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# ANALYTICAL PERFORMANCE OF PROCESS-SCALE LIQUID CHROMATOGRAPHY COLUMNS

T. J. GENTILUCCI*, S. I. SIVAKOFF and G. B. COX
*E. I. du Pont de Nemours & Co., Inc., Medical Products Department, Glasgow Site, Mailbox 122, Wilmington, DE 19898 (U.S.A.)*
and
S. D. STEARNS and M. W. HUTCHINSON
*Valco Instruments Co. Inc., P.O. Box 55603, Houston, TX 77255 (U.S.A.)*

## SUMMARY

The design of process columns with efficiencies equal to those of analytical columns is discussed, including advantages of the use of the same packing material in both. A new design in column hardware has been developed which allows process columns of 50 mm internal diameter to be packed with 10-$\mu$m, silica-based materials and to be routinely produced with reduced plate heights of 2.5 or less, yielding column efficiencies of > 40 000 plates/m in 25-cm-long columns. A description of the column design, focused on flow distribution and high-pressure safety considerations is presented. The scale-up from analytical to process chromatography is facilitated by the use of high-efficiency chromatographic materials.

## INTRODUCTION

Recent advances in biotechnology, specifically recombinant DNA technology, have given impetus to the production of highly desirable drugs on the gram and kilogram scale. As these products are mostly peptides and proteins, the problems of their purification are complex. This is especially true with regard to the stringent purity requirements imposed upon products of recombinant DNA. As a result, there is an increasing need for high-resolution separation techniques of peptide and protein purification. In many cases, the final purification of these compounds requires the high resolving power of liquid chromatography.

Recent advances in the theory of preparative liquid chromatography have obviously focused on analytical efficiency[1]. For the optimum production rate of a given component in a mixture, it has been demonstrated by Knox and Pyper[1] that the analytical efficiency of the process column should be three times the number of plates required for the preparative separation at the optimum sample load. Thus, for relatively easy separations the efficiency requirements of preparative columns are modest. When more complex separations with small selectivities between the components of interest are considered, the requirement for high plate counts becomes increasingly important. In order to provide this resolving power and to keep column

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dimensions within reasonable bounds[1], it is necessary to use particle sizes in the 5–10-$\mu$m range. Such materials are capable of producing high-performance, high-efficiency columns with at least 40 000 theoretical plates/meter.

Although the operating pressures required for large-scale columns, packed with 10-$\mu$m particles can be modest, it is often possible to increase the throughput of such columns by operating them at very high flow-rates, trading off their intrinsic high efficiency for speed[2]. In addition, the requirements for packing process columns efficiently dictate the redesign of the hardware to improve the pressure resistance in both packing and operational use of these columns. The maximum pressure tolerance of the currently available hardware is 2000 p.s.i.g. High-performance silica packings routinely require loading pressures in excess of 6000 p.s.i.g.

A cooperative arrangement was set up between E. I. du Pont de Nemours & Co. and Valco Instruments to develop column hardware which meets these requirements. This paper describes the results of that collaboration to develop process-scale columns.

## EXPERIMENTAL

### *Materials and methods*

*HPLC columns.* The analytical and semi-preparative liquid chromatography columns (25 cm × 4.6 mm, 9.4 mm, and 21.2 mm I.D.) were obtained from DuPont (Wilmington, DE, U.S.A.).

The 25 cm × 50 mm I.D. column hardware were manufactured by Valco Instruments (Houston, TX, U.S.A.). These tubes were packed by a proprietary high-pressure slurry technique. A 25 cm × 50 mm I.D. Modcolumn™ was obtained from H. T. Chemicals (St. Louis, MO, U.S.A.). All tubes were packed with two reversed-phase materials, Zorbax® PRO-10 $C_8$, and Zorbax® PRO-10/300 Protein Plus ($C_3$) packings. The particle size of both packing materials is 10 $\mu$m. These materials were obtained from DuPont. The same lot of packing material was used for all columns of the same type.

Size-exclusion chromatography was performed on Zorbax BioSeries GF-250 columns from DuPont. The columns used were GF-250 (25 cm × 9.4 mm) and GF-250 XXL (25 cm × 50 mm). The GF-250 columns were packed with 4.5-$\mu$m, 150-Å PSM-150 Diol material.

*HPLC procedure.* The reversed-phase $C_8$ columns were evaluated with a standard test sample of uracil, phenol, 4-chloronitrobenzene, and toluene. The chromatography was performed isocratically with a mobile phase of 80% aq. methanol. Theoretical plates and skew were calculated from the last peak (toluene). The flow-rate of the 4.6-mm column and the 50-mm column were 1.0 ml/min and 90 ml/min, respectively.

The reversed-phase Protein PLUS columns were evaluated for toluene only, with 100% methanol as the mobile phase. The flow-rates of the 4.6-mm column and 50-mm columns were 1.2 ml/min and 110 ml/min, respectively.

Size-exclusion chromatography was performed on the GF-250 columns with 0.2 *M* sodium phosphate buffer (pH 7.0) as the mobile phase in all cases. The flow-rates were 1 ml/min for the 9.4-mm-I.D. column, 4 ml/min for the 21.2-mm-I.D. column, and 18 ml/min for the 50-mm-I.D. column. The injection volumes were varied from 10 $\mu$l for the analytical column to 1.5 ml for the process column. The protein test mixture

consisted of thyroglobulin, immunoglobulin G (IgG), bovine serum albumin (BSA), ovalbumin, and ovalbumin, and myoglobin, and it contained sodium azide. The calculated column efficiency was based on the next-to-last peak, myoglobin.

*Instrumentation*

Chromatography at flow-rates in excess of 40 ml/min was performed on a Varex PSLC-100 (Rockville, MD, U.S.A.) instrument.

Size-exclusion chromatography on the 50-mm-I.D. columns was performed on a DuPont 8800 or SM 80 liquid chromatograph, equipped with a 4X prep-head assembly, capable of flow-rates up to 40 ml/min. The system included a DuPont variable-wavelength detector and a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.).

Chromatography at flow-rates up to 10 ml/min was performed on a DuPont 8800 liquid chromatograph, equipped with a Rheodyne 7125 injector, a DuPont variable-wavelength detector, and a DuPont 2-pen recorder. A second system, consisting of a LKB (Gaithersburg, MD, U.S.A.) GTI 2150 pump, 2158 LKB detector, Rheodyne 7125 injector, and a LKB 2210 2-pen recorder was also used.

All detector analog data were digitized and archived by a Nelson Analytical (Cupertino, CA, U.S.A.) data system, using software modified in house to generate theoretical plate and skew measurement.

*Reagents*

Reference proteins were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were reagent-grade or HPLC-grade materials, purchased from J. T. Baker (Phillipsburg, PA, U.S.A.). The reference samples and reagents were used as received, unless otherwise stated.

## RESULTS AND DISCUSSION

The decision to make a high-pressure (8000 p.s.i.g. ASME [American Society of Mechanical Engineers] designed) column, which was easy to seal and could mimic the characteristics of an analytical column, was pursued after a thorough review of the available process-scale hardware. The products offered were limited in pressure resistance to 2000 p.s.i.g. These columns also exhibited some or all of the following: recessed frits, double frits, metal or Teflon™ O-rings, poor surface finish, and welded-on flanges. Columns of these types were tested and found to be difficult to use. The Teflon O-rings were easily pinched, and recessed frits made cleaning the end after column packing extremely difficult. A summary of available column hardware is shown in Table I[3].

The safe use of these columns at high pressures for loading and operation in laboratory or plant environments was of the utmost importance. Emphasis on this design was placed in several areas. The sealing mechanism and the wall thickness dictared the pressure ratings. The inlet design, frit thickness, frit placement, and sample distribution were carefully evaluated to optimize column performance. These aspects will be examined in further detail below.

The basic concept of the new column design originated from the oil and gas industry[4]. The Grayloc™ hub is a high-pressure fitting, designed to make joints

TABLE I

COLUMN HARDWARE COMPARISON

| | *DuPont analytical column* | *H.T. Chemicals* | *Amicon* | *DuPont Valco* |
|---|---|---|---|---|
| Inlet distribution | Cone | Double frit | Dispersion plate | 1° Cone |
| Frit thickness | 0.032 | 0.120 | – | 0.062 |
| Frit position | Tube end | Recessed | – | Tube end |
| Pressure rating | 8000 | 2000 | 3000 | 4000–8000 |
| ASME design | N/A | No | Yes | Yes |
| Sealing mechanism | Ferrule | Teflon O-ring | – | Metal taper |

between pipes leakproof at pressures in excess of 8000 p.s.i.g. It consists of two shaped collars which are pressed together by a formed clamp. The joint is rendered leakproof by a metal taper seal, machined on the tube of the hub body (see Fig. 1). A backup seal ring of PEEK (polyetheretherketone) is provided to ensure proper sealing.

The first trial involved the use of an externally threaded tube onto which the hub was threaded and capped with a blind hub, containing the frit and end-fitting of the column. This design was tested and found to have adequate sealing capabilities but lacked the precision required for holding the packed bed in place. The high pressures used in loading caused stresses in the threads, which allowed the end hub (cap) to pull away from the frit. Small amounts of packing then escaped from the tube and became lodged between the tube end and the frit and, subsequently, caused column voids and failure.

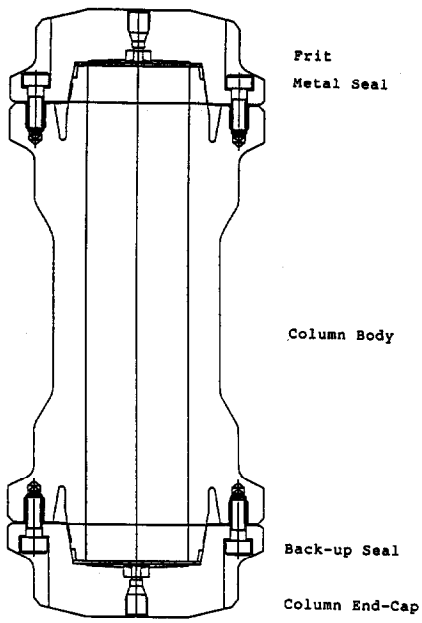


Fig. 1. Drawing of 25 cm × 50 mm I.D. column.

The final design of the ASME high-pressure column consists of a column end-cap, nozzle assembly, flow-distributor plate, 2-$\mu$m frit, sealing ring, and tube. The end cap is constructed of 316 stainless steel and designed to withstand pressures of 8000 p.s.i.g. per ASME design codes. The inlet to the end cap is designed to be used with 0.25-in. O.D. tubing with a male nut and ferrule assembly during the high-pressure packing of the column. For chromatography, the 0.25-in. inlet is then converted to 0.125-in. O.D. tubing in order to keep band spreading to a minimum with an internal reducing distribution nozzle assembly.

The distribution nozzle was designed to spread the eluent and sample uniformly to the 2-in. I.D. column. Extensive experimentation on early prototypes showed that jets of mobile phase passing through the frit deformed the packing bed at the top of the column and degraded the column performance. The nozzle has an I.D. of 0.07 in. and directs the flow 65° from the inlet flow through six evenly spaced ports of 0.03 in. I.D. This design effectively prevents the mobile phase from forming jets through the frit and disturbing the packing.

The flow distributor is sandwiched between the column end cap and the frit and is used to distribute the eluent and sample uniformly to the head of the column. The flow distributor was designed to support the frit and keep it flat with respect to the column end. A 1° cone is formed in the distributor to minimize the dead-volume and to mimic the distribution cone commonly used in the Valco 1-in. column end-fittings. Column efficiency, measured as a function of the sample load injected into the column is shown in Fig. 2A and B. These data indicate that the entire cross-section of the 50-mm-I.D. column is being utilized.

The frit used in this column is 0.062 in. thick and sits on the end of the column tube. The frit is compressed between the tube end and the flow-distributor end-cap. The sealing force is sufficient to mark the frit with the impression of the tube end, as is commonly seen in analytical columns. The frit is sufficiently thin to minimize band spreading. The ratio of the thickness to diameter is 0.031 compared to 0.177 for a 4.6-mm analytical column.

The wall thickness of the column tube was selected per ASME code to sustain a pressure of 8000 p.s.i.g. The column seal is made on the metal taper of the end of the tube. This paper seal behaves like the tubing ferrule used in the analytical columns. The column is held together with bolts and clamps to sustain the maximum pressure of 8000 p.s.i.g. The column weight can be reduced by 42 lb by removing the clamps. It can then be used with the bolts only at pressures up to 4000 p.s.i.g., which is more than most current instrument pumps are capable of delivering. The inside wall of the column is polished to a 15 $\mu$m RMS (root mean square) finish.

The final inlet design was reached through extensive experimentation. The original design had a straight in-flow through a 1/8-in. tube. The eluent entered the column at a cone-shaped space above the frit. Many columns were loaded and tested, but their performance rapidly declined.

A nozzle was designed with six equally spaced ports to distribute the force of the flow 65° from incoming eluent path. With this inlet design, consistently stable columns were produced.

The distribution of sample across the column was investigated by comparison of the 50-mm column tubes with narrower column tubes under conditions of sample overload. The performance of preparative columns has been extensively evaluated[2] in

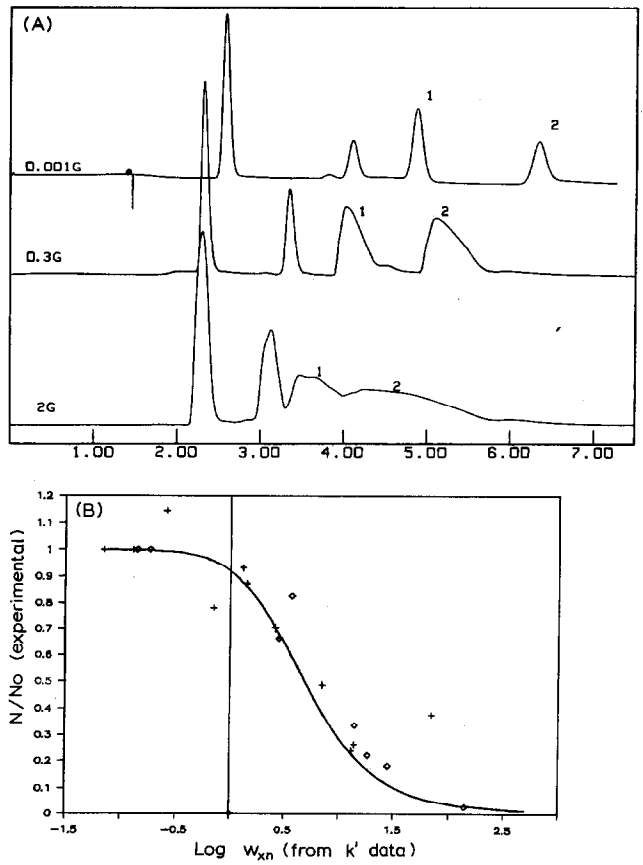


Fig. 2. (A) Comparison of sample loading for 2-phenylethanol (1) and 3-phenylpropanol (2) at sample loads of 0.001 g, 0.3 g and 2.0 g on a PRO-10 $C_8$ 250 mm × 50 mm column. The eluent was 60% aq. ethanol at 145 ml/min. (B) Plot of predictive loading on column with experimental loading for the 50-mm Pro-10 $C_8$ column used for Fig. 4.

the course of the development of theories relating column performance with sample loads in the domain of non-linear chromatography. That work showed that it is possible to predict the efficiency and retention time of a component in a given column, provided the analytical retention and efficiency are known, as well as the saturation capacity of the column for the particular solute. The latter parameter, usually derived from chromatographic experiments at analytical and preparative loads, is the value of maximum uptake of solute by the column and corresponds to a monolayer of solute molecules on the surface of the packing. In addition, normalizing factors have been found, which allow plotting of all data from columns of quite different sizes on axes of the same scale. For the efficiency, for example, it was shown that the ratio of plate number for the overloaded peak to the analytical plate number was given by the equation

$$\frac{N}{N_0} = \frac{1}{1 + w_{xn}}$$

where

$$w_{xn} = N_0[k'_0/(1 + k'_0)]^2 wx/ws$$

and $wx$ is the sample load, $ws$ the saturation capacity of the column and, $N_0$ and $k'_0$ are the analytical efficiency and capacity factor for the peak, respectively. Plotting the plate number ratio against log $w_{xn}$ yields a graph which is normalized such that data from all columns fit the same curve. Hence, if the sample introduced into the preparative column is not well distributed across the cross-section of the column, the points from loading experiments will show a premature overloading effect with a consequent decrease in column efficiency. Alternatively, a good distribution of sample should show a normalized performance close to that of the smaller column sizes.

In order to investigate this aspect of performance, a mixture of two aromatic alcohols, 2-phenylethanol and 3-phenylpropanol, was used as a sample. Columns of 25 cm in length and of 4.6, 9.4, 21.2, and 50 mm in I.D. were used with a range of sample loads. Fig. 2 shows the chromatograms obtained with various loads on the 50-mm column. Data from these experiments were compared with extensive data derived from columns of different dimensions and with different samples. A plot of the ratio of preparative to analytical efficiencies against log $w_{xn}$ is shown in Fig. 2B. It is clear from this plot that the plate numbers for the components under overload conditions are slightly higher for the 50-mm column than for the narrower columns. This implies that the sample is, in fact, spread fully over the diameter of the column. The higher efficiency is probably due to a reduction in the interference by the column walls, the so-called wall effects, on the wider-diameter-column[5].

The analytical performance of the 50-mm-I.D. column was determined for several varieties of silica-based packings and was compared with that of conventional analytical columns, packed with the same batches of packing material. The 50-mm-I.D. columns displayed efficiencies similar to or higher than the equivalent analytical columns operated at the same linear velocity. Thus, it can be concluded that these

TABLE II

COLUMN PERFORMANCE

| *Column type* | *I.D. (mm)* | *Flow-rate (ml/min)* | *Plates* | *Skew* | *h* |
|---|---|---|---|---|---|
| PRO-10 $C_8$ | 4.6 | 1.0 | 10 496 | 1.05 | 2.4 |
| PRO-10 $C_8$ | 50.0 | 90.0 | 13 335 | 0.19 | 1.8 |
| PRO-10/300 $C_3$ | 4.6 | 1.0 | 10 712 | 1.03 | 2.4 |
| PRO-10/300 $C_3$ | 50.0 | 90.0 | 14 238 | 0.06 | 1.7 |
| GF-250 Diol | 9.4 | 1.0 | 17 628 | 0.58 | 2.8 |
| GF-250 Diol | 50.0 | 18.0 | 16 108 | 0.76 | 3.1 |

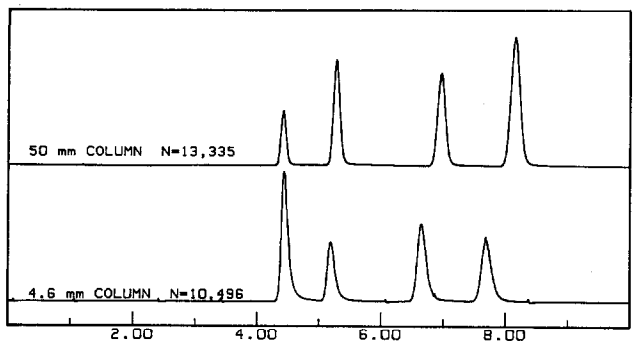


Fig. 3. Scale-up from 250 × 4.6 mm to 250 × 50 mm PRO-10 $C_8$ columns. Chromatographic conditions as previously described in Experimental.

columns are packed at least as well as, and in some cases, much better than the 4.6-mm-I.D. columns. This equivalence of performance enables simple scale-up of preparative chromatograms from exploratory experiments with small columns to the 50-mm-I.D. columns for large-scale purifications. A summary of the column performance, for columns of 25 cm in length, is shown in Table II.

Fig. 3 shows a direct scale-up separation of PRO-10 $C_8$, 4.6-mm-I.D. and 50-mm-I.D. columns utilizing a common, small-molecule test sample. The columns were eluted with a mobile phase of 80% aq. methanol at a rate of 0.7 ml/min and 90 ml/min, respectively. The 50-mm-I.D. column efficiency ($N$) for toluene was in excess of 50 000 plates/m.

The column geometry was tested on a second type of packing to show that the analytical performance of a 50-mm-I.D. column could be used for scaling up from an analytical column of similar performance. A gel column (GF-250), containing a 5-$\mu$m, 150-A° pore-size, Diol-bonded silica-based packing, was used to purify a protein mixture. Fig. 4 shows the direct scale-up of a protein separation from a 9.4-mm-I.D. column to a 50-mm-I.D. column. The proteins used for this separation were thyroglobulin, immunoglobulin, bovine serum albumin (BSA), ovalbumin, and

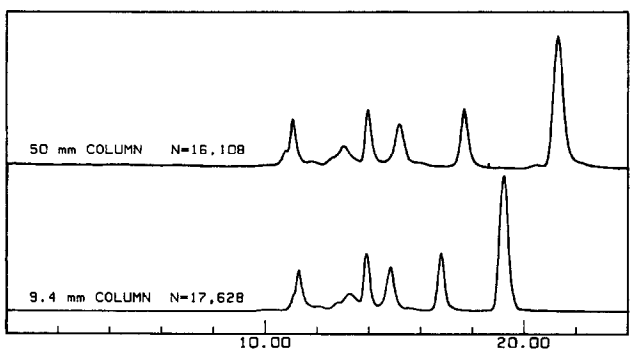


Fig. 4. Scale-up from 25 cm × 9.4 mm to 25 cm × 50 mm GF-250 columns. Chromatographic conditions as previously described in Experimental.

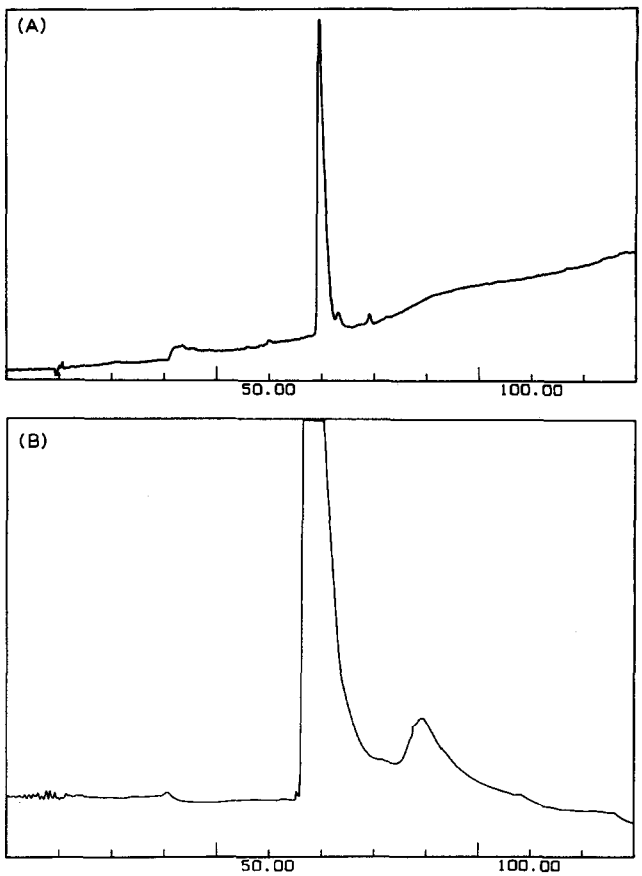


Fig. 5. (A) Analytical chromatogram for purification of bovine insulin (0.01 mg) on a 250 × 4.6 mm PRO-10 $C_8$ column. Eluent A, 0.1% aq. trifluoroacetic acid (TFA); eluent B, 0.1% TFA in acetonitrile; flow-rate 0.33 ml/min; linear gradient, 18% B to 80% B in 110 min. (B) Process chromatogram for purification of 5.4 g of bovine insulin on a 250 × 50 min PRO-10 $C_8$ column. Eluent A, 0.1% aq. TFA; eluent B, 0.1% TFA in acetonitrile; flow-rate, linear gradient, 18% B to 80% B in 110 min.

myoglobin. A small molecule, sodium azide, was used as a permeation volume marker. The packing lots were different for the two sizes of columns used in this experiment and account for the difference in relative retention times of the small molecule.

A bovine insulin scale-up under overload condition was performed on a 50-mm-I.D. PRO-10 $C_8$ column. The analytical chromatogram is shown in Fig. 5A and the preparative chromatogram is depicted in Fig. 5B. The bovine insulin sample was eluted with a 110-min linear gradient of acetonitrile, containing 0.1% trifluoroacetic acid (TFA). The scale-up was accomplished by simply increasing the flow-rate from 0.33 ml/min for the 4.6-mm-I.D. column to 37 ml/min for the 50-mm-I.D. column.

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