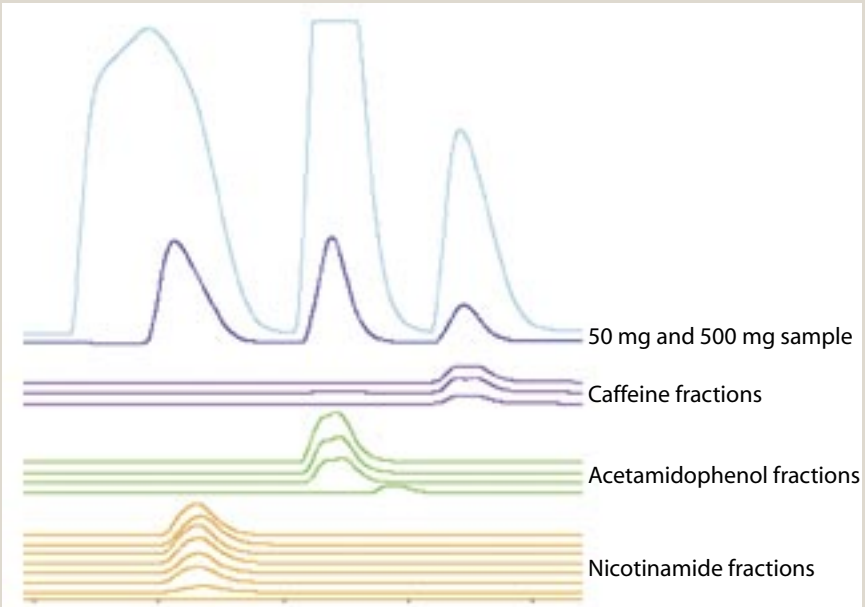
As columns are added to the semiprep series configuration, the resolution is increased for the 500-mg sample. The resolution starts at <0.5 for the single column and the two column configuration. It increases to a maximum of 0.95–1.2. The plate number increases with addition of each column. The plate number is not discernible for the single column because of peak splitting in the chromatogram. Caffeine was used as a standard measure for the other configurations. The plate number increased from 830 for two columns to 1,540 for three columns and to 3,250 for four columns.

Chromatography improved as each column was added to the series (Figure 1). Pressure increased with each column addition up to 1,200 psi observed on four columns. The pressure increase was linear to column-length increase. The 150-mm prep column was also injected with 500 mg of compound mixture, displaying poor chromatography for the first peak due to column overloading. Peak two and three were acceptable for prep injection chromatography. Decreasing sample load on column from 500 mg to 150 mg produced discernible peaks (Figure 2). Pressures observed on the 150-mm prep column were 30% higher than the similar length of stacked columns (1,560 psi). The lower pressure of the columns in series provides the ability to add more columns to the series and load more sample onto the column bed in the series of columns.

The mean coefficients of variation (CVs) for five replicates' retention time per peak are under 0.2%, with the exception of nicotinamide, which undergoes peak splitting caused by column overload. The area CVs are also under 0.2%. Acetamidophenol is not included because its absorbance extended past the UV detection limit (Table 1). Fractions collected for each peak in 3,000-mL increments calculated 106% to 149% for total percent recovery. All calculations

relate to the four column set injections. The fractions collected for each peak were devoid of any contaminants. Upon reinjection of each fraction, only single peaks were observed. The percent of recovery for each fraction closely matched that of the area of the peak for which the fraction was collected except for acetamidophenol, which surpassed the UV detection limits, accounting for the 149% recovery average.

Figure 3: Reinjected fractions



Eliminating the first stacked column and introducing another column at the end of the series provided no discernible variations in the retention time of the peaks or the peak width. The four columns were conditioned for 15 minutes, and the sample was loaded after one blank injection.

The three 50-mm columns in series and the 150-mm column were compared with analytical injections. On both columns, peak resolution, plate number, retention time, and peak shape were similar (Table 2). Plate number averages per injection were as follows:

- Caffeine: 30,586 for the 150-mm column and 30,127 for the three columns in series (a difference of 1.5%)
- Nicotinamide: 25,308 for the 150-mm column, and 26,004 for the three columns in series (a difference of 2.7%)
- Acetamidophenol: 45,325 for the 150-mm column, and 45,609 for the three columns in series (a difference of 0.6%).

All were very similar with only a small percentage of difference. No discernible drift in retention times combined with similar plate counts produce a comparable resolution between peaks for each system.

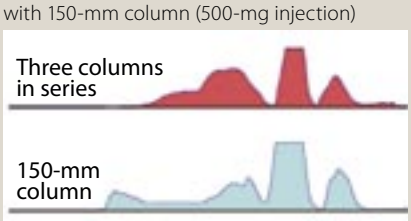
Discussion

The columns were overloaded with 500 mg to increase recovery amounts of analyte. Thus, 2.5 g of analyte was recovered per hour. A 0.95–1.2 resolution observed on the four stacked columns allows for an increase in column load over 500 mg. The poor chromatography exhibited by the 150-mm prep column demonstrates the inability of that column to accept sample quantities greater than 500 mg on column to provide adequate separation to recover uncontaminated analyte fractions. This would compare with the three 50-mm columns in series. Performance on both column configurations was similar. Peak resolution was poor, and peak splitting was observed for both configurations. The plate number of 3,250 (caffeine) for the four stacked columns surpasses the 1,540 observed on the three stacked columns and the 1,000 observed on the 150-mm column, demonstrating higher efficiency within the four-column system.

The compound solution was loaded by drawing it out of the vial and directly into the sample loop. This eliminated 1.5 minutes from the injection stage, providing shorter runs and decreasing the volume of solvent consumed during the run. 150 µL of PEEK tubing volume from the sample loop to the column prevented sample precipitation.

Analysis of the fraction reinjection in the column series exhibits little drift in retention time (Figure 3) and recovery percentages close to 100% (Table 3). Fractions were reinjected immediately after the initial run finished. The chromatography of the 150-mm prep column could facilitate fraction collection for sample load of 500 mg on column but was not attempted because of peak splitting. The range of time that the peaks spanned was nine minutes. This compares with a seven-minute range of retention time with the columns in series (Figure 4). With 3 mL collected per fraction and 15 mL per minute, the 150-mm column would collect 45 fractions, and the three columns in series would collect 35 fractions. There is a 22% difference between the 150-mm column and the three columns in series. This would congest work areas and extend drying time.

Figure 4: Three columns in series compared with 150-mm column (500-mg injection)



A 0.3-mL volume was injected into each system to provide verification of the three compounds in the sample solution and that peak spreading and splitting was exacerbated by column overloading. This compares to the 3-mL volume injected to provide

500 mg to overload the column.

Analytical injections of 100 µL/300 µg samples were conducted on each system, producing similar chromatograms. Comparing each system with analytical injections establishes that the systems are similar when not influenced by the combined stresses of high flow rates, pressures, and maximum sample capacity. These injections were completed after the columns were subjected to multiple prep injections, indicating good bed stability in both columns. The systems provide analogous chromatograms by similar plate numbers, resolution, and peak shape. With several hundred prep injections that overloaded each column configuration (columns in series and 150 mm column), no change in chromatography was observed. Both configurations performed similarly. Over more time, columns may fail due to stresses placed on them, but analytical injections confirm the stability of the of the column beds in this study.

Substituting a column in the stacked column system has little effect on the chromatography, allowing change of a defective column while using the remaining columns in the system to expedite system equilibration. The stress on the series of columns would be focused on the first column in the series. Sample impurities built up on the column, pressure spikes absorbed by the column (and so on) would all contribute to the stresses on the column, resulting in poor chromatography. Removing the first column of the series and adding a new one in the last position would quickly eliminate that. Because the three columns at the head of the series are previously equilibrated, system equilibration is rapid. The cost of replacing one column in a series would be less than replacing a large prep column (such as the 150-mm column, if subjected to the same stresses). Column equilibration would be prolonged, necessitating higher amounts of solvent and laboratory time.

Both column configurations' 500-mg injections exhibited similar chromatograms. The first peak was spread over a greater amount of time on the 150-mm column, but peak spreading and splitting were evident on both systems. Peaks two and three were both similarly shaped on both systems (Figure 4). The 500-mg prep injection into each system demonstrates how each reacts when maximum load stress is applied through injection. Each manages the sample load comparatively well with enough resolution to collect sample.

Table 1: Four columns in series

	Retention Time	Area (mVs)
Nicotinamide	6.9099	197,782,196
	6.8931	197,437,317
	6.8192	197,360,217
	6.791	198,228,694
	6.7744	197,602,742
mean	***6.8375***	***197,682,233***
st dev	***0.0609***	***345,907.9***
% CV	***0.8900***	***0.175***
Acetamidophenol	8.2525	(See note)
	8.2863	
	8.2739	
	8.2681	
	8.2758	
mean	***8.2713***	
st dev	***0.0124***	
% CV	***0.1500***	
Caffeine	9.4205	53,110,766
	9.4537	53,102,433
	9.4362	52,887,497
	9.4396	53,024,003
	9.4507	53,130,422
mean	***9.4401***	***53,051,024***
st dev	***0.0132***	***99,984.1***
% CV	***0.1398***	***0.188***

Note: No measurements here for area because the acetamidophenol peak height extends past the range of the detector

Table 2: Three columns compared with 150-mm column analytical injections

Plate Number	Caffeine	Nicotinamide	Acetamidophenol
Three columns in series	30,127	26,004	45,609
150-mm column	30,586	25,308	45,325
Retention time (min.)	**Caffeine**	**Nicotinamide**	**Acetamidophenol**
Three columns in series	5.08	6.23	6.67
	5.07	6.22	6.68
	5.05	6.22	6.67
	5.05	6.23	6.68
	5.07	6.22	6.68
150-mm column	5.08	6.23	6.68
	5.06	6.23	6.68
	5.09	6.23	6.67
	5.05	6.23	6.67
	5.05	6.22	6.68

Easy and Versatile

Configuring multiple columns in series provides an easily managed system in which to separate compounds. System versatility allows more columns to be added to increase sample loading capacity. Multiple packing materials can provide better resolution and ease of equilibration when a column needs replacement. Our evaluation demonstrates the robustness and efficiency of a stacked column system to deliver precise and accurate data for prep analyte recovery and separation of compounds.

References

1 Scott RPW. Preparative Chromatography. *Chrom-Ed. Series, On-line Reference Library*; www.chromatography-online.org.

2 Janson JC, Hedman P. Large-Scale Chromatography of Proteins. *Advances in Biochemical Engineering/Biotechnology* (Vol. 25). Fiechter A, Ed. Springer: Berlin, 1982; 43–99.

3 Trail D, Thomason V, Urh J. Column Stacking to Improve Resolution of Normal Phase Flash Chromatography. *Teledyne*

Table 3: Percent recoveries collected in four column series

Nicotinamide	Acetamidophenol	Caffeine
107%	149%	106%

Application Note AN12; www.isco.com/WebProductFiles/Applications/101/Application_Notes/AN12_RediSep_Normal_Phase_Column_Stacking.pdf.

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