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Note

Re-packing reversed-phase high-performance liquid chromatography columns as means of regenerating column efficiency and prolonging packing life

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The longevity of high-performance liquid chromatography (HPLC) columns, and the cost of their replacement, has been a subject of criticism since the advent of HPLC. The problem has given rise to numerous technical notes and papers concerned with prolonging column life either by protecting the columns from extremes of pH, pressure and solvent impurities (see for example refs. 1–3) or by regenerating the active groups on the packing[3–6].

We have reported[7] that the deterioration of Zorbax 10 μm NH_2 packing, involving the release of amino groups from the packing, resulted in a high background when using a post-column fluorescence detection method. Consequently we were unable to quantify reliably the minor, cross-linking amino acids of collagen from small amounts of tissue by this method. More recently we developed a method for the simultaneous analysis of both amino acids and the reducible collagen cross-linking amino acids as their Dns products using a 25 × 0.46 cm Techsil 5 μm C_{18} column[8]. However, although care was taken to filter and adjust the pH of samples prior to injection and a suitable guard column was always used, we observed a surprisingly rapid decrease in column efficiency which eventually resulted in an unacceptable loss of resolution of some of the amino acids.

We report here a means of regenerating at least 95% of the best resolving capacity of these reversed-phase columns by carefully monitoring efficiency and repacking the bed.

EXPERIMENTAL

Materials

The chromatography was carried out using a DuPont 8800 programmer, 870 pump and 860 column compartment (DuPont U.K., Stevenage, U.K.). The reversed-phase test mixture was supplied by HPLC Technology (Macclesfield, U.K.) and contained benzamide, benzene, benzophenone and biphenyl. Column effluent was monitored with a DuPont UV spectrophotometer, set at 254 nm for the test mixture, or

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a Gilson Spectra-Glow fluorimeter for the Dns-amino acids (Gilson Medical Electronics, Middleton, WI, U.S.A.) and recorded on a Spectra-Physics SP 4100 computing integrator (Spectra-Physics, San Jose, CA, U.S.A.). An HPLC slurry packer (Shandon) with an ultra-sonic bath (Sonicor) was supplied by HPLC Technology and used to pack 25 × 0.46 cm columns with Techsil 5 μm C_{18} at either 300 or 500 bar.

The guard columns were always 5 × 0.2 cm dry-packed Whatman Co:Pell, 30–38 μm, C_{18} (Whatman, Clifton, NJ, U.S.A.).

Chromatography

An indication of the column status was obtained by running the reversed-phase test mixture isocratically at 1.5 ml/min in 55% (v/v) acetonitrile (Rathburn Chemicals, Walkerburn, U.K.) in 40 m*M* sodium phosphate pH 6.3 at 35°C. This was the same solvent as used for the Dns-amino acid chromatography[8]. As the peaks from the standard mixture were symmetrical we used the following commonly accepted approximation for calculating the column plate number *N*;

$$N = 16\left(\frac{t_R}{t_w}\right)^2$$

where t_R is the peak retention time and t_w is the baseline width formed by the peak tangents intersection the baseline[9]. The column was then used on a daily basis for separation of the Dns-amino acids from hydrolysates of purified proteins as well as whole tissues. The short running time of the test mixture allowed us to assess the column condition either during the column re-equilibration for normal use, after overnight storage, or during preparation for overnight storage.

Column regeneration

When the theoretical plate value fell to a point which corresponded to an unacceptable loss in resolution of the Dns-amino acids the packing was removed, a small amount of fresh material (0.2 g) added and the column re-packed. To do this the packing was suspended in propan-2-ol and de-gassed in the ultra-sonic bath for 5 min. Then it was poured into the packer "bomb" and the column packed vertically at 300 or 500 bar, generated by either 70 or 120 lb/sq. in. respectively of nitrogen gas from a cylinder. After 100 ml of propan-2-ol had passed through the column, it was inverted and a further 50 ml each of propan-2-ol, methanol and finally, unless the column was to be stored for a time, acetonitrile–water (50:50) passed through. The pressurizing gas was then turned off and the column allowed to stabilise for approximately 20 min before the inlet connections were fitted.

RESULTS AND DISCUSSION

Using the programme we have developed, it is possible to resolve all the amino acids present in a collagen hydrolysate as their Dns derivatives (Fig. 1a). However, collagen contains in excess of 300 residues per 1000 of glycine. On a fresh column Dns-glycine, although eluting very close to Dns-threonine, is resolved from it. With repeated column use, however, these components ceased to be resolved (Fig. 1b).

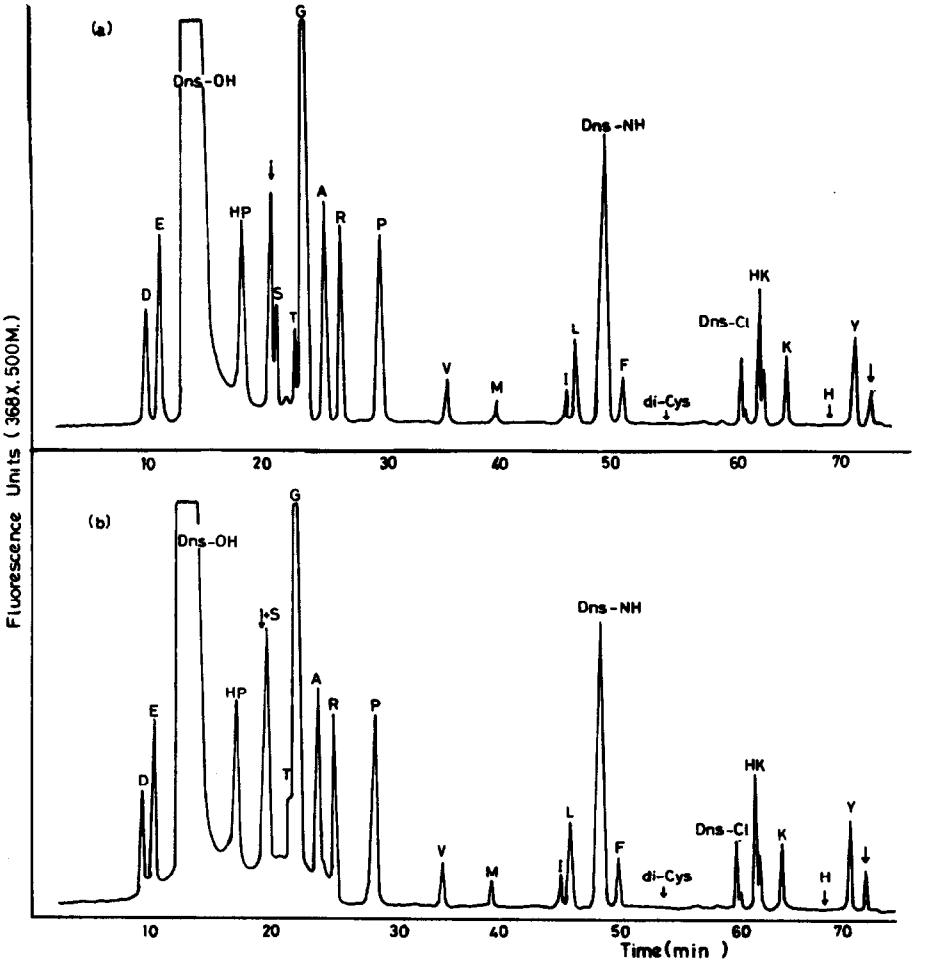

Fig. 1. (a) Resolution of the Dns-amino acids present in an acid hydrolysate of purified collagen from squid tunica. The arrows mark unknown Dns products present in the reagent blank. (b) Sample (a) analysed on the same column after the column plate count had fallen to 80% of the original. Elution is by stepwise gradient from 10 to 60% acetonitrile in 40 m*M* sodium phosphate pH 6.3. Flow-rate = 1.5 ml/min.

Many methods have been reported in recent years for the separation of derivatised amino acids by reversed-phase HPLC but, whether using *o*-phthalaldehyde or Dns chloride as derivatising agents, separation of these two amino acids poses a problem[10,11]. We have also found that a reagent blank Dns product migrates very close to Dns-serine and, when column efficiency drops only very slightly, this component precisely comigrates with Dns-serine (Fig. 1b). Obviously this can be compensated for by subtracting the reagent blank value but the comigration of Dns-glycine and Dns-threonine caused by decreased column efficiency poses a more intractable problem. The loss of resolution of Dns-glycine and Dns-threonine obviously marks the limit of the acceptable loss of resolving capacity of the column.

TABLE I

THE EFFECT OF PACKING PRESSURE AND RE-PACKING ON COLUMN INITIAL PLATE COUNT AND PLATE COUNT RECOVERY RESPECTIVELY

Columns were packed at 500 bar except 1 and 2 which were packed at 300 bar. Figures in parentheses represent percentage values of original number of plates (*N*).

Column	*Original plate No. (N)*	*1st Minimum (N)*	*1st Repack (N)*	*2nd Minimum (N)*	*2nd Repack (N)*	*3rd Minimum (N)*	*3rd Repack (N)*
1	5004	4111 (82)	4785 (96)	4332 (87)	5432 (109)	4307 (86)	
2	5031	4332 (86)	5051 (100)	4109 (82)			
3	8691	7741 (89)	10232 (118)	3026 (35)	3419 (39)		
4	9060	7558 (83)	7908 (87)	6183 (68)			
5	9771	9484 (97)	12061 (123)	7395 (76)	9269 (95)	7235 (74)	9173 (94)

Table I shows by calculating the column plate number, and then expressing it as a percentage of the original best value for the column, that a reduction in plate number to *ca.* 80% of the original resulted in an unacceptable loss of column resolving capacity. Not surprisingly the plate number recovery on re-packing can exceed the previous best for that column.

We found that repacking the column at this stage with the same material, plus a small amount of fresh (*ca.* 5%), would return most of the original resolving power thereby re-resolving Dns-serine and the reagent blank and Dns-threonine and Dns-glycine (Fig. 2). In fact we found that the same packing could be re-used up to four times with minimum reduction of column original efficiently and substantial savings of column packing. However if the plate count fell excessively (*e.g.* 3rd column, Table I) then the repacking procedure did not regenerate the lost efficiency.

Other authors have discussed a variety of methods for regenerating bonded-phase packings. These include prolonged washing of reversed-phase columns with a range of solvents of increasing hydrophobicity to remove organic impurities[3], or dilute acids and chelating agents to remove inorganic contaminants[4], or re-derivatising packings with commercially available silylating agents[6]. All these methods are time consuming and often require especially dried and purified solvents, also Wilson[6] reports limited success in regenerating 5-μm packings due to a build up of high back-pressure.

We have used the washing procedure outlined by Rabel[3] with some success and consequently tried using the column packing buffers to regenerate our C_{18} columns by washing. This latter procedure was less successful than that of Rabel and neither compared with the repacking procedure already described which only takes 2 h from removing a "spent" column to replacing the newly repacked column.

The likely explanation for the efficacy of re-packing is the exposure during re-suspension and re-packing of areas of bonded phase previously shielded from solvents and solutes by the close proximity of neighbouring packing material plus the physical and chemical removal of contaminants.

We observed a greater original efficiency, as defined by increased plate numbers (extra 58%) in analytical columns packed at 500 bar than in those packed at 300 bar. This difference was greater than variations in original column efficiency due to use of different batches of packing material. Since 500 bar is close to the safe working

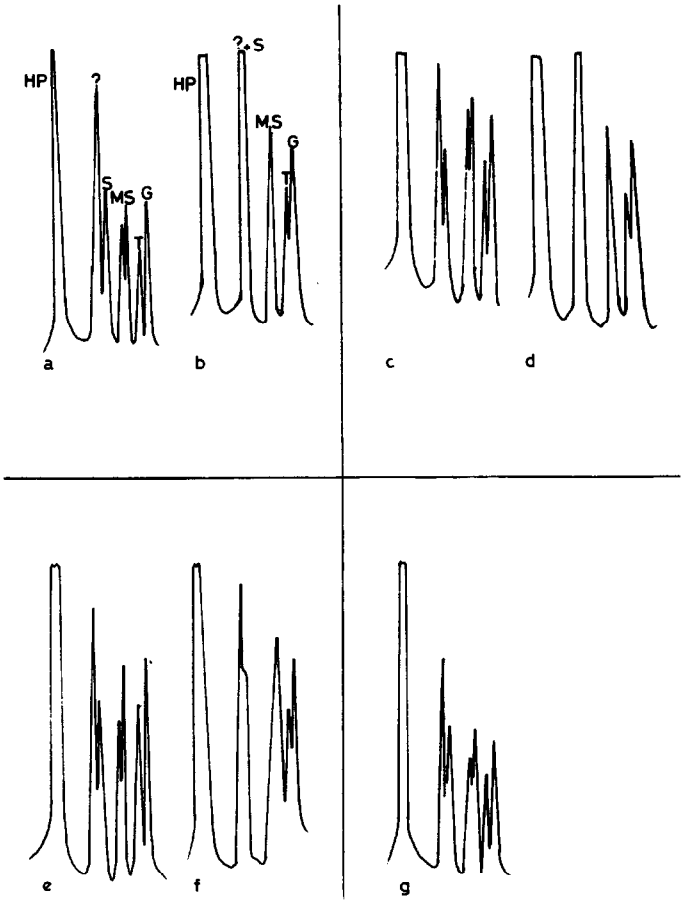

Fig. 2. Effect of re-packing (5th column, Table I) on the resolution of the Dns-amino acids hydroxyproline (HP), serine (S) and the reagent blank (?), methionines sulphoxide and sulphone (MS), threonine (T) and glycine (G) from a standard mixture. a = Original packing, b = 1st minimum, c and d = 1st repack and 2nd minimum, e and f = 2nd repack and 3rd minimum, g = 3rd repack.

limit of the column packer, we could not make comparisons at even higher pressures. However packing at ever higher pressures does not necessarily achieve significant increases in the number of injections, merely a more rapid decrease in plate number early in the column life. By using the simple expedient of running the test mixture at the start of each day we were able to save time, solvents and samples by forecasting the effectiveness of the Dns-amino acid chromatography and also to make direct comparisons between different columns and column packings. More importantly we found that by unpacking an unsatisfactory column and resuspending the packing with a small amount of fresh material, we could regenerate at least 95% of the original column efficiency which in turn resulted in a substantial increase in the number of analyses that could be carried out.

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