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DETERMINATION OF POLYMER AND PURIFICATION OF ALBUMIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic (HPLC) method is described for determining the polymer, dimer and monomer distribution in commercial albumin preparations. This method is a satisfactory substitute for conventional gel chromatography. HPLC can also be used to purify the albumin monomer from partially purified preparations. All fractions (monomer, dimer and polymer) still showed the presence of impurities when sensitive analysis by crossed immunoelectrophoresis was used, so that accurate extinction coefficients for protein determination cannot be assigned to these fractions.

INTRODUCTION

The usefulness of high-performance liquid chromatography (HPLC) for the separation of proteins by molecular sieving has been demonstrated[1–5]. Earlier methods involving gel chromatography on cross-linked dextrans, polyacrylamide or agarose took at least 16 h for a complete analysis. The use of a TSK-250 (300 x 7.5 mm I.D.) HPLC column has reduced analysis time to 20 min. Analysis for the polymer, dimer and monomer content of commercial albumin preparations has been successfully achieved by HPLC. A comparison of HPLC with gel chromatography on Sephacryl S-200 showed a good correlation by analysis of variance. Percentage compositions of the various fractions were calculated by measuring the areas under the peaks generated by monitoring the UV absorbance at 280 nm for both systems.

Most commercial preparations of albumin contain octanoic acid and N-acetyl-DL-tryptophan as stabilizer. We have tested the suitability of using the albumin monomer peak eluted from an HPLC column to establish the purity of albumin preparations by several methods: cellulose acetate (CA) electrophoresis, crossed immunoelectrophoresis (CIEP), HPLC of radioactive tracers and HPLC of the monomer peak fraction, Freezing and thawing as well as controlled lyophilization of the monomer peak fraction did not seem to alter its composition, as judged by reinjection into the HPLC column.

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EXPERIMENTAL

HPLC

Chromatography was carried out with the following components: a Waters Assoc. (Milford, MA, U.S.A.) Model 440 fixed-wavelength (280 nm) detector, a Waters Assoc. Model 6000A solvent delivery pump, a Houston Instruments Omniscribe Model B5-237-15 recorder and a TSK-250 (Bio-Rad Labs., Richmond, CA, U.S.A.) analytical column (300 x 7.5 mm I.D.) with a pre-column, packed with the same sorbent as the analytical column. The chart speed was 1 in./min and the sensitivity was 10 mV. Chromatography was performed at ambient temperature at a flow-rate of 1 ml/min. Approximately 20 min were required per separation.

Gel chromatography

Chromatography was carried out on a 100 x 2.6 cm I.D. column, packed with Sephacryl S-200, in conjunction with an LKB Uvicord-S detector (280 nm), an LKB Multiperpex Model 2132 pump and a Houston Instruments Omniscribe Model B5 237-15 recorder, chart speed 1 in./h, sensitivity 10 mV. Chromatography was performed at ambient temperature at a flow-rate of 25 ml/h and took at least 16 h per separation.

Reagents und solvents

HPLC equilibration and elution buffer: 0.07 *M* sodium phosphate 0.05 % sodium azide (pH 7); ionic strength, 0.15; filtered prior to use through a 0.22-μm membrane filter.

Gel chromatography equilibration and elution buffer: 0.5 A4 sodium chloride–0.1 *M* Tris–0.1% sodium azide (pH 8).

HPLC procedure for molecular weight determination

A vial of lyophilized gel-filtration standard (thyroglobulin, immune serum globulin, ovalbumin, myoglobin, vitamin B,,) was reconstituted with 1 ml of distilled water, and 10 μl of the mixture were injected into the HPLC column. After a 20-min development of the chromatogram, the retention times were measured for each individual protein. The theoretical molecular weight of each individual protein was plotted against its corresponding retention time on semi-logarithmic graph paper. The molecular weight of the sample was read from the graph.

Gel chromatography for molecular weight determination

Two vials of a reconstituted (1 ml of water per vial) gel-filtration standard (Bio-Rad Labs.) were pooled. A 2-ml volume of the reconstituted standard was injected on to the Sephacryl S-200 column. After development for at least 16 h, the retention time was measured for each individual protein. The theoretical molecular weight of each individual protein was plotted against its corresponding retention time on semi-logarithmic graph paper. The molecular weight of the sample was read from the graph.

Sample preparation

Bradford protein analysis. The collected peak fraction (i.e., monomer, dimer and polymer) were concentrated to approximately 10% of the original volume by

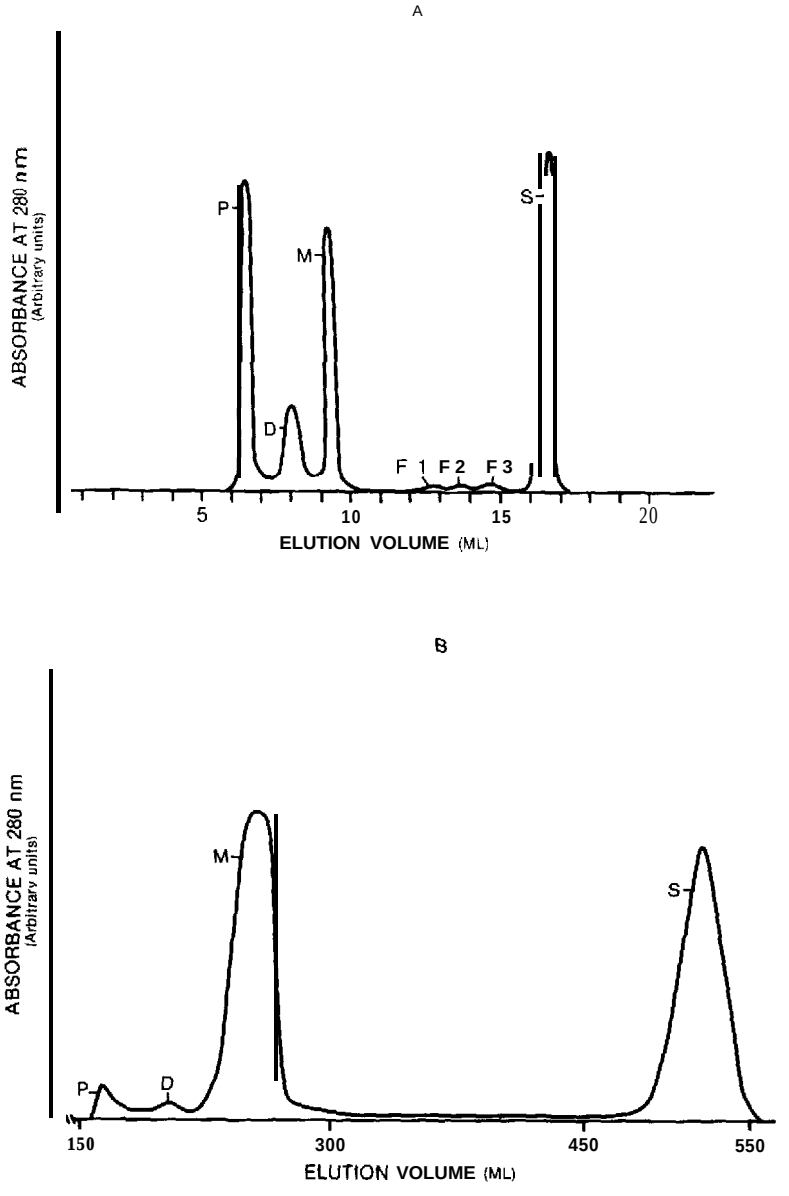

Fig. 1. Albumin in Chromatograms. (A) HPLC. Column, TSK-250 (300 × 7.5 mm I.D.); eluent, 0.07 *M* sodium phosphate buffer (pH 7), ionic strength 0.15; flow-rate, 1 ml/min; sample volume, 10 μl; chart speed, 1 in./min; detector, 280 nm; attenuation, polymer and dimer fractions = 0.1 a.u.f.s., fragments, monomer and stabilizer 2.0 a.u.f.s. M = monomer; D = dimer; P = polymer; F = fragment; S = stabilizer. (B) Gel chromatography. Column. Sephacryl S-200 (90 × 25 cm): solvent. Tris–NaCl buffer (pH 8); flow-rate. 25 ml/h; sample volume. 4 ml; chart speed, 1 in./h; detector. 280 nm; attenuation. 2 a.u.f.s. Abbreviations as in (A); No F detected.

TABLE I

ANALYSES OF VARIANCE FOR THE DIFFERENT FRACTIONS OF A COMMERCIAL ALBUMIN PREPARATION

Fraction	Mean (%)	*Standard deviation*	*Coefficient of variation (%)*	*F ratio*	*p*	*95% Confidence interval*
Monomer	88.82	2.253	2.536	0.36	0.5494	-0.228 to 0.930
Dimer	3.08	0.910	29.515	7.46	0.0081	0.243 to 1.111
Polymer	8.08	1.867	23.102	0.44	0.5103	-0.384 to 1.110

means of a Centriflo CS-25 membrane (Amicon, Lexington, MA, U.S.A.). The concentrated samples were used for protein determination by the Bradford method[6].

Cellulose *acetate and crossed-immunoelectrophoresis.* The samples from the Centriflo concentrator were further concentrated to at least 2 % protein for the polymer and dimer and to 5 % protein for the monomer fractions by means of a Minicon Concentrator CS 15 (Amicon).

Cellulose acetate electrophoresis

A small amount of each sample was applied to the surface of a cellulose acetate membrane (Beckman Microzone cell). The sample was electrophoresed for 17 min with barbital buffer (pH 8.6) at a constant voltage of 250 V and a current of between 3.5 and 6.0 mA per membrane, The electropherogram was stained with Ponceau S for IO min, then rinsed with 5 % aqueous acetic acid. The membranes were cleared with 22 % ethanolic acetic acid for 2 min, then the plate was allowed to dry in a pretreated oven, set at 90°C, for at least IO min or until the membrane was dry and clear. A Beckman Model R 112 densitometer was used for scanning.

Crossed immunoelectrophoresis

The sample was applied to the appropriate sample well (3 μl for the monomer, 6 μl for the dimer and polymer fractions) on 1 % agarose gel. After electrophoresis for 1.5 h with Tris-tricine buffer (pH 8.6) at 10 V/cm, 250 mA and 100 W, the gel was cut and transferred to another gel plate. The empty part of the plastic plate was filled with 8 ml of 1 % agarose gel, containing 0.9 ml of a polyvalent antibody (antibody to whole human serum). The conditions for electrophoresis in the second dimension were the same as in the first, except that the voltage was reduced to 2 V/cm. After overnight electrophoresis, the gel was washed with distilled water, rinsed in 0.9 %

TABLE II

MOLECULAR WEIGHTS OF DIFFERENT FRACTIONS DETERMINED BY HPLC AND SEPHACRYL S-200 METHODS

Fraction	*HPLC*	*Sephacryl S-200*
Monomer	65,000	63,200
Dimer	131,000	147,000
Polymer	> 1,000,000	> 1,000,000

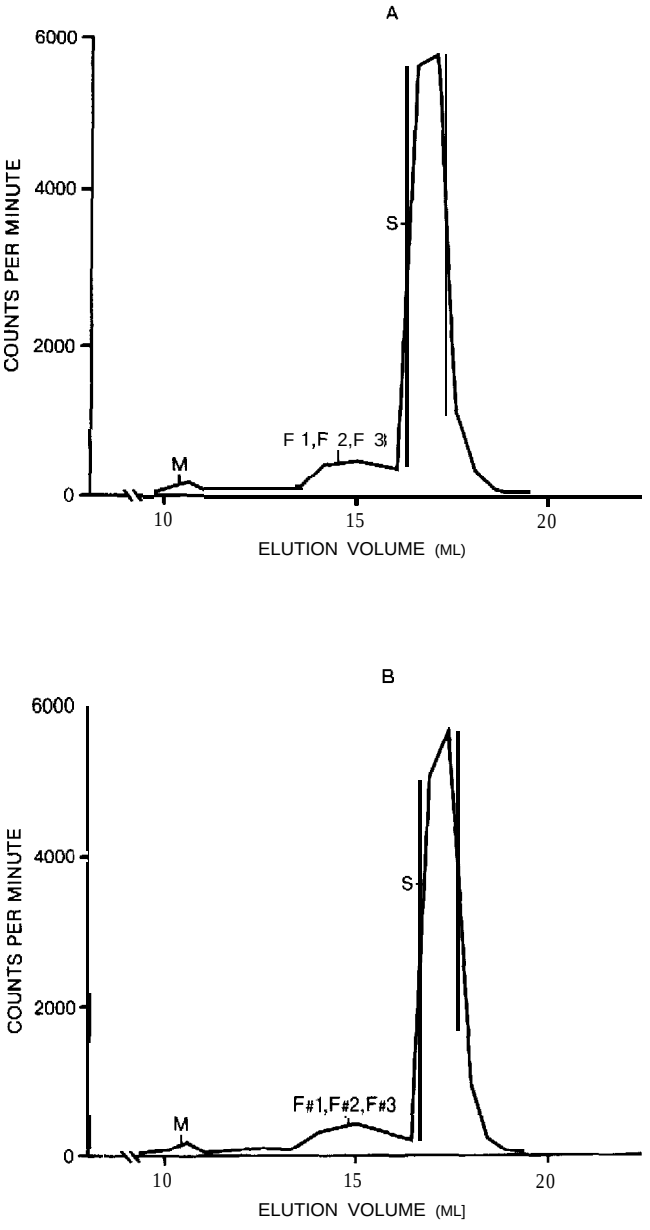

Fig. 2. (A) Chromatogram of unheated 5 % normal serum albumin, spiked with [^{14}C]octanoic acid. Conditions as in Fig. 1A, except chart speed, 0.5 in./min; detector, beta scintillation counter; scintillation cocktail, Aquasol. The radioactivity counts were mostly in the stabilizer region (between 15 and 20 min retention time). (B) Chromatogram of heated 5 % normal serum albumin, spiked with [^{14}C]octanoic acid. Conditions as in (A), except heating condition, 10 h at 60°C. The radioactivity counts were mostly in the stabilizer region (between 15 and 20 min retention time).

sodium chloride solution for 30 min and pressed. After staining with Coomassie Blue **R250** for 1 min, the electropherogram was destained with ethanol-acetic acid-water (9:9:2), pressed and finally dried with a hair dryer.

RESULTS AND DISCUSSION

Typical HPLC and gel chromatograms are presented in Fig. 1A and B. Several

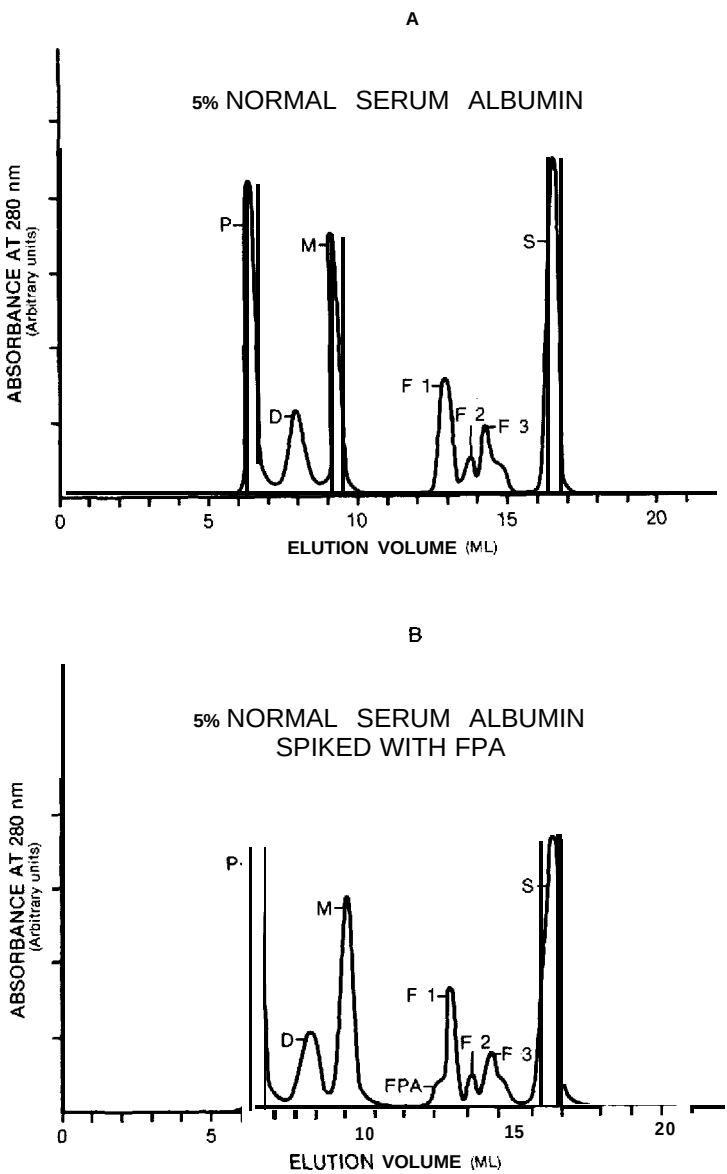

Fig. 3. (A) Chromatogram of 5 % normal serum albumin. Conditions as in Fig. 1A. (B) Chromatogram of 5 % normal serum albumin, spiked with FPA. Conditions as in Fig. 1A; FPA = fibrinopeptide A.

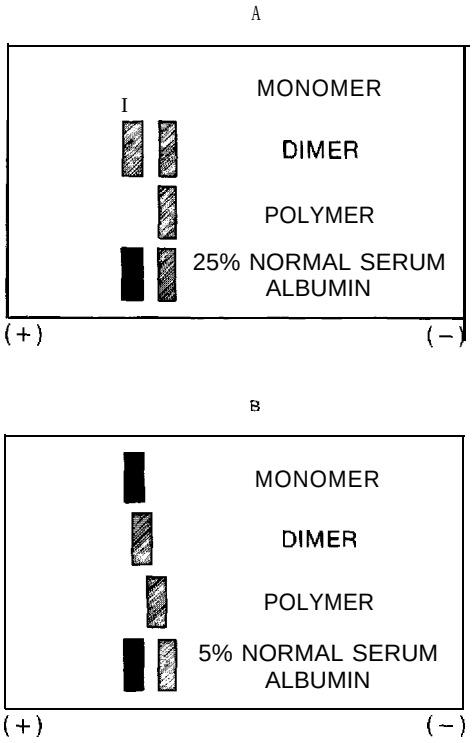

Fig. 4. (A) Cellulose acetate electropherogram of 25% normal serum albumin. (B) Cellulose acetate electropherogram of 5 % normal serum albumin. For conditions, see Cellulose *acetate electrophoresis.*

different commercial protein solutions (5 % normal serum albumin, 25 % normal serum albumin, 5 % plasma protein fraction) were repeatedly analyzed on different days for statistical evaluation of both methods (Table I). The results showed good correlation for the monomer and polymer determinations, whereas very slight discrepancies were noted in the dimer content. The molecular weights of the albumin peaks, as determined by HPLC and gel chromatograms, are compared in Table II.

The albumin monomer fractions, obtained by HPLC, were collected, pooled and lyophilized or frozen, The monomer peak did not show any change in retention time and the peak showed no shoulder, even after freezing or lyophilization.

Radiolabeled bradykinin ([^{125}I]BK) and octanoic acid ([^{14}C]octanoic acid) were added to the albumin solution and chromatographed to determine their retention times. The results showed that only 1–2% of the added radioactivity from [^{14}C]octanoic acid remained with the monomer peak, whereas the bradykinin peptide was completely separated from the monomer peak. There was no difference in results, whether the albumin was heated or not heated after the labeled octanoic acid addition (Fig. 2A and B). Fibrinopeptide A, a peptide also known to be present in commercial albumin preparations, showed as a leading shoulder of fragment peak 1. (Fig. 3A and B).

Fig. 4A and B show the purity of the monomer peak, as determined by cellulose acetate electrophoresis. The albumin monomer fraction from commercial al-

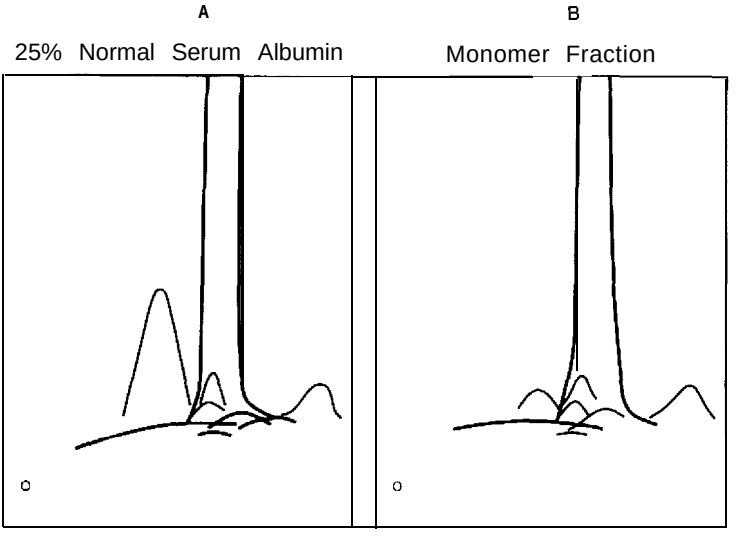

Fig. 5. (A) CIEP pattern of 25 % normal serum albumin. (B) CIEP pattern of 25 % normal serum albumin monomer fraction. For conditions, see *Crossed immunoelectrophoresis*.

bumin preparations when electrophoresed showed the absence of α- and /&globulin-like material. Although analyses showed homogeneity of the monomer peak by CA electrophoresis and rechromatography by HPLC, determination by CIEP still showed the presence of non-albumin materials (Fig. 5A and B).

The relative polymer, dimer and monomer contents of the albumin preparation were determined by either the cut-and-weigh or the integration method. The pooled monomer. dimer and polymer fractions were subsequently analyzed for protein con-

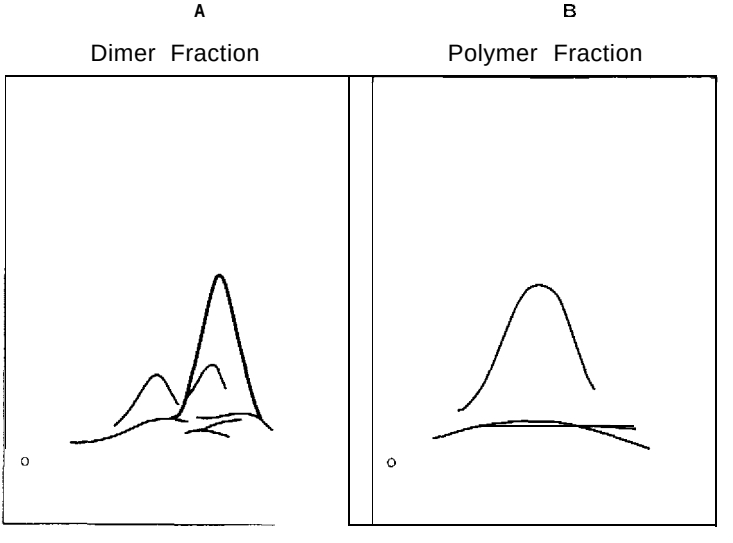

Fig. 6. (A) CIEP pattern of 2.5 % normal serum albumin dimer fraction. (B) CIEP pattern of 25 % normal serum albumin polymer fraction. For conditions, see *Crossed immunoelectrophoresis*.

TABLE III

RELATIVE MONOMER, DIMER AND POLYMER CONTENTS OF A 5% NORMAL SERUM ALBUMIN, AS DETERMINED BY THE BRADFORD AND HPLC METHODS

Fraction	*Relative content (%)*	
	Bradford	***HPLC***
Monomer	93.5	93.6
Dimer	5.5	2.8
Polymer	1.0	3.6

tent by the Bradford method, The Bradford method is based on the formation of a complex between the protein and Coomassie Blue R250 dye. The results obtained are compared in Table III. While the monomer content was in agreement with the results of the Bradford method, the polymer and dimer peak analyses were not. This discrepancy indicates heterogeneity of the polymer and dimer peaks as shown by the CA and CIEP results (Fig. 6A and B). This heterogeneity of the polymer and dimer would account for the differences in extinction coefficient and consequently protein content.

It can be concluded that the HPLC method is a suitable substitute for the conventional gel chromatography as well as a method for further albumin purification

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