Selective Precipitation of Interleukin-4 Using Hydrophobic Ion Pairing: A Method for Improved Analysis of Proteins Formulated with Large Excesses of Human Serum Albumin

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In order to ensure the stability of protein pharmaceuticals, human serum albumin (HSA) is often added as an excipient, frequently in large excess. This makes chromatographic analysis of the stability of the active protein difficult. In the case of interleukin-4 (IL-4), separation from HSA can be achieved to some degree by size exclusion chromatography, but some HSA co-elutes with the IL-4. Hydrophobic ion pairing provides a method for selective precipitation of IL-4 from HSA. Hydrophobic ion pairing involves the electrostatic interaction of ionic detergents with oppositely charged polypeptides. Even when HSA is present in fifty-fold excess (w/w), the resulting precipitate contains greater than 70% of the IL-4. Selective precipitation with SDS produces enhancements in IL-4 over HSA of more than 2000-fold. This approach permits subsequent facile analysis of IL-4 by conventional reverse phase HPLC.

KEY WORDS: hydrophobic ion pairing; interleukin-4; protein analysis; HPLC; human serum albumin; formulation.

INTRODUCTION

One of the major difficulties with analysis of protein products is interference from excipients which may have hydrophobic, ionic and spectrophotometric characteristics similar to the active ingredient. An interference-free analysis is particularly difficult when the major excipient is a protein, such as human serum albumin (HSA). The amount of HSA used in protein products is sometimes as much as fifty times greater than that of the active material. Therefore, the removal of HSA prior to analysis may be required for the analysis of protein products. Separation of the protein from HSA should be possible, but must be as efficient as possible. Chromatographic separations based on size exclusion, reversed-phase high performance chromatography, high performance ion exchange chromatography, and high performance capillary electrophoresis should provide resolution of the two proteins when they differ greatly in their physicochemical properties. However, some sample preparation is usually required to remove large amounts of HSA prior to chromatographic analysis.

Addition of low concentrations of sodium dodecyl sulfate (SDS) or another ionic detergent leads to formation of ions pairs with oppositely charged groups on the protein, in a process we term hydrophobic ion pairing (HIP). The precipitation of proteins using low concentrations of SDS is well established (1–5). However, it has been only recently recognized that these complexes also display altered solubility properties as well (6,7). In this study, the ability of ionic detergents to achieve selective precipitation prior to chromatographic analysis of interleukin-4 (IL-4) is examined by exploring effects of pH, ionic strength, and SDS molar ratios leading to optimal formation of the IL-4 and/or HSA HIP complex.

MATERIALS AND METHODS

Human serum albumin was purchased from Sigma as an essentially globulin and fatty acid free lyophilized powder. Interleukin-4 was a gift from Schering-Plough. Sodium dodecyl sulfate was obtained from Sigma. All solvents and reagents were analytical or HPLC grade in purity.

HPLC Assay

The LC consisted of a dual pump system with a variable wavelength detector set at 214 nm. The column was an Aquapore RP-300 (100 mm × 2.1 mm). Injection loop size was 50 μl. Mobile phase A was 0.1% TFA in water and mobile phase B was a mixture of 10% TFA in water (0.1%) and 90% acetonitrile. The flow rate was 2 ml/minute with the pumps operating at pressures of about 2500 psi. The gradient was linear, beginning with 30% of phase B and increasing to 50% of B in 8 minutes. The IL-4 elutes at approximately 5.7 minutes and HSA elutes at approximately 6.9 minutes using this protocol. Peak widths were typically about 0.15 minutes for IL-4 and about 0.38 minutes for HSA. Standard curves were generated using serial dilutions of both IL-4 and HSA.

Sample Preparation

All samples were prepared so the final theoretical concentration of IL-4 would be 0.1 mg/ml. Sample size was 0.5 or 1.0 ml in all cases. For samples containing varying ratios of IL-4 and HSA, SDS was added based on molar ratios to IL-4. After addition of SDS, the pH of the solutions was adjusted with HCl. The samples were spun at 4000 r.p.m. for 10 minutes. The supernatant was decanted and saved. The pellets were washed with 1 ml water and then spun again at 4000 for 10 minutes. The wash was discarded and pellets were resuspended in either 0.5 ml or 1.0 ml of methanol (corresponding to the original sample volume), and then injected into the HPLC for analysis.

RESULTS AND DISCUSSION

Baseline separation of HSA and IL-4 were attained, even at 50:1 HSA/IL-4 ratios with an overall assay time of

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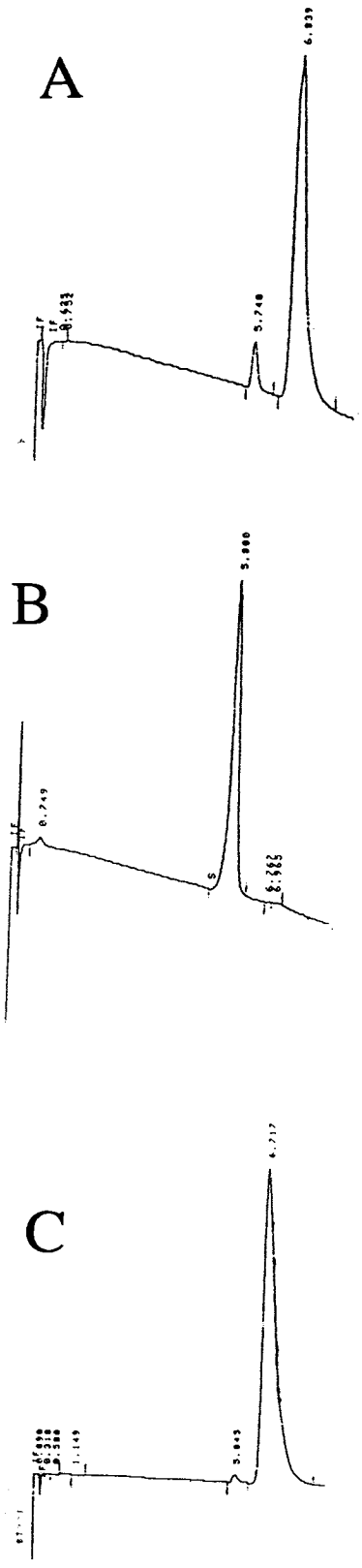

Figure 1. HPLC chromatograms (100 mm × 2.1 mm Aquapore RP-300 column, mobile phase A: 0.1% TFA in water and mobile phase B: 10% TFA in water (0.1%) and 90% acetonitrile, flow rate of 2 ml/minute, the gradient was linear, beginning with 30% of phase B and increasing to 50% of B in 8 minutes) for a mixture of IL-4 (0.1 mg/ml) and HSA (2 mg/ml): (A) before precipitation, (B) protein in the pellet after precipitation, and (C) protein in the supernatant. The SDS to IL-4 ratio was 40:1 and the pH was 5.4. The scales for traces A and B have been adjusted for clarity.

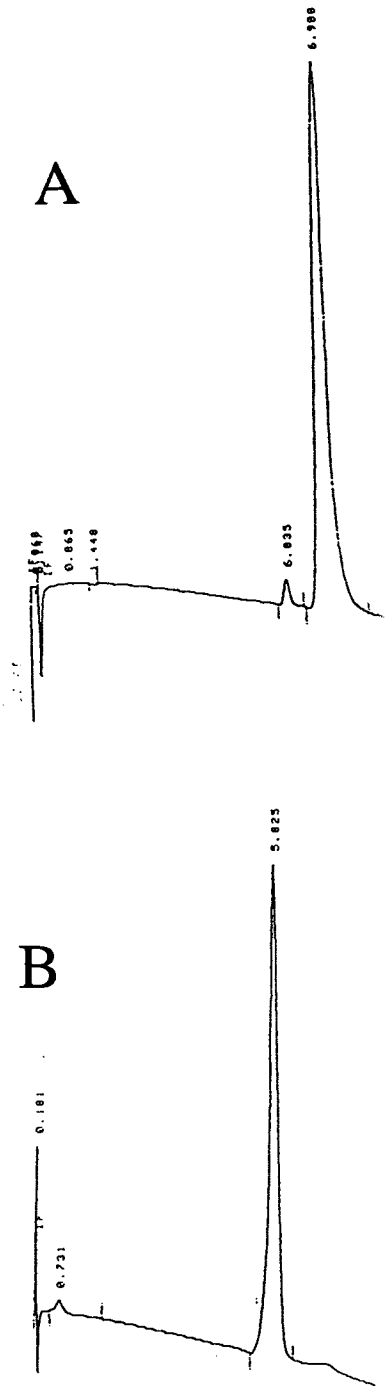

Figure 2. HPLC chromatograms (100 mm × 2.1 mm Aquapore RP-300 column, mobile phase A: 0.1% TFA in water and mobile phase B: 10% TFA in water (0.1%) and 90% acetonitrile, flow rate of 2 ml/minute, the gradient was linear, beginning with 30% of phase B and increasing to 50% of B in 8 minutes) for a mixture of IL-4 (0.1 mg/ml) and HSA (5 mg/ml). Panels (A) is before precipitation, and panel (B) is after precipitation. The SDS to IL-4 ratio was 100:1 and the pH was 5.2. The scales for traces A and B have been adjusted for clarity.

Table I. Summary of Precipitation Efficiency of IL-4 from HSA

		Initial conc. (μg/ml)		Pellet conc. (μg/ml)[b]		Super. conc. (μg/ml)[b]	
pH	SDS ratio[a]	[HSA]	[IL-4]	[HSA]	[IL-4]	[HSA]	[IL-4]
4.5	20:1	0	100	na	84	na	0.5
5.0	40:1	1000	100	1.5	77	1075	0.3
5.4	40:1	1000	100	<1	77	990	<1
6.0	40:1	1000	100	<1	52	nd	nd
5.0	60:1	1000	100	10	30	nd	nd
6.0	60:1	1000	100	<1	3.8	nd	nd
5.2	40:1	2000	100	0.4	45	nd	nd
5.2	50:1	2000	100	0.6	76	1700	14
5.2	70:1	2000	100	0.5	11	nd	nd
5.2	100:1	2000	100	2.1	4.9	nd	nd
5.2	40:1	5000	100	<1	<1	4050	65
5.2	70:1	5000	100	2.3	1.5	nd	nd
5.2	100:1	5000	100	1.8	71	3960	<1

na = Not applicable.
nd = Not determined.
[a] SDS ratios are on a molar basis relative to IL-4.
[b] Measured concentration in the precipitate. Small peaks which could be detected but not integrated are listed as <1 μg/ml.

approximately twelve minutes. A typical chromatogram is shown in Figure 1A. A standard curve was generated for both IL-4 and HSA samples. Samples were typically extracted into methanol for injection onto the column. Pellets were redissolved in 500–1000 μl of methanol, while supernatants were injected directly onto the column.

Initial experiments were aimed at identifying conditions under which HSA would not precipitate upon addition of SDS, but which would precipitate IL-4. At pH 5 and with no added buffer, addition of SDS to a HSA solution does not produce a precipitate to any detectable level. Under these same conditions, 20:1 SDS (molar ratio of SDS to protein, based on the protein) produces a fine precipitate of IL-4 in the absence of HSA. Analysis of the precipitate and the supernatant shows that more than 99% of the IL-4 has been removed from solution, although only approximately 70–75% of the total IL-4 was recovered in the pellet (Table I). Presumably, the IL-4 precipitate binds irreversibly to the container or column and so is not detected.

Since various levels of HSA could be used as an excipient, three solution mixtures of HSA and IL-4 (HSA/IL-4 solutions A, B, and C) were prepared with 10-, 20-, and 50-fold excess of HSA to IL-4 (0.1 mg/ml), respectively. For HSA/IL-4 solution A, the conditions which produced precipitation for IL-4 alone (addition of 20:1 SDS) did not produce any visible precipitate. The failure to achieve selective precipitation of IL-4 under these conditions was found to be due to partial binding of SDS by the HSA. Further addition of SDS (bringing the ratio to 40:1) to solution A produced a precipitate which could be isolated by centrifugation. Dissolution of the pellet in methanol and analysis by HPLC indicated that more than 75% of the IL-4 was recovered and less than 1% of the HSA was present in the pellet (Table I). The precipitation seemed to be slightly more effective at pH 5.4 than pH 5, as judged by the amount of residual HSA in the pellet, although in both cases there was at least a 500-fold enhancement in the ratio of IL-4 to HSA (Table I).

Increasing the pH to 6.0 still led to selective precipitation of IL-4, but the amount of IL-4 recovered was only about 50%. In addition, at pH 5, increased amounts of SDS (60:1) produced some precipitation of HSA as well as IL-4 (Table I). Apparently, the excess SDS binds to the HSA, forming some amount of a HIP complex at this ratio, leading to some precipitation of the HSA. The phenomenon of HIP is a stoichiometric binding of detergent (based on the number of basic residues). Beyond that ratio, little additional detergent binding is observed, and the remaining SDS associates with the HSA rather than the IL-4. Also, excess detergent can lead to reduced insolubility of a HIP complex, possibly due to some micelle formation.

Similar results were obtained for HSA/IL-4 solutions B and C. As with solution A, increased amounts of SDS were necessary to produce optimal precipitation of IL-4 due to interference from HSA. For HSA/IL-4 solution B, the best conditions appear to be pH 5.2 and a SDS ratio of 50:1. This produces a pellet which contains 126 times more IL-4 than HSA, representing a 2500-fold enrichment in IL-4 (Figure 1B and 1D). Analysis of the supernatant indicated that 14% of the IL-4 remained (Figure 1C).

In the case of HSA/IL-4 solution C, similar results were obtained. After precipitation at a ratio of 100:1 (SDS to IL-4), the ratio of IL-4 to HSA in the pellet was approximately 40:1 and the amount of IL-4 recovered was 65–75%. For HSA/IL-4 solution C, this represents a nearly 2000-fold enhancement in IL-4 from the original formulated product.

CONCLUSIONS

Selective precipitation of IL-4 from formulations containing large amounts of HSA demonstrates that HIP is a

valid method for achieving significant enhancement of the active protein in subsequent chromatographic analyses of these preparations. In the case of a formulation which initially contains less than 2% IL-4 relative to HSA, analysis of the protein recovered in the precipitate showed it to be more than 97% IL-4. This precipitation method should provide easier determination of the chemical stability of IL-4 mixtures containing large amounts of HSA.

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