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Note

High-performance liquid chromatographic separation of hepatitis B immunoglobulin and aggregates

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The majority of infants infected with hepatitis B virus during gestation become chronic carriers themselves, and can subsequently develop potentially fatal chronic hepatitis, cirrhosis and primary hepatocellular carcinoma[1]. Treatment of these infants at birth with a combination of heptatitis vaccine and heptatitis B immunoglobulin (H-BIG) has been found to be highly effective in preventing transmission of the infection[1,2].

Current assay procedures for H-BIG specify not less than 80% monomeric immunoglobulin G[3], a value determined by time-consuming ultracentrifugation. High-performance liquid chromatography (HPLC) has been shown to provide separation of protein aggregates using TSK gel permeation columns[4] which correlate well with separations obtained by column chromatography. The following describes the separation of H-BIG under similar conditions and correlation of the analytical results generated with ultracentrifugation data for seventeen stability samples.

EXPERIMENTAL

Sodium chloride, sodium azide, and potassium hydrogen phosphate were reagent-grade and used as received. Blue dextran, ovalbumin, bovine serum albumin and α-chymotrypsinogen A molecular weight standards (Sigma, St. Louis, MO, U.S.A.) were used as received and dissolved in distilled water in appropriate proportions. Highly purified H-BIG of known protein content was used as a retention time marker and prepared by repetitive centrifugation and alcoholic precipitation at low (−6 to +2°C) temperatures from plasma from human donors.

A TSK G3000SW column (30 cm × 7.5 mm I.D.) was used (Alltech Assoc., Deerfield, IL, U.S.A.). The mobile phase consisted of 0.1 *M* potassium hydrogen phosphate, 0.1 *M* sodium chloride and 0.005 *M* sodium azide adjusted to pH 6.0 with orthophosphoric acid, then filtered through 0.45-μm polycarbonate membranes (Nuclepore, Pleasanton, CA, U.S.A.) and degassed before use. The flow-rate at ambient column temperature was 0.5 ml/min with typical column backpressures of 200 to 300 psi. The chromatographic system consisted of a Beckman 112 pump, 210 injector, and 165 variable-wavelength detector (Beckman Instruments, Fullerton, CA, U.S.A.) set at 280 nm (UV). A Hewlett-Packard 3390A integrator was used to calculate integrated areas; peak height measurements were obtained manually. Pur-

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TABLE I

PRECISION OF MEASUREMENT OF H-BIG AND POLYMERIC FORMS BY HPLC AND ULTRACENTRIFUGATION

Trimer is equivalent to the 12S form, approximately 389 000 MW; dimer is equivalent to 10S form, approximately 208 000 MW; H-BIG monomer is equivalent to the 7S form, approximately 108 000 MW; less than monomer is equivalent to the 3–5S forms, approximately 41 000 MW. R.S.D. = Relative standard deviation (%).

	Peak area (%)				*Peak height (%)*			
	Trimer (12S)	*Dimer (10S)*	*H-BIG (7S)*	*LT Monomer (3.5S)*	*Trimer (12S)*	*Dimer (10S)*	*H-BIG (7S)*	*LT Monomer (3.5S)*
Mean	1.31	10.54	77.58	10.58	3.94	12.55	72.14	11.37
S.D.	0.11	0.08	0.45	0.44	0.25	0.26	0.33	0.26
R.S.D.	8.2	0.8	0.6	4.2	6.4	2.1	0.5	2.31
Range	0.36	0.29	1.26	1.16	0.79	0.70	0.96	0.77

ified H-BIG at a level of 0.3% protein/ml was injected as a retention time marker.

Ultracentrifugation data were obtained on a Beckman Model E analytical centrifuge at controlled temperature and a rotor speed of 60 000 rpm (260 000 *g*). Sodium phosphate buffer of nominal pH 7 and ionic strength (μ) = 0.20 was placed in one sector of a 12-mm double sector cell fitted with schlieren window holders and sapphire windows, while the other sector contained sample at a concentration of 10–15 mg/ml H-BIG. Protein movement was photographically recorded in 16-min intervals, and peak area percent values were obtained from the average of five replicate planimeter measurements of the areas under each peak.

RESULTS AND DISCUSSION

The precision of the method was determined by calculating peak area and height percent values for eight replicate injections of a single sample. These data are

TABLE II

LINEARITY OF DETECTOR RESPONSE TO H-BIG

*% Protein**	*Peak area response (counts)*	*Peak height response (mm)*
0	0	0
3.42	1 731 800	10.0
6.85	3 296 400	19.1
10.27	5 338 700	29.4
13.70	6 627 800	39.7
17.12	8 477 100	49.0
20.54	10 016 000	57.0
Corr. Coeff.	0.9993	0.9995
Slope	$2.04 \cdot 10^{-6}$ %/counts	0.36%/mm
Y-Intercept	−0.08% protein	−0.09% protein
X-Intercept	41 178 counts	0.26 mm

* %Protein calculated from Kjeldahl nitrogen determination.

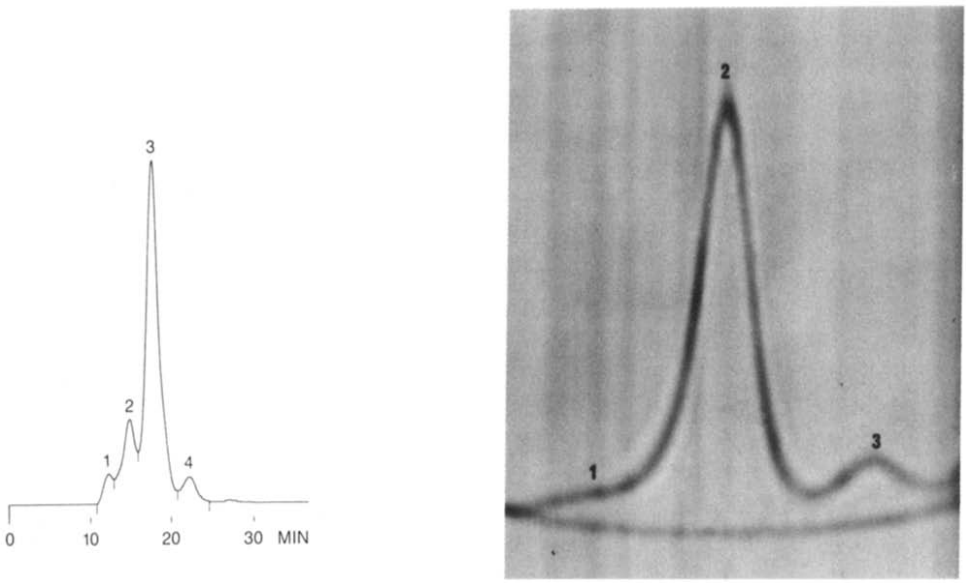

Fig. 1. Comparison of HPLC and ultracentrifugation results. (A) HPLC chromatogram showing trimer (1), dimer (2), monomer (3) and less than monomer (4) forms of H-BIG. (B) Ultracentrifugation trace of the same material showing 3–5S (1), 7S (2) and 10S (3) forms of H-BIG.

presented in Table I, while Table II gives the results of a linearity study performed on the basis of percent protein in the samples injected. From these studies the height measurements emerged as slightly more precise than area values, presumably a reflection of the integrator's difficulty in discerning the fairly broad, low-level peaks present in these samples. These results are consistent with those seen for similar measurements of other protein systems[5].

Seventeen samples of H-BIG stressed under different temperatures for different periods of time were analyzed by both HPLC and ultracentrifugation; comparative traces for the two methods can be found in Fig. 1. Table III contains HPLC peak area percent values for the predominant fractions of each sample with the corresponding data obtained by ultracentrifugation. Comparison of the dimer and monomer values by a paired t-test yielded -0.04 and 0.09, respectively, for LC *vs.* ultracentrifugation ($t = 1.33$; $p = 0.20$).

Beyond the dimer and trimer values, agreement between the two methods suffers. Because the HPLC column has an optimal range of separation, components with molecular weights at the high and low extremes will not be well resolved. Therefore, any component with molecular weight larger than approximately 300 000 will elute at the void volume of the column and be calculated as trimer. This artifact of the separation explains why only one sample was reported to contain trimer by the ultracentrifugation method while trimer was reported in almost all samples by HPLC. Should more detailed information concerning the distribution of high-molecular-weight components be desired, a more approximately gel permeation column would be in order.

When viewed in the context of a stability indicating assay, the HPLC data confirm the results expected: at higher temperatures, greater aggregate formation

TABLE III

COMPARISON OF HPLC PEAK AREA (%) AND ULTRACENTRIFUGATION MEASUREMENTS

N.D. = Not detected; RT = room temperature.

Sample No.	*Time (months)*	*Temp. (°C)*	*HPLC*				*Ultracentrifugation*			
			12S	*10S*	*7S*	*3.5S*	*12S*	*10S*	*7S*	*3.5S*
1	9	2–8	0.7	17.9	81.4	ND	ND	20.0	76.2	3.8
2	9	RT	1.5	13.0	72.1	13.4	ND	11.7	68.7	19.6
3	9	37	0.9	12.0	75.1	8.9	3.5	11.2	70.3	15.0
4	48	2–8	1.5	18.0	76.6	3.8	ND	17.8	75.3	6.9
5	48	RT	1.4	10.3	70.3	18.0	ND	10.0	70.1	19.9
6	6	2–8	1.6	19.4	79.0	ND	ND	17.8	76.3	5.9
7	6	2–8	1.3	17.7	81.0	ND	ND	17.8	78.1	4.1
8	3	2–8	1.2	17.3	81.4	ND	ND	18.1	81.9	ND
9	3	RT	1.0	16.0	82.9	ND	ND	13.1	86.9	ND
10	3	37	1.9	13.9	80.7	3.0	ND	9.6	87.8	2.6
11	0	2–8	1.1	16.3	82.5	ND	ND	15.1	84.9	ND
12	1	2–8	0.2	12.7	87.1	ND	ND	15.4	84.6	ND
13	1	RT	0.2	12.2	87.6	ND	ND	13.2	86.8	ND
14	1	37	0.4	10.9	88.8	ND	ND	11.8	88.2	ND
15	0	2–8	0.3	12.9	86.6	ND	ND	15.1	84.9	ND
16	0	2–8	1.9	10.4	86.7	ND	ND	16.7	83.3	ND
17	96	2 8	0.7	9.8	71.9	17.6	ND	14.3	68.6	17.1

occurs with a concomitant decrease in monomer. At lower temperatures this trend is less noticeable, in some cases more aggregation occurs in a short time period at higher temperatures than in a far longer period at lower temperatures. In addition, increases in degradation products are found at all temperatures with time, in some cases, at the expense of both monomer and trimer peaks.

The high correlation between HPLC and ultracentrifugation results indicates that HPLC can be used as a substitute for the more expensive and time-consuming ultracentrifugation method for determination of protein composition. Sample throughput using the proposed technique is far higher than can be obtained for ultracentrifugation, and quantitation can be performed easily with high precision.

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