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Serum protein determination by high-performance gel-permeation chromatography[1]

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Abstract

A general high-performance gel-permeation chromatography (HPGPC) method was developed to determine protein in human serum with improved sensitivity and speed. The optimum UV wavelength for protein detection was found to be 210 nm, by comparing the protein values obtained by varying the UV wavelength of the HPLC detection system with the protein values obtained from spectrophotometric protein assays, i.e., the bicinchoninic acid (BCA) method and the biuret method. The analysis time was less than 1 min. Since this HPGPC serum protein assay method is simple and rapid, it is expected to be particularly well adapted for use in clinical laboratories. © 1997 Elsevier Science B.V.

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1. Introduction

To date, protein assays have been performed mainly according to the method of Lowry et al. [1], which has been shown to be applicable in almost every area of biochemical research. However, various reagents in the protein solution interfere with this assay. A method was developed by Smith et al. [2] to overcome the problems of interference caused by non-ionic detergents, using bicinchoninic acid (BCA). However, interferences caused by sulfhydryl reagents (2-mercaptoethanol, dithioerythritol, dithiothreitol, thioglycolate and thimerosal), N-acetylglucosamine, serotonin, epinephrine and ascorbic acid, which occur with the method of Lowry et al., and the BCA method, remain to be overcome. Another spectrophotometric biuret method [3] has also been known to be subject to interferences caused by the turbidity of serum, bilirubin, ammonium ions and sucrose.

Previously, we developed a high-performance gel-permeation chromatographic (HPGPC) assay for urinary proteins and albumin using a non-ionic

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detergent, Brij-58 (polyethyleneglycol 1000 monocetylether), and a high concentration of glycerol (50%, v/v) in the eluent [4]. This high-performance liquid chromatographic (HPLC) protein assay method is simple, i.e., only filtration and dilution are required before analysis. Furthermore, since detection of the protein is performed by direct measurement of the intrinsic UV absorbance of proteins and does not require chemical or derivatization reactions, a wide range of linear correlation curves [from 10 to 4000 ng of bovine serum albumin (BSA)] could be obtained with this HPGPC urinary protein assay method [4].

In order to apply this HPLC assay to the determination of urinary proteins in serum, we further studied the wavelength of UV absorbance detection using human serum from healthy children ($n=16$). Spectrophotometric protein assays using the BCA and the biuret methods were used as the reference assay methods.

2. Experimental

2.1. Chemicals and reagents

Bovine albumin (Cohn fraction V), human albumin (Cohn fraction V), bovine gamma-globulins (Cohn fractions II and III) and human gamma-globulins (Cohn fractions II and III) were purchased from Sigma (St. Louis, MO, USA). Brij-58, copper (II) sulfate pentahydrate, glycerol and potassium tartrate were from Wako Pure Chemicals (Osaka, Japan). A BCA protein assay kit was from Pierce (Rockford, IL, USA). The HPGPC column was a Develosil 100 Diol-5 column (35×8 mm I.D., 10 nm average pore diameter, 5 μm average particle size; Mfg. No. 1405331) from Nomura (Aichi, Japan). Standartips (100 and 1000 μl) were from Eppendorf-Netheler-Hinz (Hamburg, Germany). Ekicrodisc 3 (0.45 μm average pore diameter) was from Gelman Sciences (Ann Arbor, MI, USA).

2.2. Specimens

Fresh human serum ($n=16$; male, 10; female, 6) from healthy children (age: mean, 11.7±2.6 years; range, 5.5–15.6 years) was kindly donated by the Division of Endocrinology and Metabolism of the National Children's Hospital (Tokyo, Japan). The stature of each of these healthy subjects was within one standard deviation from the average stature for children of the same age. These healthy subjects were not treated with any drugs. Serum specimens were stored at −80°C.

2.3. High-performance liquid chromatography

A Model LC-10AT or 10AD pump (Shimadzu, Kyoto, Japan) was used. The injector was a Model U-6K (diaphragm type; 2 ml or 0.05 ml sample loading loop, Waters, Milford, MA, USA). Detection was carried out using a Model L-4000 UV detector (Hitachi, Tokyo, Japan). A Model 561 recorder (Hitachi) and a Chromatopac C-R6A data processor (Shimadzu) were also used. A line filter (GL Sciences, Tokyo, Japan) was inserted between the injector and the column. Eluent (2.0 l) was prepared as follows: first, 1.0 l of 0.2 *M* sodium phosphate buffer (pH 5.3) containing 0.6 *M* sodium chloride was made. Then, 1.0 l of glycerol and 20 ml of Brij-58 (dissolved in a water bath at 60°C) were added and mixed thoroughly (this usually took 30 min or more), to give final concentrations of sodium phosphate (0.1 *M*), sodium chloride (0.3 *M*), glycerol (50%, v/v) and Brij-58 (1%, v/v). The flow-rate was 2.0 ml/min and the column inlet pressure was 140 kg/cm^2 at a column temperature of 35°C. A column oven, Model CTO-10 AC (Shimadzu) was used. Detection of serum protein was performed at various wavelengths in the ultraviolet region and these were then compared.

2.4. HPLC serum protein assay

Fresh serum (0.04 ml) was dissolved in 0.36 ml of the serum dilution solution (0.1 *M* sodium phosphate, pH 7.0, containing 1 m*M* EDTA and 10% (v/v) glycerol). A 0.04-ml volume of this ten-fold diluted serum was further dissolved in 0.76 ml of the neutral HPGPC eluent (adjusted to pH 6.8). This 200-fold diluted serum sample was filtered through Ekicrodisc 3, and a 0.005-ml volume of the filtrate was injected into the HPLC system. Standard BSA (2.0 mg/ml; BSA standard solution included in the Pierce BCA protein assay kit or BSA from Sigma,

dissolved in an aqueous 0.9% NaCl, 0.02% sodium azide solution) was diluted ten-fold with this neutral HPGPC eluent, and 0.01 ml (2.0 μg of BSA) was injected into the HPGPC system (external standard). The analysis time was less than 1 min. The serum protein concentration was calculated as follows;

$$p = \frac{h_2}{h_1} \cdot b \cdot F \cdot \frac{1}{v}$$

where, p = concentration of serum protein; h_1 = peak height (mm) of standard BSA; h_2 = peak height (mm) of sample; b = amount (mg) of standard BSA; F = dilution factor (200) and v = injected volume (ml) of sample (0.005 ml).

2.5. Spectrophotometric serum protein assays

In order to compare the values obtained by the HPLC method with the spectrophotometric values, serum protein contents were independently assayed either by the BCA method [2] or the biuret method [3]. A Model U-3200 (Hitachi) spectrophotometer was used.

(1) BCA method: a BCA protein assay kit from Pierce was used. The stock albumin standard (2 mg/ml) supplied with this kit was used as the standard protein. Serum was diluted 100-fold with the serum dilution solution, as described above. A 0.01-ml volume of diluted serum was used. The calibration curve was prepared by adding 0.01 ml of the serum dilution solution in order to correct for the blank. The calibration curve was prepared from 0 to 0.20 mg/ml (0, 0.05, 0.10, 0.15 and 0.20 mg/ml).

(2) Biuret method: the method of Gornall et al. [3] was used. BSA (10 mg/ml dissolved in the serum dilution solution, Sigma) was used as a standard protein. A 0.02-ml volume of serum was used directly. The calibration curve was prepared from 0 to 5 mg/ml (0, 1, 2, 3, 4 and 5 mg/ml).

2.6. Statistics

Statistical parameters were calculated using a personal computer with Statflex Lite Version 2 software (J.I.P., Tokyo, Japan). Both parametric and non-parametric statistics were used; i.e., Pearson's correlations (r) and Spearman's rank correlations (rS) for correlating the variables, respectively. Difference plot analysis was also performed according to Pollock et al. [5].

3. Results and discussion

In order to achieve rapid analysis of serum proteins, we used a short column (35×8 mm I.D.). As an example, diluted standard albumin (0.01 ml; 2 μg) and a diluted serum sample (0.005 ml) were injected into the HPLC system. The results are shown in Fig. 1, chromatograms 1 and 2, respectively. As shown in Fig. 1 (chromatograms 1 and 2), the excluded standard albumin (front peak in chromato-

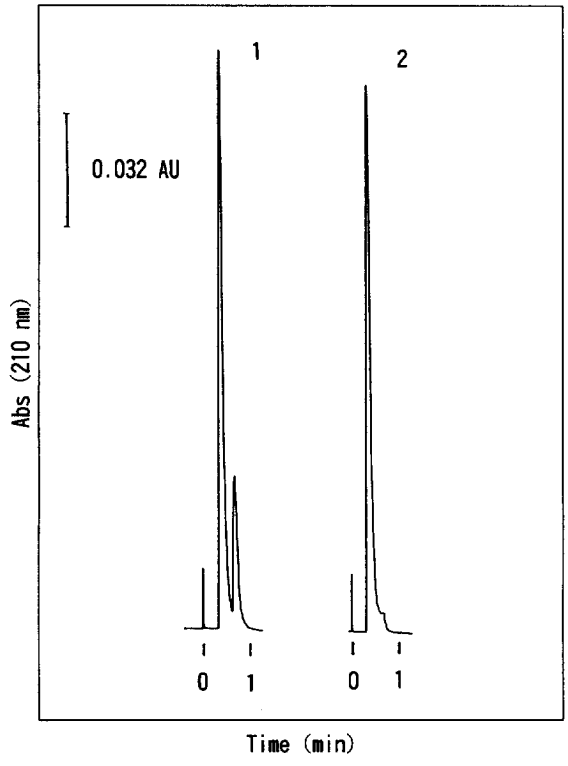

Fig. 1. Typical example of serum protein analysis using the HPGPC method with a 35×8 mm I.D. Develosil 100 Diol-5 column (10 nm average pore I.D.). Chromatogram 1, 2.0 μg of BSA standard was injected. Chromatogram 2, 0.005 ml of a 200-fold diluted serum sample (75.5 mg/ml) was injected. Other conditions are as described in Section 2.

gram 1) and proteins (front peak in chromatogram 2) appeared at a retention time of 21.6 s at the flow-rate of 2.0 ml/min. Further, chromatogram 1 of Fig. 1 showed the separation of the interference peak due to azide (rear peak) and of the albumin peak (front peak). The peak height of albumin, detected at 210 nm, correlated linearly with the amount of BSA injected, between 0 and 4 μg (0, 1, 2, 3 and 4 μg). The method used to calculate the serum protein concentration for Fig. 1 is described in Section 2.

Repeatability was tested by injecting 2.0 μg and 40 ng of BSA seven times; peak heights at 210 nm were 117±1.29 mm [0.32 AUFS, coefficients of variation (C.V.)=1.1%] and 66.9±1.56 mm (0.01 AUFS, C.V.=2.3%), respectively. Day-to-day repeatability was also tested by injecting 2.0 μg of BSA seven times on five consecutive days; average peak heights were 118.8±2.28 mm (C.V.=1.9%). The lower limit of detection was 2 ng of BSA (signal-to-noise ratio=3). Therefore, this method is sufficiently sensitive and reproducible to measure the protein concentration of serum.

Since serum protein concentrations were relatively high, a suitable dilution method for the serum is essential. Previously, Chauhan and Dakshinamurti [6] found that human serum biotinidase was stable at high concentrations (more than 0.05 *M*) in neutral phosphate buffer. We considered that this stability of human serum biotinidase in phosphate buffer to be due to homogeneous dispersion of the protein enzyme and/or prevention of protein aggregation. Then, we successfully used neutral phosphate buffer containing glycerol and EDTA as the dilution solution for serum (see Section 2).

In order to find the optimum wavelength for serum protein analysis, we measured the serum protein content at various wavelengths in the UV absorbance region, i.e., at 200, 205, 210, 215, 220, 225, 254 and 280 nm using sixteen sera from healthy children. The serum protein values obtained by the HPLC method were statistically compared with those obtained with the spectrophotometric biuret and BCA methods. The results obtained at shorter wavelengths are summarized in Table 1. The longer UV wavelengths of 254 and 280 nm were unexpectedly useless, since serum protein values at these wavelengths were very high. The protein concentration in hemolyzed serum could be measured at shorter UV wavelengths.

Table 1
Comparison of serum protein determinations using a HPLC method at different wavelengths and spectrophotometric biuret and BCA protein assays[a]

Methods	Average±S.D. (mg/ml)
Spectrophotometric	
Biuret method	83.5±5.64
BCA method	75.1±5.68
HPLC–UV method	
at 200 nm	76.9±7.54
at 205 nm	70.9±7.10
at 210 nm	70.2±6.82
at 215 nm	69.0±6.25
at 220 nm	63.7±6.15
at 225 nm	69.8±7.07

Correlations (*r* and *r*S) between biuret and BCA values, and HPLC values

	Biuret		BCA	
	r	*r*S	*r*	*r*S
at 200 nm	0.732[c]	0.774[c]	0.658[c]	0.596[d]
at 205 nm	0.813[b]	0.823[c]	0.752[b]	0.650[c]
at 210 nm	0.863[b]	0.860[c]	0.753[b]	0.662[c]
at 215 nm	0.804[b]	0.767[c]	0.835[b]	0.732[c]
at 220 nm	0.784[b]	0.668[c]	0.777[b]	0.750[c]
at 225 nm	0.820[b]	0.804[c]	0.792[b]	0.734[c]

[a] Fresh healthy serum ($n=16$) was used. Spearman's rank correlation coefficient (*r*S) and Pearson's correlation coefficient (*r*) were used. [b] $p<0.001$; [c] $p<0.01$; [d] $p<0.05$.
Conditions were as described in Section 2.

However, contaminating hemoglobin from the hemolyzed serum elevated the protein value tremendously at 254 or 280 nm (data not shown). As shown in Table 1, HPLC analysis at 210 nm showed the highest correlation to values obtained using the biuret method ($r=0.863$, $p<0.001$; Fig. 2) and a high correlation to values obtained using the BCA method ($r=0.753$, $p<0.001$; Fig. 3). It was also found that the relatively lower correlations (*r* and *r*S) between spectrophotometric protein values obtained using the BCA and biuret methods were 0.746 ($p<0.001$) and 0.667 ($p<0.01$), respectively (Fig. 4). Although HPLC values obtained at 215 nm may be equally useful from a statistical point of view, the average protein value obtained by HPLC at 210 nm (70.2 mg/ml) was closer to that obtained using the BCA method (75.1 mg/ml) and the biuret method (83.5 mg/ml) than the HPLC value obtained at a

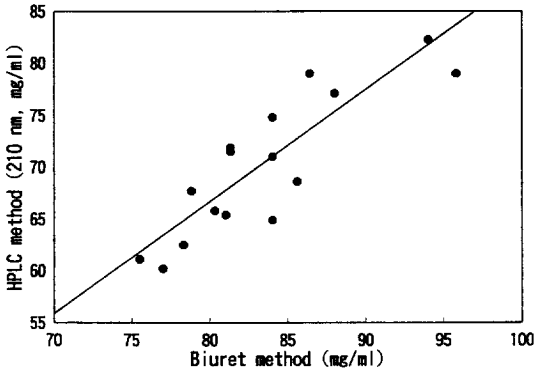

Fig. 2. Correlation between the HPLC method (at 210 nm) and the biuret method ($n=16$). $r=0.863$ ($p<0.001$); $y=-16.91+1.043x$. $rS=0.860$ ($p<0.01$).

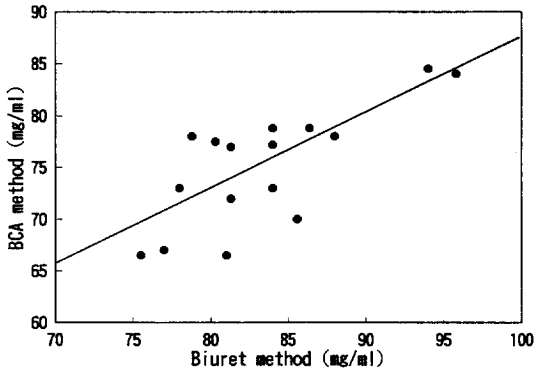

Fig. 4. Correlation between the biuret method and the BCA method ($n=16$). $r=0.746$ ($p<0.001$); $y=12.41+0.7513x$. $rS=0.677$ ($p<0.01$).

wavelength of 215 nm (69.0 mg/ml). Difference plot analyses were performed and essentially no differences were observed between the HPLC values obtained at 205, 210 and 215 nm, and the values obtained with the BCA and biuret methods (data not shown). Therefore, we concluded that the optimum wavelength for serum protein determination was 210 nm.

Thus, it is shown that this HPLC method for serum protein measurement is simple and applicable to clinical protein analysis of human serum.

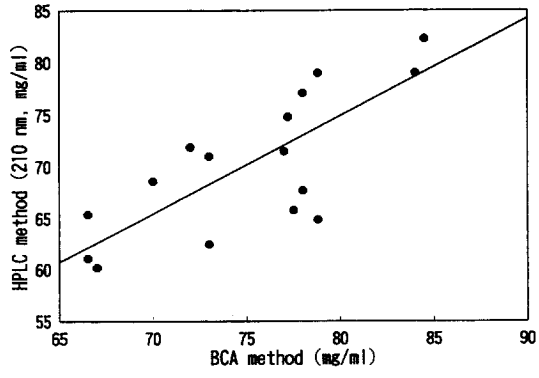

Fig. 3. Correlation between the HPLC method (at 210 nm) and the BCA method ($n=16$). $r=0.753$ ($p<0.001$); $y=2.248+0.9043x$. $rS=0.662$ ($p<0.05$).

Acknowledgments

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