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Note

Preparation of efficient and stable reversed-phase microbore columns for high-performance liquid chromatography

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Much attention has recently been focussed on the applicability of microbore columns in high-performance liquid chromatography[1–6]. The theoretical considerations, the instrumental requirements and the economic aspects have been extensively elucidated in these papers. However, relatively little information has been given about the preparation of microbore columns[1,7,8].

In this paper we describe a detailed procedure for the preparation of highly efficient and stable reversed-phase microbore columns with internal diameters in the range of 1.2–1.5 mm. The influence of the packing pressure, the packing flow and the column length and the lifetime have been studied.

EXPERIMENTAL

Equipment

The set-up for the column packing is schematically shown in Fig. 1. Two types of pumps were tested to pump the slurry into the microbore columns: a syringe pump Model 8500 (Varian, Palo Alto, CA, U.S.A.) and a constant-pressure pump (Burdosa, Rödgen, F.R.G.).

The chromatographic set-up for column testing is shown in Fig. 2. It consisted of a constant-flow pump Model 303 (Gilson, Villiers Le Bel, France) equipped with a pump head for a flow range of 5–5000 μl/min, a Model 7413 injection valve with an internal loop of 0.5 μl (Rheodyne, Berkeley, CA, U.S.A.) and a Model 440 UV detector (Waters, Milford, MA, U.S.A.). The make-up flow was delivered, via a T-piece constructed from a zero-dead-volume Swagelok® connector, by a second constant-flow pump (Eldex, Menlo Park, U.S.A.).

The stability tests were performed with electrically controlled air-actuated injection valves which were adjusted to inject a sample every 60 sec.

Chemicals and materials

All solvents were of analytical grade (Baker, Phillipsburg, NJ, U.S.A.), used without pre-treatment except for filtration over a 0.2-μm filter (Millipore, Bedford, MA, U.S.A.). The alkyl-modified silica gels were: Zorbax BP ODS, 7–8 μm (Dupont, Wilmington, DE, U.S.A.) and CP-Spher C_{18}, 7–8 μm (Chrompack, Middelburg, The

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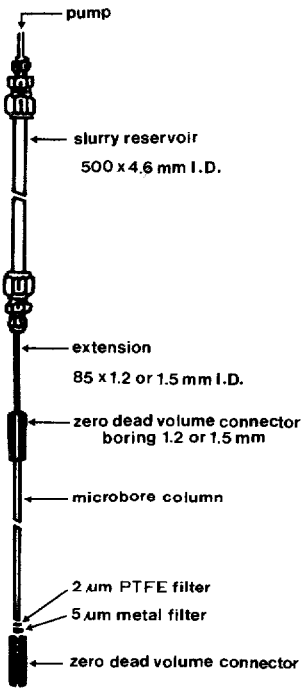

Fig. 1. Schematic representation of the slurry packing set-up.

Netherlands). The columns were made of stainless steel (1.2–1.5 mm I.D., 1/4 or 1/6 in. O.D.). The terminators were obtained from Swagelok® (1/16 in. zero-dead-volume union) or from Valco® (1/4 in. zero-dead-volume union). The 1/4 in. O.D. tubes were obtained from Chrompack.

Procedures

Polishing of the inner walls. Preliminary experiments showed that the smoothness of the inner walls of the columns was of paramount importance for producing efficient columns. Because the smoothness seemed to vary from tube to tube, all columns were extensively polished before packing. A tightly fitting cotton thread coated with a polishing agent (Perspex Polish No. 1; ICI, U.K.) was pulled back and forth through the column for about 15 min. The columns were then rinsed with water and ethanol and the remainder of the polishing agent was removed by pulling a

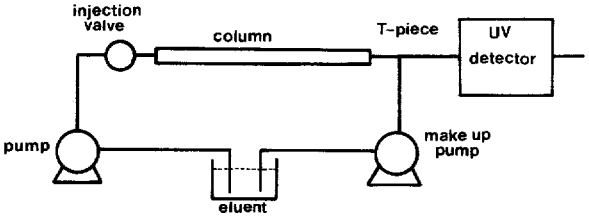

Fig. 2. Schematic representation of the chromatographic set-up for microbore column testing.

cotton thread, soaked with ethanol, back and forth through the column. This last procedure was repeated with new cotton thread, soaked with ethanol, until no dirt was visible on the cotton thread. The polishing procedure was judged complete if upon visual examination the inner surface of the column appeared to be shiny.

Column packing. Packing material (300–400 mg) was wetted with 0.5 ml of methanol and then suspended in 6.5 ml of tetrachloromethane. All solvents must be filtered over a 0.2-μm Millipore filter in order to remove any dust particules. Also the packing material has to be examined for the possible presence of coarser (dust) particles. The slurry was placed in the slurry reservoir to which the microbore column was connected (see fig. 1). The end-terminator contains a thin stainless-steel frit (pore size 5 μm) used to support a 2-μm glass fibre filter (SM 13400, Sartorius, Göttingen, F.R.G.) to hold the packing. The slurry was pressed into the column at constant flow-rate and a maximum pressure of 600 bar, using methanol as displacer liquid. After pumping through about 100 column volumes, the pump was switched off. After waiting about 30 min for release of the pressure, the microbore was disconnected and the excess of packing material on the top of the column was removed with a microspatula. Then the inlet terminator, equipped with a 2-μm glass fibre filter, was mounted and the column was equilibrated with the mobile phase.

Column evaluation. The efficiency of the microbore columns was calculated from the peak widths of some test solutes (uracil, $k' = 0$; toluene, $k' = 2$) at 0.607 of the peak height, using methanol–water (8:2, v/v) as mobile phase. The asymmetry factors were calculated from the peak widths at the front and back of the peak at 0.1 of the peak height.

The column stability was tested at an inlet pressure of 50 bar, a sample of 20 μl being injected every 60 sec.

RESULTS AND DISCUSSION

Extra column peak broadening

Because we did not have a low-volume detector cell at our disposal, compatible with microbore columns, we decided to apply a make-up flow at the end of the column in order to minimize peak broadening at the detection stage. The eluate is continuously mixed with an extra amount of mobile phase (see fig. 2) by which a peak is transported through the detector in a much shorter time. The peak broadening caused by the detector is thus diminished. Unfortunately the use of a make-up flow destroys the most important benefit of microbore columns, the higher mass sensitivity, since the peak is diluted again by the make-up flow. However, for the present investigation, it offered the possibility to obtain undisturbed results pertaining to the column phenomena.

The total time variance of a chromatographic set-up, $\sigma^2_{t(tot)}$, is mainly determined by the time variances of three independent broadening processes

$$\sigma^2_{v(tot)} = \sigma^2_{t(col)} + \sigma^2_{t(inj)} + \sigma^2_{t(det)} \quad (1)$$

where $\sigma^2_{t(col)}$ = the variance caused by the column, $\sigma^2_{t(inj)}$ = the variance caused by the injection system and $\sigma^2_{t(det)}$ = the variance caused by the detection system. The total volume variance, $\sigma^2_{v(tot)}$, is related to the time variance, $\sigma^2_{t(tot)}$, according to

Influence of the packing flow

The influence of the packing flow on the column performance was investigated with two commercially available reversed-phase packings, by measuring the theoretical plate height at a given linear velocity. The results of these experiments (the means from three columns) are shown in Fig. 4. The optimum flow-rate at the minimum corresponds to 1.2 ml/min. The occurrence of an optimum packing flow-rate might be attributed to an unacceptably loose packing at low packing flow-rates and to cracking of the particles at large packing flow-rates. We found no difference in column performance when using the syringe or membrane pumps, provided the flow of the latter was kept constant during the packing procedure.

The slightly better performance obtained with CP-Spher, in a freshly packed column, was also found in subsequent experiments.

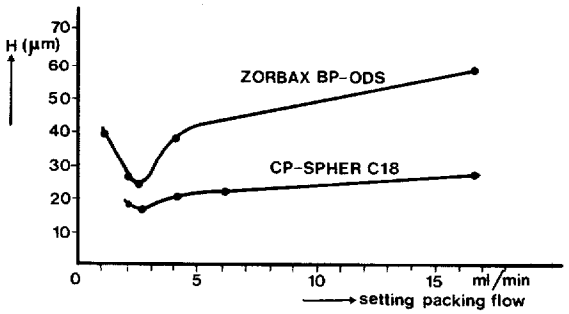

Fig. 4. Influence of the packing flow on the column efficiency, H. Column dimensions: 250 × 1.2 mm. Mobile phase: methanol–water (8:2, v/v). Solute: toluene, $k' = 2$. Linear velocity: $v = 1.2$ mm/sec.

Influence of column length

In order to determine the maximum column length which still can be packed efficiently by the described procedure, columns with different lengths were packed. Columns up to 250 mm long were packed at the optimum flow-rate of 1.2 ml/min and a maximum inlet pressure of 600 bar. However, longer columns were packed at a significantly higher inlet pressure (up to 950 bar). Fig 5 shows the results of these measurements. The described packing procedure allows the preparation of efficient columns up to 250–350 mm long. With longer columns the efficiency is considerably lower.

Column life time stability

The stability of freshly packed microbore columns was investigated with both reversed-phase packings. The efficiency of the columns was measured before and after a few thousand injections (see Figs. 6, 7). Very efficient microbore columns can be obtained with both supports. The reduced plate height, h, at the minimum of the H/v curve was found to be about 1.8. The asymmetry factors, defined as the ratio of the peak width at the front and back of the peak at 0.1 of the peak height, ranged from 1.00 to 1.05. On both types of supports the efficiency drops by about 40% after 8000 injections, resulting in a reduced plate height of about 2.5, still considered as

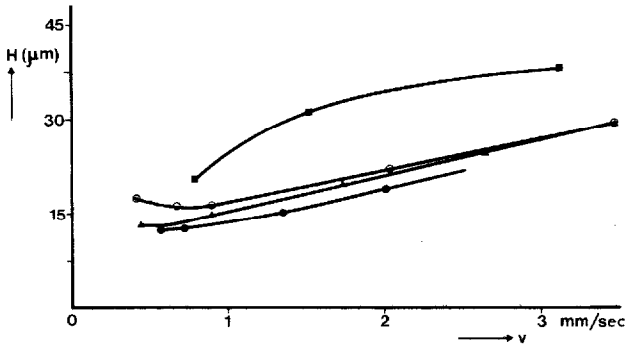

Fig. 5. Influence of the column length on the column efficiency, H. Column diameter: 1.2 mm. Stationary phase: CP-Spher C_{18}. Mobile phase and solute as in Fig. 4. Column length: 100 (●), 200 (▲), 250 (○) and 550 mm (■).

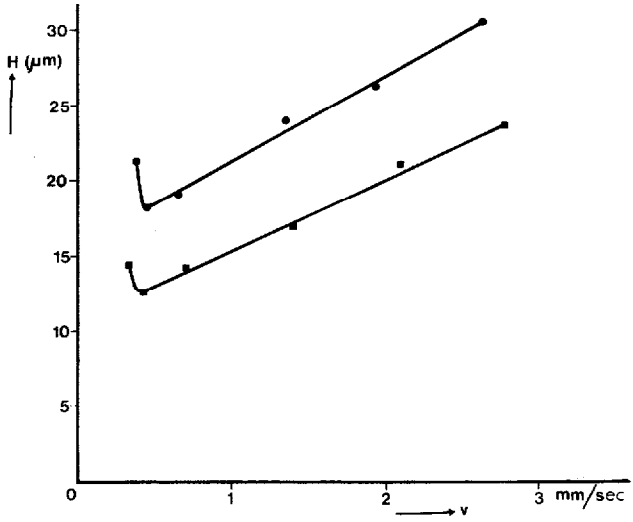

Fig. 6. Theoretical plate height *versus* linear velocity, v, before (■) and after stability test (●, 8000 injections). Column dimensions: 250 × 1.5 mm. Stationary phase CP-Spher C_{18}. Mobile phase and solute as in Fig. 4.

efficient. Also the asymmetry factors increase somewhat ($\approx$1.10) after 8000 injections.

The change in efficiency with the number of injections was followed in more detail at two linear velocities (Figs. 8 and 9). The efficiency of the CP-Spher C_{18} drops rather steeply during the first 1000 injections and then decreases more gradually. A similar behaviour was found with the 1.2 mm I.D. Zorbax BP ODS columns. However, the 1.5 mm I.D. Zorbax BP ODS columns showed a completely different behaviour. The efficiency improved significantly during the first 4000 injections and then started to decrease after a larger number of injections. Up to now we have not been able to find an explanation for this advantageous behaviour. The results of the

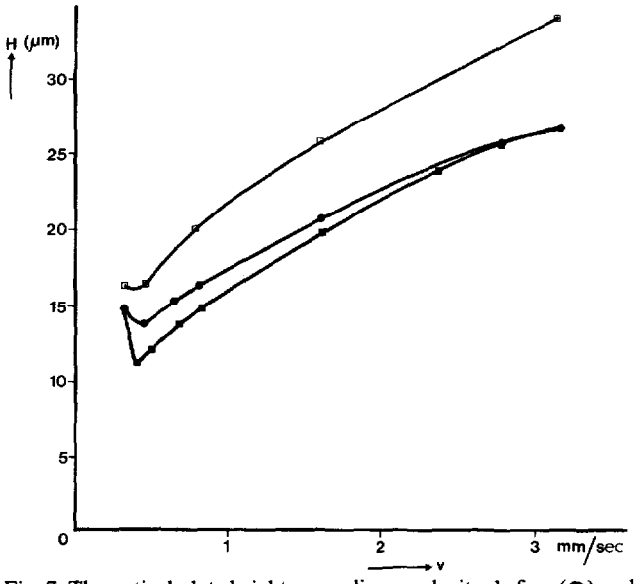

Fig. 7. Theoretical plate height *versus* linear velocity, before (●) and after stability test (■, 3000; □, 8000 injections). Stationary phase: Zorbax BP ODS. Other conditions as in Fig. 6.

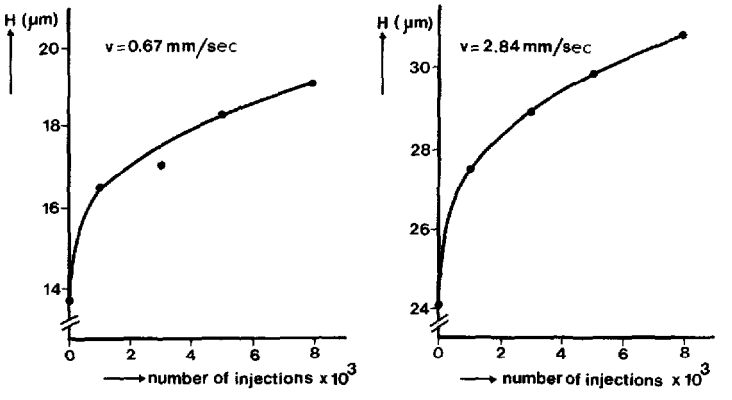

Fig. 8. Influence of the number of injections on the theoretical plate height at two linear velocities. Column dimensions: 250 × 1.5 mm. Stationary phase: CP-Spher C_{18}. Other conditions as in fig. 6.

lifetime tests show that, although loss in efficiency occurs, very efficient columns remain after quite a large number of injections. The excellent stability of microbore columns was also found when applying these columns to real samples such as catecholamines in serum[9]. More then 1000 serum samples were analyzed on such a microbore column without a significant drop in efficiency.

CONCLUSIONS

The preparation of reversed-phase microbore columns appears to be no more complicated than of conventional columns, provided the inner walls of the column

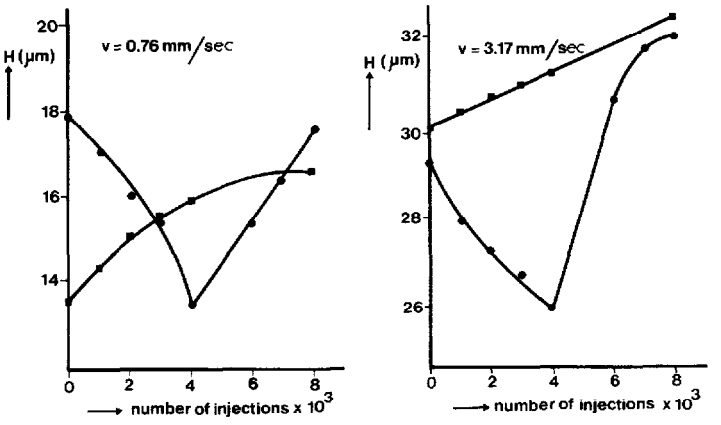

Fig. 9. Influence of the number of injections on the theoretical plate height at two linear velocities. Column dimensions: 250 × 1.5 mm (●) and 250 × 1.2 mm (■). Stationary phase: Zorbax BP ODS. Other conditions as in Fig. 6.

tubes are extensively polished. Further care should be taken to remove any dust particules from the slurry liquids and supports.

There is an optimum packing flow when packing the column at constant flow. For Zorbax BP ODS and CP-Spher C_{18} this is 1.2 ml/min. However, the optimum may be different when using other supports.

Under the aforementioned conditions, reduced plate heights of 2 can be realized with microbore columns. This is at least equal to or even better than those obtained with conventional columns. In our experience, microbore columns exhibit greater lifetimes than conventional columns.

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