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## Note

# Simple method for increasing the life-time of 3-μm particulate columns for reversed-phase liquid chromatography

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Microparticulate columns for high-performance liquid chromatography (HPLC) are becoming increasingly popular. Short columns (10 cm) packed with 3—5 μm ODS material are often superior with respect to performance to the conventional columns (25—30 cm) containing 10 μm particle size material. These short columns carry the advantage of rapid, efficient analysis and low solvent consumption [1—3]. One disadvantage is the high back pressure obtained with the columns containing 3—5 μm packing material [4].

The microparticulate columns for reversed-phase chromatography are often mechanically fragile, which may partly be explained by the high back pressure [5]. We observed peak broadening and double peaks when using 5-μm ODS columns from commercial sources and 3-μm ODS columns packed in our own laboratory. Attempts have been made to avoid this problem by reducing the pressure surge on the top of the column by modification of the valve injector [6].

The present report describes procedures employed in our laboratory to stabilize short (10 cm) 3-μm ODS columns for HPLC. This was obtained by mounting a short guard column packed with 40-μm pellicular material as an extension of the analytical column. The guard column was subjected to mechanical compression using a stainless-steel piston, followed by refilling.

## EXPERIMENTAL

### *Materials*

ODS Hypersil 3-μm packing material for HPLC, and empty stainless-steel columns (10 × 0.5 cm), guard columns (2.5 × 0.5 cm), column adaptor for valve

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injection and accessories (seals, mesh and distributor) were purchased from Shandon Southern Products, Cheshire, Great Britain. Packing material for guard columns, Pelliguard LC-18, 40 μm, was from Supelco, Houston, TX, U.S.A. Adenosine, S-adenosyl-L-homocysteine and 2′-deoxyadenosine were obtained from Sigma, St. Louis, MO, U.S.A.

*Instruments*

A Spectra-Physics SP 8700 solvent delivery system was connected to a Perkin-Elmer ISS 100 autosampler for HPLC. The effluent from the column was monitored at 254 nm using a fixed-wavelength detector from Beckman, Model 160. The time constant was set at 0.2 sec. The chromatographic profiles were recorded using a reporting integrator from Hewlett-Packard, Model HP 3390 A.

*Packing of the analytical column and guard column.*

The analytical column was slurry packed with 3-μm ODS Hypersil material at 60 MPa, using a Shandon column packer. The solvents were changed in the sequence isopropanol, methanol, and the procedure was as recommended by Shandon.

The pellicular material for the guard column was dry-poured into the pre-column.

*Preparation of samples*

Tissues (liver, kidney, brain, spleen and thymus from mice) were homogenized in 0.8 *N* perchloric acid (1:4, w/v), and the precipitated protein removed by centrifugation. The perchloric acid extract was either subjected directly to HPLC analysis, or the acid was neutralized to pH 7.0 by the addition of 1.44 *N* KOH—1.2 *N* $KHCO_3$ [7]. The insoluble potassium perchlorate was allowed to precipitate for 30 min at 0° C, and then removed by centrifugation.

*Column efficiency*

The number of theoretical plates of the column ($N$) was calculated according to the equation [8]

$$N = 5.54 \left( \frac{t'_R}{W_h} \right)^2$$

The test mixture used for assessment of column performance contained benzamide, biphenyl, acetophenone and benzophenone, and the mobile phase was 70% methanol in water. The flow-rate was 1 ml/min. The efficiency of the 3-μm ODS columns was 90,000—110,000 theoretical plates per meter.

## RESULTS

*Protection of the analytical column by guard column*

Samples (25 μl) of neutralized tissue extract were injected into a 3-μm ODS Hypersil column eluted with 15 m*M* acetate buffer, pH 4.5, containing 4.9% methanol. After 20—60 injections peak broadening and double peaks were

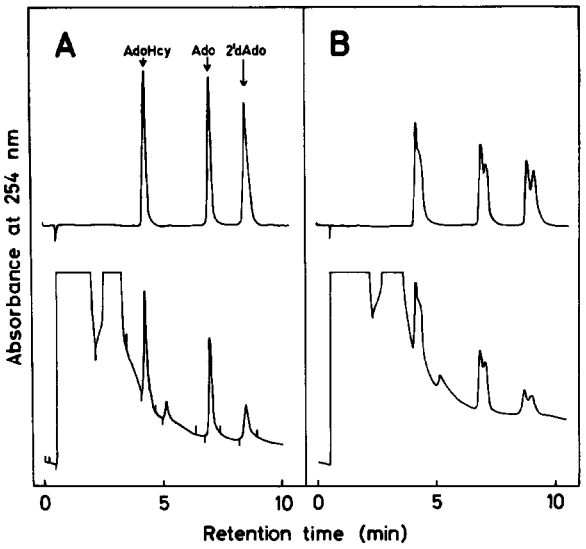


Fig. 1. Chromatograms of standards and liver extract before (A) and after (B) damage of the column. (A) Samples of 25 μl were analyzed on a 3-μm ODS Hypersil column (10 × 0.5 cm). The mobile phase was 15 m*M* acetate buffer, pH 4.5, containing 4.9% methanol, and the flow-rate was 2 ml/min (18.4 MPa). The upper panel shows the elution profile of standards, and the lower panel the chromatogram of neutralized extract from mouse liver. (B) The same samples were analyzed on the column damaged after 43 injections. AdoHcy = S-adenosylhomocysteine; Ado = adenosine; 2'dAdo = 2'-deoxyadenosine.

observed (Fig. 1). The performance of the column was often partly restored after elution of the column for 5—10 min in the opposite direction, but peak splitting soon (after 5—10 injections) reappeared.

The analytical column was equipped with a guard column (2 cm, Supelco), which was connected to the top of the analytical column by a 2-cm tube (0.254 mm I.D.). The performance of the system was markedly reduced (the number of theoretical plates decreased to about 60%). Only a slight protective effect on the analytical column was observed, i.e. after 40—70 injections peak broadening and splitting was observed. The performance was not restored by replacement of the guard column, showing that the damage was localized to the analytical column.

The guard column (2.5 cm, Shandon) was mounted on the top as an extension of the analytical column, and the two columns were separated by a thin (0.15-mm) mesh (2 μm). Broad peaks were observed after about 100 injections. After replacement (or compression, see below) of the guard column, the performance of the system was restored. The guard column extended the life-time of the analytical column to more than 1000 analyses. A defective guard column was not repaired by replacement of only the upper (0.5 cm) part of the packing material.

*Mechanical compression of the guard column*

Attempts were made to inject samples (25 μl) containing 0.8 *N* perchloric acid into ODS Hypersil columns equipped with guard column (end to end). The mobile phase contained 100 m*M* ammonium formate buffer, pH 3.5,

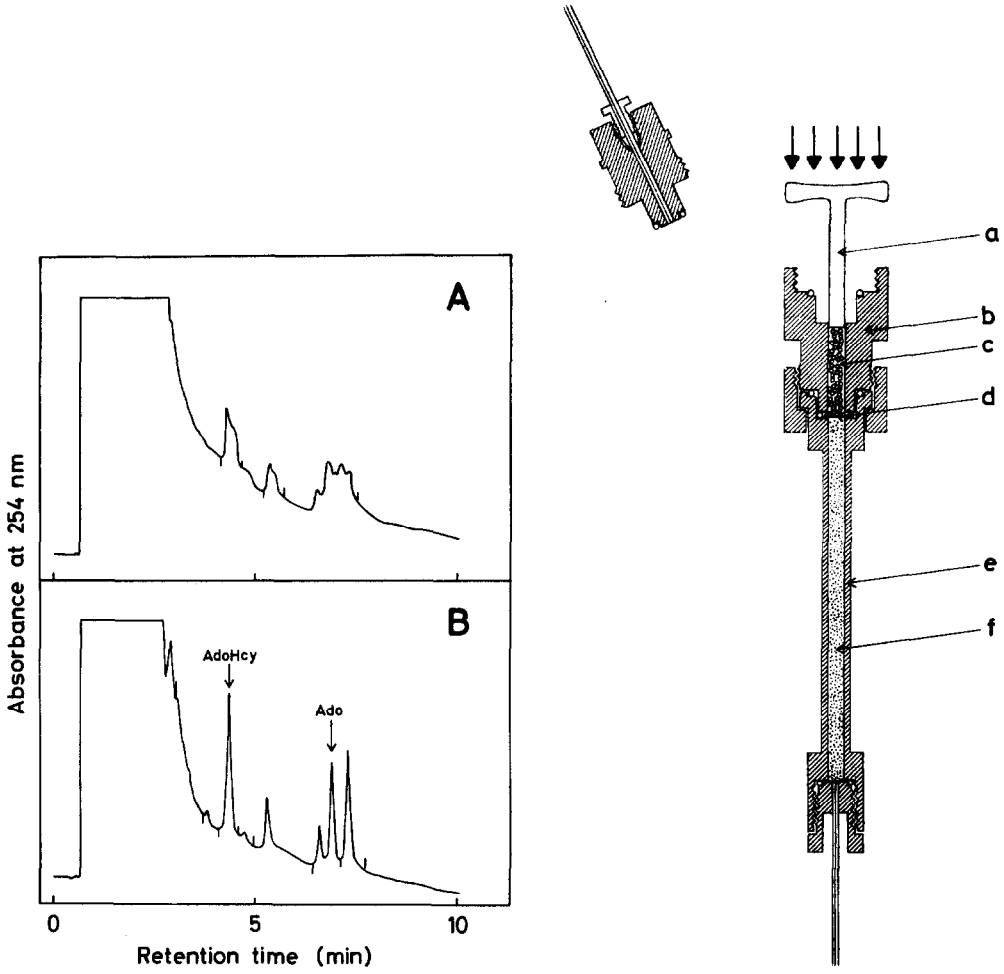


Fig. 2. Chromatograms of liver extract before (A) and after (B) compression of the guard column. (A) Liver extract (in 0.8 *N* perchloric acid) was analyzed on a 3-$\mu$m ODS Hypersil column equipped with a guard column forming an extension of the analytical column. The guard column was damaged prior to analysis by injecting ten samples. The mobile phase was 100 m*M* ammonium formate, pH 3.5, containing 1% acetonitrile, and the flow-rate was 2 ml/min. (B) Chromatogram of the same extract after compression of the guard column with a stainless-steel piston followed by refilling, as described in the text.

Fig. 3. Column design and compression of the guard column. a = Stainless-steel piston; b = guard column; c = 40-$\mu$m pellicular ODS material; d = mesh (0.15 mm), e = analytical column; f = 3-$\mu$m ODS material.

to prevent the detrimental effect of low pH on the bonded phase [9]. Peak splitting was observed after about ten injections (Fig. 2). The guard column was then compressed using a stainless-steel piston, fitting the internal diameter of the precolumn (Fig. 3), and the dead-volume created was filled with 40-$\mu$m pellicular material. The performance of the system was restored (Fig. 2). After a further 75 analyses (corresponding to 1.5 l of mobile phase) band broadening reappeared, but the compression was successfully repeated 5—10 times. The

life-time of the analytical column corresponded to 500—800 injections under these conditions.

*Comments on column design*

The procedure involving repetitive compressions and refilling of the guard column required opening of the guard column inlet. The use of a swaged connection at the column head was unsatisfactory because it was often damaged after only 10—20 dismantlings. The columns used in the present experiments (Fig. 3) were tightened by hand and could be opened repeatedly without damage. Eventually, only the O-ring needed to be replaced.

## DISCUSSION

A guard column which forms a direct extension of the analytical column greatly improves the mechanical stability of the 3-$\mu$m ODS column. This may be explained by the protection given to the inlet of the analytical column against the high-speed inlet jet of the mobile phase and/or the pressure surge on the top of the column during injection.

When the microparticulate ODS column equipped with a guard column was loaded with samples containing strong acid and eluted with a mobile phase of high ionic strength, the guard column rapidly deteriorated. This resulted in the appearance of peak broadening and peak splitting. The mechanical nature of this phenomenon is indicated by the fact that the guard column was repaired by compression and refilling (Fig. 2). The damage of the guard column may be explained by formation of microchannels leading to multiple flow paths.

The performance of the guard column was restored several times by compression, and the life-time of the column was limited by the chemical stability of the packing material.

Attempts were made to slurry pack the precolumn at 60 MPa. This procedure showed no advantage to dry packing. This may be related to the low flow resistance offered by the 40-$\mu$m material used for the guard column.

Repetitive compression of the guard column forms the basis for the HPLC method developed in our laboratory for measurement of purines (adenosine, 2'-deoxyadenosine and S-adenosylhomocysteine) in several tissues and cell types from mammals [7, 10, 11]. The biological material was extracted in perchloric acid. Neutralization of the extract is not required, which ensures simple sample processing, and no further dilution of the sample. Furthermore, neutralization of perchloric acid with potassium hydroxide leads to a continuous precipitation of insoluble potassium perchlorate at low temperature. The precipitate is trapped in the column inlet filter, leading to a progressive increase in back pressure.

The highly efficient 3-$\mu$m ODS columns connected to a UV detector with low noise ($2 \times 10^{-5}$ a.u.f.s.), rapid response (time constant of 0.2 sec) and 1 cm light path, makes possible the detection of less than 0.2 pmol of these purines. Furthermore, mechanical compression of the guard column allows the unattended analysis of 75 samples.

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