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## Note

# *In situ* silylation of an octadecylsilyl-silica stationary phase applied to the analysis of peptides, such as secretin and glucagon

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Reversed-phase high-performance liquid chromatography has become a routinely used tool for the analysis of peptides. The use of perfluoroalkanoic acids as lipophilic ion-pairing reagents for the analysis of peptides on octadecylsilyl-silica[1] was developed in this laboratory. We separated some closely related 27 peptides, secretin and its analogues, using these acids as counter-ions. After repeated use of, especially, perfluorooctanoic acid (0.005 *M*) in the eluent, we observed a decrease in the efficiency of the column in the chromatography of large peptides, such as secretin, as a result of strong tailing of the peaks (Fig. 1). We found, in experiments with secretin and its analogues, that the histidine residue plays an important rôle in the adsorption process. Regeneration of the column with various organic solvents did not eliminate the phenomena observed. The column showed no deterioration during the analysis of protected peptides and alkaloids, for example.

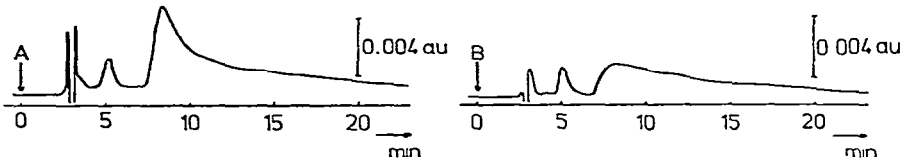


Fig. 1. Chromatograms of secretin (A) and glucagon (B) on a deteriorated column. Column, Polygosil $C_{18}$, 10 $\mu$m (30 × 0.4 cm I.D.); eluent, methanol–water–TFA (65:35:0.1); flow-rate, 1.2 ml/min; detection, UV, 205 nm; injection, 2.5 $\mu$l.

The phenomena described were possibly caused by the hydrolysis of siloxane bonds, *e.g.* by acids or traces of fluoride. The hydroxyl groups formed thus might affect the interaction of the peptides with the stationary phase. Perfluorooctanoic acid is adsorbed more strongly on the stationary phase than is trifluoroacetic acid (TFA), therefore the surface concentration of the former acid is higher. Consequently, the pH near the surface is lower if perfluorooctanoic acid is added to the eluent instead of TFA. Although we routinely use perfluorooctanoic acid at lower concentration than TFA (0.005 *M* and 0.013 *M* (= 0.1%, v/v), respectively) the former could be much more aggressive to the stationary phase.

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We have now solved the problem by *in situ* silylation of the deteriorated octadecylsilyl-silica, by eluting a solution of chlorodimethyloctadecylsilane in toluene through the packed column. This treatment results in a complete regeneration of the stationary phase.

## EXPERIMENTAL

### *Apparatus*

Two Waters Assoc. Model 6000 A pumps with a Model U6K injector in combination with a Model 660 solvent programmer were used. UV detection was performed with a Schoeffel Spectroflow-monitor SF 770.

### *In situ silylation of the stationary phase*

The silylation was performed on a column (30 × 0.4 cm I.D.) packed[2] with Polygosil $C_{18}$, 10 $\mu$m (Macherey, Nagel & Co., Düren, G.F.R.). This column was flushed, via a linear gradient, from methanol, the solvent in which the column is usually stored, to toluene. An empty column (20 × 0.8 cm I.D.) was coupled in a vertical position, with the inlet on top, between the pump and the $C_{18}$ column, and the detection system was uncoupled. The auxiliary column was filled with a solution of chlorodimethyloctadecylsilane in toluene (0.1 g/ml)[3]. This solution was pumped (0.2 ml/min) through the $C_{18}$ column. After silylation the column was flushed via a linear gradient (1 ml/min) successively with methanol, methanol–water–TFA (50:50:0.1), methanol, and methanol–chloroform (50:50). Finally it was flushed overnight with methanol via a gradient (0.2 ml/min). After removal of the auxiliary column the $C_{18}$ column could be used for chromatography.

### *Analysis of peptides*

Synthetic porcine secretin (Squibb, New Brunswick, NJ, U.S.A.), containing cysteine hydrochloride (0.5 mg/mg peptide), dissolved in water (1 mg/ml), and natural porcine glucagon (Eli Lilly, Indianapolis, IN, U.S.A.), dissolved in dilute hydrochloric acid (1 mg/ml), were used for testing the column. The eluents were obtained by mixing the components on a volume-to-volume basis and were degassed by careful filtration under reduced pressure, after which TFA or perfluorooctanoic acid was added. The column was kept at 30°C during chromatography.

## RESULTS AND DISCUSSION

The condition of the $C_{18}$ column after prolonged use becomes clear from Fig. 1, which shows chromatograms of secretin and glucagon, with methanol–water–TFA as the eluent. The use of perfluorooctanoic acid as counter-ion resulted in even worse peak forms. Silylation of the column at 30°C gave a considerable improvement of the chromatographic properties of the column (Fig. 2a), with methanol–water–TFA as the eluent. A subsequent treatment at 48°C gave a further improvement (Fig. 2b). Satisfactory results were also obtained with methanol–water–perfluorooctanoic acid as the eluent (Fig. 3).

In preliminary experiments we used chlorotrimethylsilane as the silylating reagent. This gave excellent results, even after repeated silylation of deteriorated

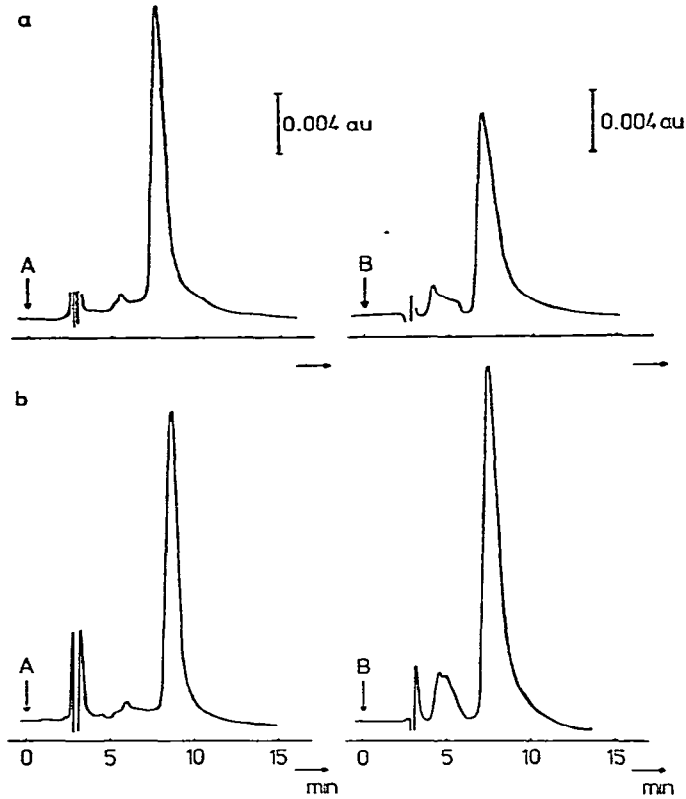


Fig. 2. Chromatograms of secretin (A) and glucagon (B) after *in situ* silylation of the column. (a) After silylation at 30°C, (b) after silylation at 48°C. Column, Polygosil $C_{18}$, 10 $\mu$m (30 × 0.4 cm I.D.); eluent, methanol–water–TFA (64:36:0.1); flow-rate, 1.2 ml/min; detection, UV, 205 nm; injection, 2.5 $\mu$l.

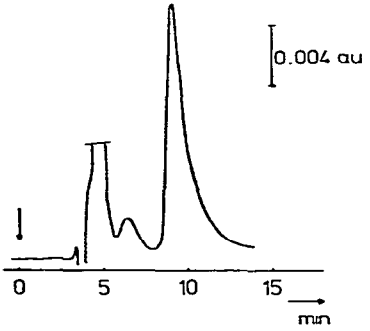


Fig. 3. Chromatogram of secretin after *in situ* silylation at 48°C. Column, Polygosil $C_{18}$, 10 $\mu$m (30 × 0.4 cm I.D.); eluent, methanol–water (82:18) with 0.005 *M* perfluorooctanoic acid; flow-rate, 1 ml/min; detection, 215 nm; injection, 10 $\mu$l.

columns. The blocking of "active adsorption sites" on the octadecylsilyl-silica by trimethylsilyl groups, however, may change the nature of the stationary phase considerably after repeated application of the silylation procedure. For this reason, we consider that silylation with chlorodimethyloctadecylsilane is a better procedure.

The results obtained show that the *in situ* silylation increases the useful lifetime of the column. The silylation step should preferably be performed at elevated temperatures, although application at ambient temperature also gives acceptable results. After silylation of the column a reasonable efficiency was obtained for large peptides, in several systems. The rinse procedure that is described in the Experimental section is essential in order to obtain good results. The use of solvents other than toluene did not improve the results of the silylation. The addition of pyridine resulted in the formation of the insoluble pyridine hydrochloride.

These results indicate that active adsorption sites are eliminated in the course of the silylation and support the hypothesis that the deterioration of the column is caused by the hydrolysis of siloxane bonds.

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