Investigation of Protein Recovery and Memory Effects in Reversed-Phase and Ion-Exchange Chromatography

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Introduction

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a useful method for protein analysis. Due to its compatibility with mass spectrometry detection, it became an important method for the characterization of pharmaceutically important proteins (LC/MS).

Reversed-phase HPLC encounters problems with recovery of hydrophobic proteins and glycoproteins, in severe cases proteins do not elute from the column. Due to incomplete recovery, ghost peaks may appear in subsequent analyses (memory effect). Published reports have shown that C4 chemistry is preferred over C18 columns and provides better protein recovery [5]. Besides aliphatic chain length, the pore size and the base sorbent also play a major role in protein recovery [3]. Polymer sorbents have been shown to provide for greater protein recovery than silica [6].

The separations of intact proteins was investigated by RP-HPLC and ion exchange chromatography using polymeric and siliceous sorbents (Fig. 1). The recovery and memory effects were monitored for very hydrophobic proteins. For RP-HPLC conditions see experimental. Anion exchange columns were investigated: reverse gradients, repetitive gradients, acetonitrile washes, and injections of concentrated acetic acid in between runs. The percentage of remaining ovalbumin after the column cleanup was measured using blank gradient elution (refer to figure 2A).

Experimental

HPLC System:
Agilent® 2745 (Waters) with a 996 PDA detector

Mobile Phases:
A: 0.1% TFA in water (RP-HPLC)
20 mM sodium phosphate pH 7.0 (Cation exchange-HPLC)
20 mM Tris-HCl pH 7.8 + 1 M NaCl (Anion exchange-HPLC)
20 mM Tris-HCl pH 7.8 + 1 M NaCl + 0.01% SDS (Anion exchange-HPLC)

B: 0.06% TFA in acetonitrile (RP-HPLC)
50% B in 15 minutes (Cation exchange-HPLC)
0-60% B in 15 minutes (Anion exchange-HPLC)
20% B in 15 minutes (Cation exchange-HPLC)

Gradient Conditions:
From 20% A to 80% A in 15 minutes (RP-HPLC)
0.05% A in 15 minutes (Anion exchange-HPLC)
0.70% A in 21 minutes (Cation exchange-HPLC)

Column Dimensions:
4.6 x 50 mm unless otherwise noted

Column Temperature:
40°C (RP-HPLC)
Ambient (IE-HPLC)

Flow Rate:
0.75 mL/minute

Injection Volume:
20 µL (Total protein load: 1.5 nmol) RP-HPLC
20 µL (Total protein load: 2.37 nmol) Anion exchange-HPLC
20 µL (Total protein load: 2.91 nmol) Cation exchange-HPLC

Figure 1: A protein mixture was chromatographed on selected reversed-phase and ion exchange columns. Recovery and memory effects were monitored for very hydrophobic proteins. For RP-HPLC conditions see experimental. Anion exchange conditions were 0.5% B in 15 minutes (mobile phase A) 20 mM Tris-HCl pH 7.8, mobile phase B: buffer A + 1 M NaCl. Symmetry 300 C4 (A) shows slightly better recovery and peak shape than Symmetry 300 C18 (B). Non-porous column (C) exhibits very poor recovery of ovalbumin. Prototype polyDVB column (D) has peak shape and recovery comparable to [A] and [B]. Anion exchange (E) is able to separate both isomers of ovalbumin. 100% recovery was observed for ovalbumin.

Figure 2: Memory effects of ovalbumin can be seen on all reversed-phase columns. The overlay labeled A shows one protein mixture injection followed by three blank injections. Ovalbumin can be seen eluting in all three blank injections. The overlay labeled B shows one protein mixture injection followed by one blank injection onto an anion exchange column. No ovalbumin memory effect was observed.

Figure 4: Effect of column cleanup on ovalbumin memory. Different cleaning procedures were investigated: reverse gradients, repetitive gradients, acetonitrile washes, and injections of concentrated acetic acid in between runs. The percentage of remaining ovalbumin after the column cleanup was measured using blank gradient elution (refer to figure 3).

Conclusions

• Limited recovery and memory effect of proteins was found to a varying degree on all reversed-phase HPLC columns.

• Memory effect can be greatly reduced by effective column cleanup.

•Unlike RP, ion exchange HPLC allows for good protein recovery and exhibits minimal memory effects.

• Ion exchange (under these conditions) is not directly compatible with MS; a derailing step is required.

•PolyDVB columns and silica-based Delo-Pack columns were found to perform well with mass spectrometry compatible mobile phases (1% formic acid). Results not shown.

References