Phase separation of acetonitrile-water mixture in protein purification

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The removal of acetonitrile (ACN) from reversed-phase high performance liquid chromatography (RP-HPLC) effluent fractions often presents a problem. High concentrations of ACN place a tough demand on the equipment in terms of solvent resistance. Energy cost is also a concern when ACN is removed by evaporation or freeze-drying. This work shows that a phase separation occurs for ACN-water solutions at −17°C. The top phase contains 88 (volume)% ACN, and the bottom phase is 65% water. Since the bottom phase contains 35% ACN, it is not frozen. Surprisingly, proteins such as human growth hormone and its analogs remain in the bottom phase 99% or more after a phase separation. This appears to be an easy and energy efficient method to remove the majority of ACN after RP-HPLC.

Keywords: purification; chromatography; acetonitrile; protein; human growth hormone

Introduction

Acetonitrile (ACN) is undoubtedly one of the most widely used solvents in Reversed Phase High Performance Liquid Chromatography (RP-HPLC).[1] It is desirable over most other organic solvents because of its low ultraviolet (UV) density, its low oxygen solubility, and its elution strength. RP-HPLC is an important tool in protein analysis and purification. In preparative or large-scale RP-HPLC purification of proteins, ACN removal after purification is a major concern.

Human growth hormone (hGH) is a protein with 191 amino acids and a molecular mass of 22,000 daltons.[2] The genetically engineered hGH analog, named hGHG120R, has one amino acid change—glycine to arginine at position 120 in the third α-helix of the hGH molecule. The hGH analog, which is secreted by stable, transfected, recombinant mouse L cells, possesses the same binding affinity to mouse liver membrane preparations as wild-type hGH. Surprisingly, transgenic mice that expressed this mutated hGH gene showed a significant growth-suppressed phenotype.[3,4] hGHG120R acts as an hGH antagonist. It may have important implications in treating humans with elevated levels of growth hormone, as found in type I diabetics. Currently, we are producing multimilligrams of hGHG120R for animal testing.

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In preparative RP-HPLC purification of hGHG120R, a C4 RP-HPLC column was used.[5] The HPLC-purified hGHG120R typically resulted in an effluent fraction containing 65% (vol.) ACN/35% water/0.1% trifluoroacetic acid (TFA). We discovered that when such a solution was stored in a freezer at −17°C for several hours (or overnight), a very clear phase separation occurred. The top phase was 88% ACN and the bottom phase was 65% water. Because the bottom phase contained 35% ACN, it remained unfrozen. Surprisingly, the water phase contained 99%+ of the total protein. Similar results were obtained for hGH solutions after phase separation.

The lyophilized form of hGH or hGHG120R is desirable for storage and formulation.[6,7] Because of the drastically reduced amount of solvent, the product can be easily lyophilized using a centrifugal evaporator with much less burden to the lyophilizer.

Experimental

The hGH and hGHG120R solutions used in phase separation experiments were the effluent fractions of the proteins from preparative RP-HPLC in a multistage purification process developed by us for the purification of hGH and its analogs from cultured animal cells.[5] They contained approximately 60–65% ACN with 0.1% TFA.

A Waters dual-pump gradient HPLC system (Millipore Corporation, Bedford, MA, USA) was used to analyze protein concentrations. This computer-controlled system has two Model 510 pumps, a Model U6K detector with a 2.5-ml sample loop, and a Model 486 variable wave-

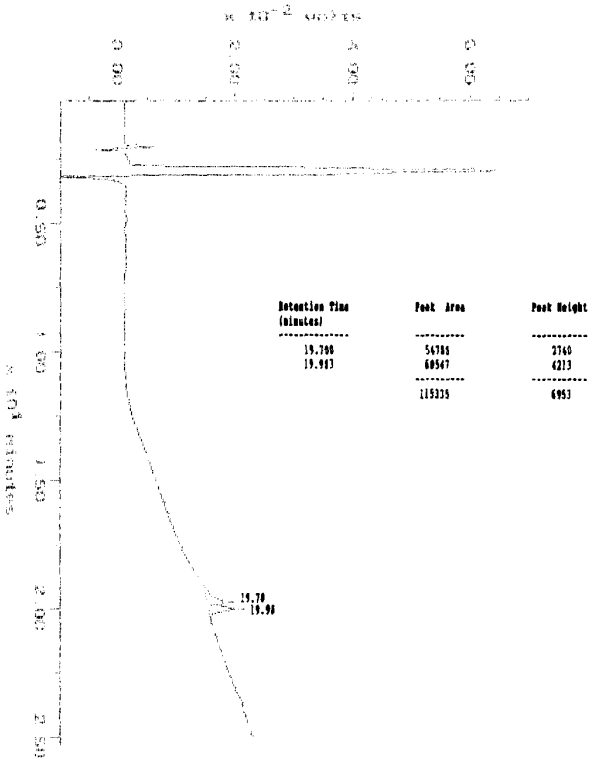

Figure 1 RH-HPLC profile of the top phase of an hGHG120R sample

length UV detector. The computer software used was Waters' Baseline 810 package. A linear gradient, from 40% ACN in water + 0.1% TFA to 80% ACN in water + 0.1% TFA in 30 minutes at 1 ml/min, was used to achieve the separation. A Vydac brand C4 RP-HPLC column (5 μm, 300 Å pore size, 0.46 × 25 cm) from The Separations Group (Hesperia, CA, USA) was used. UV detection was set at a 220-nm wavelength. The sample injection size was 50 μl.

Phase separations of blank ACN-water mixtures were carried out by placing ACN-water solutions with different ACN volume percentages in 15-ml test tubes at −17°C for 24 hours. The volumes of the top and bottom phases were then measured.

Results and discussion

Figures 1 and *2* are the analytical C4 RP-HPLC chromatograms of the top and the bottom phases of a concentrated hGHG120R sample obtained from a preparative RP-HPLC separation. *Figure 3* is the HPLC profile of the hGHG120R sample before the phase separation. Comparison of Figures 1 and 2 shows that the total protein concentration (based on peak areas) in the top phase (8.3 ml) is only ~0.5% of that in the bottom phase (17.5 ml). Roughly 80% of the ACN was removed from the hGHG120R sample by getting rid of the top phase. The very small 20.98 minute peak in Figure 2 seems to be a protein closely related to the hGHG120R produced by the recombinant mouse L cells. It is currently under investigation. For hGH samples, similar results were obtained from a phase separation.

Table 1 shows the results obtained from phase separation experiments using blank ACN-water solutions. Experiments demonstrated that the phase separation occurs *only* in the ACN concentration range of 35–88%. The range limits are actually the bottom- and top-phase ACN concentrations. This is because there is only one set equilibrium concentration for the phase separations of the binary system (ACN-water) at −17°C. This was confirmed by measuring the ACN concentrations of the top and bottom phases using a reflective index meter. These two "critical concentrations" were also confirmed by doing mass balances of data given in Table 1.

We found the phase separation method to be a very convenient way of removing about 80% of the ACN in an hGH or hGHG120R sample obtained from a preparative RP-HPLC purification. We have been routinely using this method before the sample is lyophilized in our production of hGHG120R.[5] Interestingly, no literature data on the phase equilibria of ACN-water systems at low temperatures were found, probably because the two compounds were considered totally miscible, which is true at room temperature.

After a phase separation, the bottom phase contains a certain amount of ACN and it is not frozen at −17°C. This makes the phase separation method more attractive, since the method will not damage freeze-sensitive proteins, such

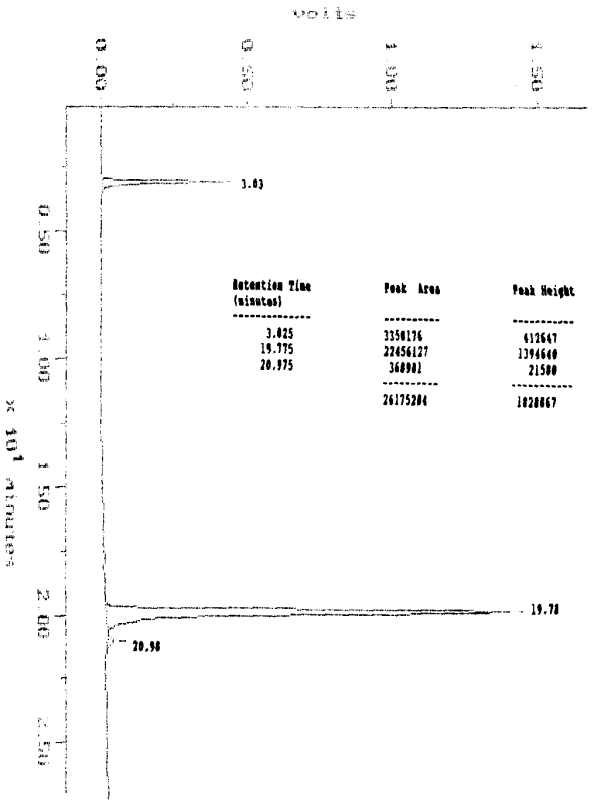

Figure 2 RP-HPLC profile of the bottom phase of the hGHG120R sample

Table 1 Phase separation of blank ACN-water mixtures at −17°C

ACN % before phase separation	30%	40%	50%	60%	70%	80%	90%
Before mixing ACN vol./water vol.	3 ml/ 7 ml	4 ml/ 6 ml	5 ml/ 5 ml	6 ml/ 4 ml	7 ml/ 3 ml	8 ml/ 2 ml	9 ml/ 1 ml
Top-phase vol. (ml)	—	1.28	2.88	4.76	6.64	8.27	—
Bottom-phase vol. (ml)	—	8.33	6.68	4.88	3.08	1.31	—
Top-phase vol./bottom-phase vol.	No*	0.15	0.43	0.98	2.16	6.13	No*

*No phase separation occurred.

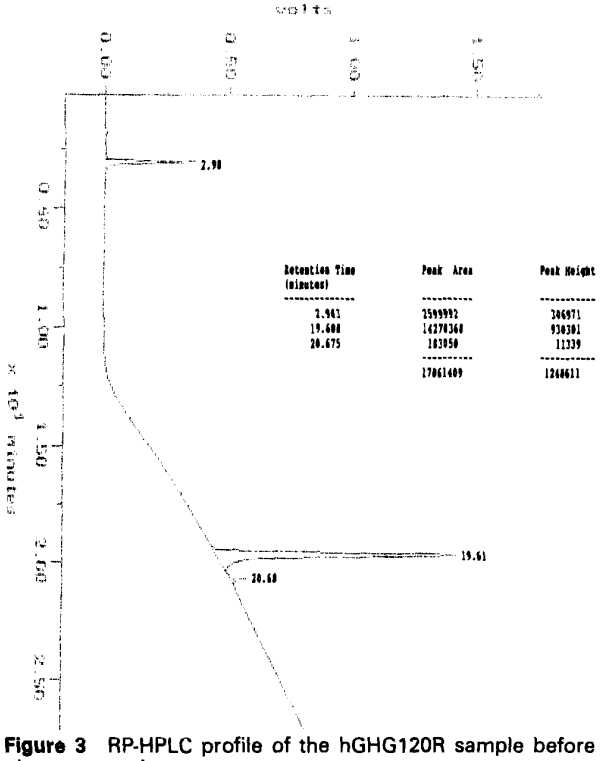

Figure 3 RP-HPLC profile of the hGHG120R sample before phase separation

as hGH and hGHG120R. For hGH and its analogs, a repeated freeze-thaw process reduces bioactivity.[6]

In some practical cases, an RP-HPLC purified protein solution may be difficult to handle because it has a high ACN content and the equipment used in buffer exchange (such as a membrane diafiltration device, a centrifugal ultrafilter, or a size exclusion column) is not resistant to this high-solvent concentration. The phase separation method may prove to be an easy way to deal with these problems. This method also helps reduce sample volume. The reduction in sample volume is especially helpful if a size exclusion column is used to carry out buffer exchange before the protein is finally lyophilized, since the sample capacity of a size exclusion column is often quite small. If the sample is to be dried directly using a centrifugal vacuum evaporator, such as a Savant SpeedVac (Savant Instruments, Incorporated, Farmingdale, NY, USA) or a CentriVap system (Labconco Corporation, Kansas City, MO, USA), the reduction of sample volume and ACN amount is still beneficial in terms of reduced equipment wear.

The reason that hGH and hGHG120R stay in the bottom phase is probably because these two relatively hydrophilic proteins prefer water to ACN, which is a highly polar solvent. The phase separation phenomenon may be applied to other similar proteins. To support this view, we performed the phase separation of a 47-mg/l myoglobin solution in 60% ACN. Similar results were obtained.

Conclusions

The removal of ACN from RP-HPLC fractions often presents a problem in lab experiments and commercial production processes. High concentrations of ACN places a much higher demand on the equipment in terms of solvent resistance. Energy cost is also a concern when ACN is removed by evaporation or freeze-drying. The phase separation phenomenon is a useful tool for the removal of ACN with almost no loss of protein in systems involving some hydrophobic proteins. This process requires little energy, because only cooling of a liquid is required. A regular freezer can be used to carry out the process. It does no damage to freeze-thaw-sensitive proteins, since the bottom phase is not frozen.

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