
Key Words

Column liquid chromatography
Capillary zone electrophoresis
Electrospray ionization-mass spectrometry
Human serum albumin

Summary

Human serum albumin (HSA) is one of the most abundant human proteins and has been shown to be heterogeneous. A RP-HPLC method has been developed to separate HSA components in commercially available preparations. Separations were carried out on Aquapore RP-300, C8 columns using gradient elution with a combination of acetonitrile/water mobile phases containing 0.05 % trifluoroacetic acid as ion-pairing agent. Optimum resolution was attained on narrow-bore columns using a stepwise, linear gradient that incorporated a shallow intermediate step of 0.20 %/min in Mobile Phase B. Under similar elution conditions, separations carried out on standard-size columns showed the expected decrease in resolution due to increased peak widths. A comparative analysis of three commercial products highlighted qualitative and quantitative differences. Capillary zone electrophoresis was used for the analysis of collected RP-HPLC fractions. Results indicated that while the HPLC separation was incomplete, one of the major HPLC peaks was primarily composed of one of the three main components typically separated by CZE. ESI-MS was used to characterize the two major RP-HPLC fractions and also showed that the HPLC separation was incomplete. The MaxEnt transform of the HPLC peaks was consistent with components all being HSA and closely related derivatives.

Introduction

Methodologies using high performance chromatographic and electrophoretic separation techniques have been increasingly used over the last two decades for the purification, characterization and quality assessment of biomolecules [1, 2]. These methods have been particularly useful for biopharmaceuticals, especially for biologically important proteins and glycoproteins produced through recombinant DNA technology. Reversed-phase HPLC (RP-HPLC) has been one of the more versatile chromatographic modes to study proteins and their impurities, owing in part to advances made in microparticulate stationary phases and to the development of a wide range of columns of differing selectivities [3, 4]. For instance, several methods have described the separation of variants differing by a single amino acid from the native protein. Capillary zone electrophoresis (CZE) has rapidly become a valuable analytical technique by virtue of the exceptional separation efficiencies attained and its capability for rapid, automated and reproducible separations [5]. These considerations make CZE particularly well suited for the separation of closely-related protein variants.

Human serum albumin (HSA) is a single chain, non-glycosylated protein [6]. However, studies have demonstrated that it is composed of a complex mixture of variants such as mercapto and non-mercapto [7], glycated [8] and polymerized [9, 10] forms. Conditions of storage and manufacture can further increase heterogeneity by introducing deamidation, oxidation or polymerization [10]. Chromatographic separations of HSA components based on size-exclusion [7, 11, 12], ion-exchange [13-16] and reversed-phase [17] HPLC have been reported. Recent studies have also demonstrated that HSA heterogeneity can be characterized by CZE on bare-silica capillaries under conditions that minimize protein adsorption on walls [18, 19] or by using coated capillaries [20]. In this study, we report on the development of a rapid RP-HPLC method that provides reproducible separation of HSA-related components in commercially available HSA products, with on-line characterization of components by ESI-MS.

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Table I. Gradient profile 1 for the separation of human serum albumin by RP-HPLC. The flow rate was that used with the narrow-bore column.

Time min	%A	%B	%C	Flow Rate in mL min ⁻¹
0	70	30	0	0.15
10	70	30	0	0.15
15	65	35	0	0.15
30	63	37	0	0.15
35	50	50	0	0.15
40	0	0	100	0.5
50	0	0	100	0.5
59	90	10	0	0.5
60	70	30	0	0.15
70	70	30	0	0.15

Table II. Gradient profile 2 for the separation of human serum albumin by RP-HPLC. The indicated flow rate was for a standard-size column.

Time (min)	%A	%B	%C	Flow Rate (mL min ⁻¹)
0	70	30	0	0.70
1	70	30	0	0.70
6	65	35	0	0.70
25	61	39	0	0.70
30	50	50	0	0.70
35	0	0	100	2.00
41	0	0	100	2.00
45	70	30	0	2.00
46	70	30	0	0.70

Experimental

Materials

Pentex® HSA, fraction V was from Miles Inc. (Kankakee, IL), Albuminar®-25 HSA preparations (lots No. M65205, M66206 and M67007) from Armour Pharmaceuticals (Kankakee, IL) and crystallized and lyophilized HSA (lot No. 126C-8070) from Sigma (St-Louis, MO). Buffer salts and additives were HPLC or Molecular Biology grade reagents. OmniSolv HPLC grade solvents were obtained from EM Science. Hen egg lysozyme was from Sigma (St-Louis, MO). Aquapore RP-300 Brownlee columns (250 × 4.6 mm i.d.) were purchased from Chromatographic Specialities (Brockville, ON).

Methods

HPLC

Mobile Phase A consisted of 0.05 % trifluoroacetic acid (TFA) in 10 % acetonitrile/90 % water, Mobile Phase B was 0.05 % TFA in 90 % acetonitrile/10 % water and Mobile Phase C was 0.05 % TFA in acetonitrile. Narrow-bore column separations were performed on Hewlett Packard 1090 Series II ternary DR-5 pumps, 6 mm SST micro 1.7 µL high pressure flow cell. The column was an Aquapore RP-300, 7 µm, 220 × 2.1 mm i.d. and was equilibrated with a mixture of Mobile phases A and B (70:30) until a stable baseline was obtained. Elutions were carried out using multi-step gradient profiles 1 and 2 as described in Table I and II respectively. The flow rate was that presented in Table I. The column was maintained at a temperature of 50 °C. The effluent was monitored at 220 nm, then was split 10:1 prior to ESI-MS analysis. Standard-size column separations were carried out on a SpectraSystem (Thermo Separation Products, Mississauga, ON) consisting of a P4000 quaternary pump system, an AS 3000 autosampler and a UV6000LP diode array detector. The column was an Aquapore RP-300, 7 µm, 250 × 4.6 mm i.d. and was maintained at 50 °C. Separations were carried out using

gradient profiles 1 and 2 with the flow rate as indicated in Table II. Chromatograms were monitored between 200 and 350 nm. In preparative runs, 100 µL aliquots of HSA (2 mg/ml) were injected and peaks were collected. The solvent was removed by lyophilization.

CZE

The CZE system consisted of a P/ACE 5500 (Beckman Instruments, Fullerton, CA) fitted with a variable wavelength UV detector. Fused-silica capillaries were from Beckman Instruments (Fullerton, CA) (57 cm × 50 µm i.d.) and Polymicro Technologies (Phoenix, AZ) (67 cm × 52 µm i.d.). Samples were injected hydrodynamically and peaks were detected on-column at 200 nm. Separation conditions were as previously reported [18]. The electrophoretic buffer consisted of 20 mM sodium phosphate/5 mM putrescine, pH 8.5. A rinse sequence of 1M HCl (3 minutes), water (3 minutes), 1 % NaOH (3 minutes), water (3 minutes) and buffer (5 minutes) was carried out after each run. A 2-minute pre-run equilibration was carried out with electrophoretic buffer.

ESI-MS

AutoSpec-Q (EBE-qQ geometry) Micromass (Manchester, LTK); electrospray ion source, spray housing at 85 °C; scan range 2000–1000 at 30 s dec⁻¹; needle voltage = 8.3 kV, sampling cone = 4.2 kV, skimmer lens = 4.2 kV, ring electrode = 4.1 kV and accelerating voltage = 4.0 kV; vacuum: source/analyzer/inlet = 3 × 10⁻⁶/6 × 10⁻⁸/0.2 mbar; a variable position fused needle (10 cm × 50 mm i.d. flame drawn out ≈ 15 µm i.d.) was used; electrical contact was made via a SS union to the 0.127 mm i.d. red Peek tubing transfer line; purified air (20 L hr⁻¹) was used as the nebulizer gas and UHP nitrogen (250 L hr⁻¹) as the bath gas. Calibration was with

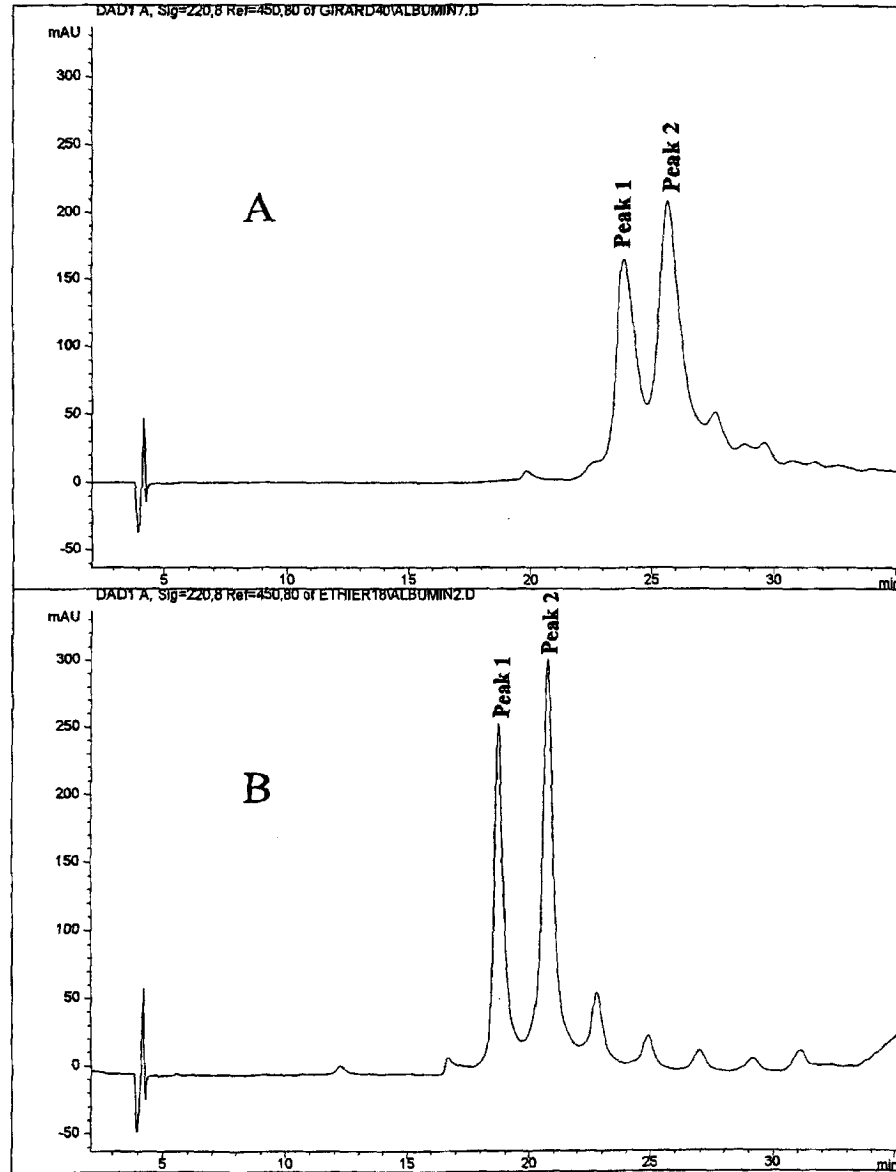


Figure 1
 RP-HPLC analysis of Pentex HSA on an Aquapore RP-300, 220 × 2.1 mm i.d., column: partial chromatograms obtained with (A) gradient profile 1 (Table I) and (B) gradient profile 2 (Table II). In both cases the flow rate was that shown in Table I.

hen egg lysozyme, molecular mass of 14303.4 ± 0.46 Da (calculated value = 14305 Da).

Results and Discussion

Analysis of HSA by RP-HPLC

Gradient elution using acetonitrile as organic modifier and TFA as ion-pairing agent has been frequently used for the separation of proteins by RP-HPLC [3, 4]. While the separation of non-related proteins usually requires gradient elution over a wide range of organic modifier, resolution of closely-related species can be maximised using a stepwise, shallow gradient, a procedure that maximizes retention factor (k) differences [4]. In preliminary experiments we examined the elution of HSA

on the moderately hydrophobic Aquapore RP-300 C8 columns using a stepwise, linear gradient over a wide range of Mobile Phase B (30–60 % over 20 minutes). Under these conditions, HSA eluted off the column as a single peak in the range of 35–40 % Mobile Phase B. Optimization of the gradient conditions by incorporating a shallow step in that critical range led to the separation of HSA into several components as demonstrated in Figures 1 and 2 for narrow-bore and conventional-size columns respectively. The column temperature was found to be an important factor in obtaining reproducible separations. When runs were carried out at room temperature, unpredictable shifts in peak retention times occurred, a likely consequence of the non-specific adsorption of HSA to the stationary phase. At 50 °C, reproducible separations were obtained (see below).

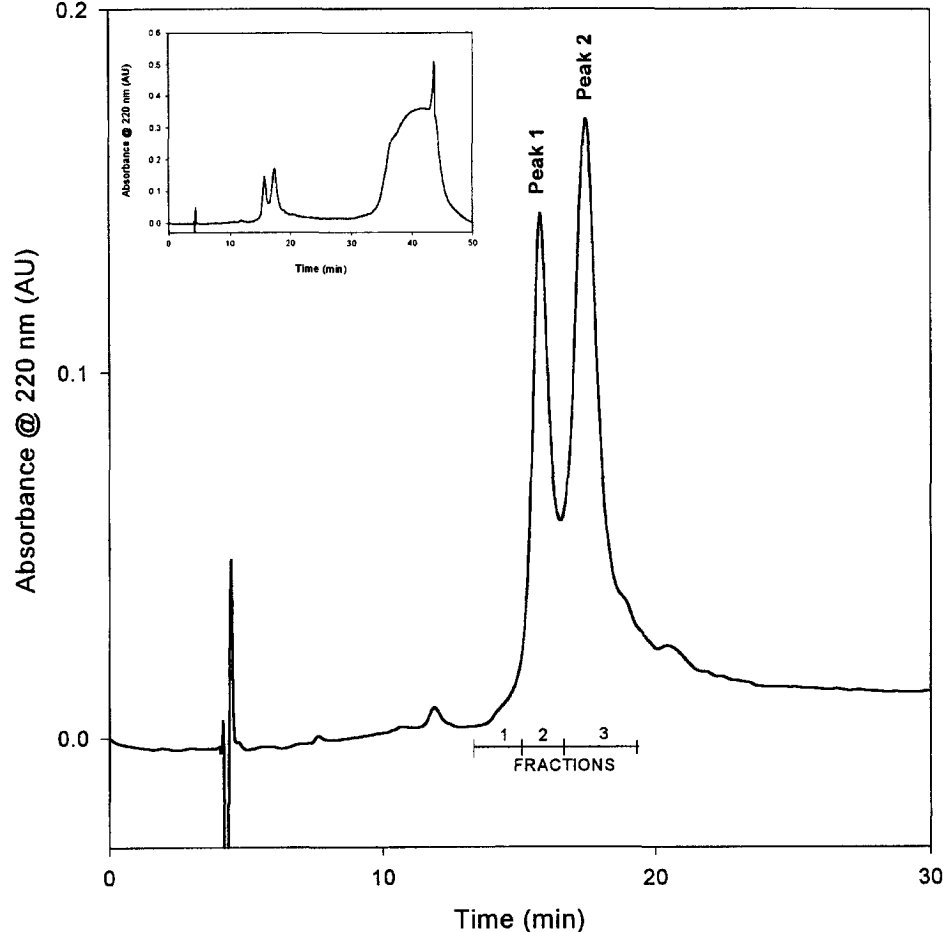


Figure 2
 RP-HPLC analysis of Pentex HSA on Aquapore RP-300, 250 × 4.6 mm i.d. using gradient profile 2 (Table II).
 Inset: full chromatogram.

Chromatograms obtained with the narrow-bore column (Figure 1) featured two major peaks, 1 and 2, and several late-eluting minor ones. Most peaks were eluted off the column during the intermediate, shallow gradient step. The resolution was found to be highly sensitive to the steepness of the shallow step. Figure 1A shows the chromatogram obtained with gradient profile 1 (Table I) consisting of three linear gradient steps where the rate of change of the shallow step was 0.13 %/min in Mobile Phase B. In gradient profile 2 (Table II) the steepness of the shallow step was increased to 0.20 %/min which gave an increase in peak separation as seen in Figure 1B.

Separations on standard-size columns were carried out with the same gradient profiles after adjusting the flow rate to 0.7 mL min⁻¹ to account for the column size difference. Peak profiles were similar to those obtained on narrow-bore columns (Figure 2). The two main peaks, 1 and 2, eluted off the column during the shallow gradient step as was the case with the narrow-bore column. The UV spectra taken at the apex of the two main peaks were typical of proteinaceous compounds with absorption maxima around 280 nm, suggesting that they were related to HSA (data not shown). While the resolution

between the two main peaks was acceptable, the late-eluting minor components were not resolved under these conditions and appeared as shoulders on the down slope of peak 2. As expected, the overall decrease in resolution on conventional-size columns was attributed to the increased dilution of the components as they traverse the column, a situation that generates larger peak volumes and increased peak widths. Despite the reduced peak resolution, the use of standard-size columns remains advantageous as they can be used with most HPLC systems presently on the market. They do not necessitate the use of specialized components such as detector microcells.

Peak retention times were highly reproducible for runs made within the same series, with relative standard deviations (RSD) of 0.7 % and 0.4 % (n = 6) for peaks 1 and 2 respectively. Peak areas were less reproducible with RSD values of 8.4 % and 13.8 % (n = 6) for peaks 1 and 2 respectively. For runs carried out on separate days, while the variation in peak areas was essentially the same as that for same day experiments, peak retention times varied by up to 2–3 minutes (RSD > 10 %).

A comparison of commercial HSA preparations is presented in Figure 3. The HSA product used in the

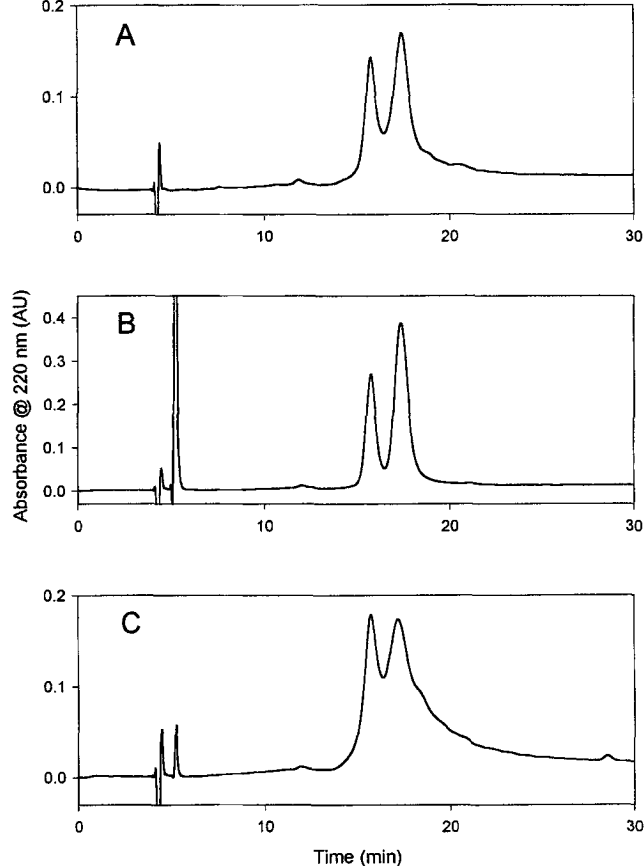


Figure 3
Comparative RP-HPLC analysis of (A) Pentex HSA, (B) Armour HSA and (C) Sigma HSA. Separation conditions as in Figure 2.

method development (Figure 3A) was compared to two other commercially available products. Although the three products contained peaks 1 and 2 as main components, substantial differences in peak widths and peak patterns suggested significant qualitative and quantitative differences in their composition. The product from Armour (Figure 3B) showed the best peak separation as well as the fewest shoulders on the down slope of peak 2. On the other hand, the product from Sigma (Figure 3C) had increased peak widths and numerous shoulders, a likely indication of the increased heterogeneity of this preparation. Similar differences had previously been observed in the CZE analysis of these preparations [18].

Analysis of HSA Fractions by CZE

Capillary zone electrophoresis separates compounds on the basis of mobility differences which are function of charge and size differences and as such it constitutes a complementary technique to RP-HPLC where hydrophobicity is the major mode of separation. In order to obtain further information on the identity of the HSA components separated by RP-HPLC, fractions were collected from consecutive HPLC runs as described in Figure 2 and were analyzed by CZE using a previously

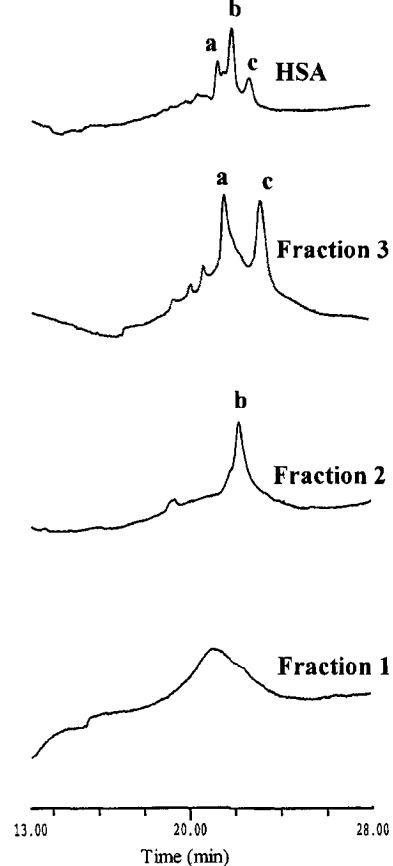


Figure 4
CZE analysis of collected RP-HPLC fractions: (from bottom to top) fractions 1, 2 and 3 and Pentex HSA (0.1 mg mL^{-1}).

reported method [18]. Figure 4 presents electropherograms of the three collected fractions and that of Pentex HSA. The electropherogram of Pentex HSA was characterized by the presence of three major peaks, a, b and c, and a number of unresolved minor peaks, most of which migrated ahead of the major components. Fraction 1, which corresponded to a small, early-eluting shoulder by HPLC, gave a broad, unresolved peak by CZE. On the other hand, fraction 2, which corresponded to HPLC peak 1, was composed mainly of peak b, one of the three major peaks observed in the HSA electropherogram (top). The remaining two major CZE peaks, a and c, were found in the fraction corresponding to the HPLC peak 2. Several minor early-migrating peaks were also found in the latter fraction. These results indicate that RP-HPLC can be effectively used to separate some of the major components present in HSA preparations.

ESI MS Analysis of HSA HPLC Components

The two major HPLC peaks 1 and 2 resolved on the narrow-bore column (Figure 1A) were characterized by ESI-MS (Figure 5) [21]. The major component of peak 1 (Figure 5A) was at 66,655 Da, which is ~ 217 higher than the calculated average mass for intact HSA. Peak 2 (Fig-

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