Serum globulins contribute to the discrepancies observed between the bromocresol green and bromocresol purple assays of serum albumin concentration

Y. XU, L. WANG, J. WANG, H. LIANG and X. JIANG
Department of Clinical Laboratories, The Fourth Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province, P. R. China

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ABSTRACT

The bromocresol green (BCG) and bromocresol purple (BCP) assays often yield discordant serum albumin results. This study seeks to test the hypothesis that bias in albumin results are influenced by the concentration of serum globulin subtypes. The concentrations of serum albumin, α1-globulin, α2-globulin, β-globulin and γ-globulin are determined in 197 human serum specimens by total serum protein quantification and protein electrophoresis, and by the BCG and BCP assays. The influence of globulins on albumin measurement is validated with protein mixtures of albumin and globulins. The BCG assay bias was directly proportional to the concentrations of α1-globulin and α2-globulin, and inversely correlated with the concentrations of β-globulin and γ-globulin (r^2=0.793). The BCP assay bias was inversely proportional to the concentration of α1-globulin and α2-globulin (r^2=0.464) but not related to the concentrations of β-globulin or γ-globulin. Among the 197 study participants, those with nephrotic syndrome had a significantly higher level of α2-globulin compared to those in other categories. Thus, the authors conclude that serum globulins contribute to the bias seen in the BCG and BCP assays, with the greatest effects observed for α-globulin on the BCG assay where higher concentrations contributed to a higher bias.

KEY WORDS: Bromcresol green.
Bromcresol purple.
Serum albumin.
Serum globulins.

Introduction

The bromocresol green (BCG) and bromocresol purple (BCP) assays are conventional methods for determining serum albumin concentration in most clinical laboratories. Although their advantages include speed, economy and precision, the two assays often yield discordant results,[1–4] which are commonly observed among samples from patients and from external quality assessment schemes.[5,6] In most circumstances, the BCG assay gives higher serum albumin concentration results than the BCP assay.[1–7] These discrepancies are clinically important because medical decisions will be made on the basis of the results of these assays.

Several solutions have been proposed to deal with the uncertainty resulting from these discrepancies, including the preferential use of one assay for certain clinical conditions.[8] For example, it has been reported that the BCP assay has a negative bias in the presence of delta bilirubin, whereas the BCG assay does not.[9] Therefore, the BCG assay may be more accurate for patients with hepatic disease that results in increased conjugated bilirubin. In sera from patients with chronic renal failure undergoing haemodialysis, the BCP method tends to underestimate the albumin concentration relative to the BCG assay.[10] Moreover, an increased percentage of oxidised albumin in serum leads to increased albumin levels measured by both assays, although the variation in the BCP assay is greater; therefore, neither assay provides accurate results for patients with cirrhosis.[11]

For these reasons, it seemed advisable to investigate whether or not other clinical conditions could be identified in which it was preferable to use one assay rather than the other. Although both assays have been reported to react with globulins as well as albumin,[12–14] to date there have been no reports comparing the effects of the different subtypes of globulin on both assays.

In this study, the authors hypothesise that changes in concentration of the globulin subtypes might characterise different clinical conditions and contribute to bias in the quantitation of albumin by the BCG and BCP assays. The study determines the concentrations of serum albumin, α1-globulin, α2-globulin, β-globulin and γ-globulin by protein electrophoresis, and the concentration of serum albumin by the BCG and BCP assays from patients with various clinical conditions. It also analyses the relationships between the bias in albumin results and each globulin subtype, with protein electrophoresis as the reference standard.

Correspondence to Xiaofeng Jiang
Email: xiaofengjiang17@163.com

Materials and methods

This study was approved by the Institutional Review Board of Harbin University, and written informed consents were obtained from all study participants. The individuals enrolled in this study comprised 61 healthy volunteers (who were undergoing routine physical examinations) and 136 in-patients. Among the in-patients, 18 had chronic renal failure, 18 had nephrotic syndrome, 31 had hepatic cirrhosis, 15 had acute hepatitis, 13 had chronic hepatitis, 15 were recovering from surgery and 26 were pregnant. Specimens with obvious monoclonal protein spikes or abnormal bands on electrophoresis were excluded. Venous blood samples were drawn from the forearm after fasting, and serum was obtained following centrifugation.

Determination of serum protein concentration

Total protein and albumin: Serum total protein and albumin were measured on the Abbott Architect C8000 analyser (Abbott Laboratories, Abbott Park, IL) using reagents for total protein, albumin BCG and albumin BCP. Multi-constituent calibrators (Abbott Laboratories) were used to calibrate the assays and values of total protein (TP), albumin BCG (AlbG) and albumin BCP (AlbP) were 40 g/L, 23 g/L and 20 g/L in CAL 1 level, and 67 g/L, 56 g/L and 54g/L in CAL 2 level, respectively.

Relative concentrations of individual serum proteins: The HYDRASYS instrument (Sebia, Norcross, GA, USA) was used to determine the relative concentrations of individual serum proteins. Electrophoresis was performed with Hydragel agarose gels at alkaline pH according to the manufacturer's instructions. Relative concentrations (percentages) of the separated albumin, α1-globulin, α2-globulin, β-globulin and γ-globulin, stained with amido black, were evaluated by densitometry and analysed using the Phoresis software package. The concentrations of individual components were calculated with reference to the total serum protein concentration determined using the Architect analyser, as described above.

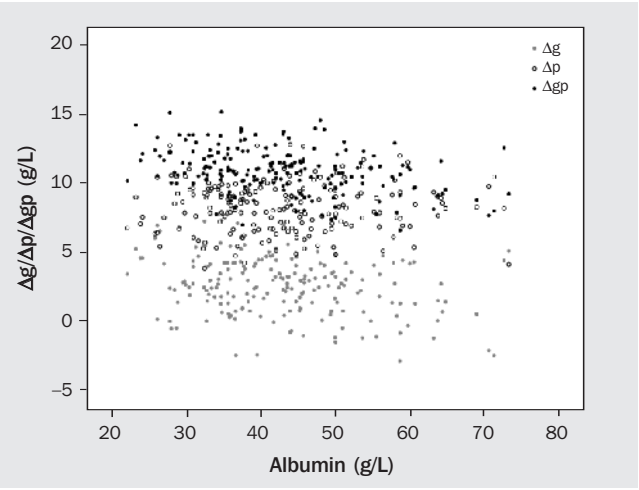

Fig. 1. Relationship between assay biases and discrepancy and the albumin concentration determined by electrophoresis in the serum specimens from 197 participants. Δg and Δp (the assay biases) represent the differences between the albumin concentrations determined by the BCG and BCP assays, respectively, and by gel electrophoresis. Δgp (the assay discrepancy) represents the difference between the results of the BCG and BCP assays.

Preparation of solutions for validation: Solutions of albumin and globulin were prepared by ammonium sulphate fractionation[15] of pooled serum samples obtained from the healthy control subjects. The globulin fraction precipitated at lower ammonium sulphate concentration and was collected by centrifugation. The albumin was collected from the supernatant by further addition of ammonium sulphate and centrifugation. Both fractions were dialysed against saline (until no ammonium ion was detected by Nessler's reagent) and adjusted to a final concentration of 70 g/L, as determined by the Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA) with bovine serum albumin as the calibration standard. Two series of mixtures were prepared with different ratios of the albumin and globulin solutions. The first series contained a constant globulin concentration of 35 g/L and varying albumin concentrations of 0, 8.8, 12.5, 26.3 and 35 g/L. The second series contained a constant albumin concentration of 35 g/L and varying globulin concentrations of 0, 8.8, 12.5, 26.3 and 35 g/L.

Statistical analysis

Three variables were defined as i) Δg = AlbG–Alb, ii) Δp = Alb–AlbP, and iii) Δgp = AlbG–AlbP, where Alb, AlbG and AlbP represent the serum albumin concentration calculated as described above on the basis of the Hydragel analysis and the total protein concentration, the value obtained from the BCG assay, and the value obtained from the BCP assay, respectively. Thus, Δg represents the bias of the BCG assay, Δp represents the bias of the BCP assay, and Δgp is the discrepancy between the BCG and BCP assays.

Values for all measurements are expressed as the mean±SD unless stated otherwise. Pearson's correlation and multiple regression were used to evaluate the relationships between the albumin results and globulin concentrations. The equations for the regression model were :

$\Delta g = 0.57 + 1.69 \times \alpha1\text{-globulin} + 0.62 \times \alpha2\text{-globulin} - 0.34 \times \beta\text{-globulin} - 0.37 \times \gamma\text{-globulin}$

$\Delta p = 7.66 - 0.99 \times \alpha1\text{-globulin} - 0.15 \times \alpha2\text{-globulin} + 0.09 \times \beta\text{-globulin} + 0.34 \times \gamma\text{-globulin}$

$\Delta gp = 8.24 + 0.70 \times \alpha1\text{-globulin} + 0.47 \times \alpha2\text{-globulin} - 0.25 \times \beta\text{-globulin} - 0.03 \times \gamma\text{-globulin}.$

The linear model was fitted without considering interaction effects because there was only a slight linear relationship, as shown in Figure 1. One-way ANOVA with Bonferroni adjustment was used to compare measurements by disease status. Data were analysed using SAS 9.0 (SAS Institute, Cary, NC, USA.). All *P* values were two-sided and were considered significant at <0.05.

Results

Relationship between discrepancy results and albumin concentration

Although considerable variation was seen, the bias of the BCG assay (Δg) tended to be less than that of the BCP assay (Δp), and the discrepancy between the two assays tended to be greater than the individual biases. However, there was no

Table 1. Regression equations and standardised equations for serum globulins and albumin assay biases and discrepancy.

	Δg	Δp	Δgp
	Coefficient (SE)	Coefficient (SE)	Coefficient (SE)
α1-globulin	1.690 (0.089)*	–0.987 (0.126)*	0.703 (0.133)*
α2-globulin	0.620 (0.050)*	–0.150 (0.071)*	0.470 (0.074)*
β-globulin	–0.338 (0.047)*	0.088 (0.066)	–0.251 (0.070)*
γ-globulin	–0.372 (0.025)*	0.342 (0.035)	–0.030 (0.037)
Constant	0.574 (0.521)	7.661 (0.744)*	8.235 (0.783)*
r^2	0.793	0.464	0.259

Δg: AlbG–Alb, Δp: Alb–AlbP, Δgp: AlbG–AlbP, where Alb, AlbG, and AlbP are serum albumin concentration determined by protein electrophoresis, BCG assay and BCP assay, respectively.

SE: standard error. *$P<0.05$.

Equations for the regression model: Δg=0.57+1.69×α1-globulin+0.62×α2-globulin–0.34×β-globulin–0.37×γ-globulin
Δp=7.66–0.99×α1-globulin–0.15×α2-globulin+0.09×β-globulin+0.34×γ-globulin
Δgp=8.24+0.70×α1-globulin+0.47×α2-globulin–0.25×β-globulin–0.03×γ-globulin

significant relationship between any of the variables and albumin concentration across the range of albumin levels measured.

Contribution of globulins to serum albumin concentration

Multivariate regression analysis was conducted on the biases and discrepancy of serum albumin measurement and the concentration of globulins (Table 1). For the BCG assay, the assay bias (Δg) was directly proportional to the concentrations of α1-globulin and α2-globulin, and was also inversely correlated with the concentrations of β-globulin and γ-globulin (r^2=0.793, $P<0.0001$). For the BCP assay, the bias (Δp) was inversely proportional to the concentrations of α1-globulin and α2-globulin (r^2 = 0.464, $P<0.0001$) but there was no significant relationship between Δp and β-globulin or γ-globulin concentration.

Validation tests

In the protein mixtures that contained a constant concentration (35 g/L) of globulin, the BCG assay was more likely to be influenced by its presence than was the BCP assay (Fig. 2). Before the addition of albumin and with only 35 g/L globulin present, the bias obtained with the BCG assay relative to protein electrophoresis was approximately 4 g/L, and that with the BCP assay was approximately 2 g/L. The biases with both assays increased when albumin was added, but they remained almost unchanged over a range of albumin concentrations from 10 to 40 g/L in protein mixtures with a constant globulin level. The values of the discrepancy between the two assays (Δgp) were similar.

A series of protein mixtures containing a constant concentration (35 g/L) of albumin were used to assess the influence of globulin on the BCG and BCP methods. The

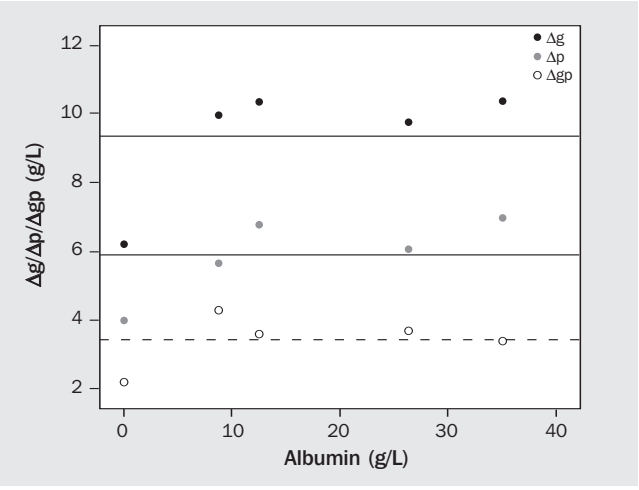

Fig. 2. Relationship between the assay biases and discrepancy and albumin concentrations among mixtures with a constant globulin concentration (35 g/L). The assays were tested in solutions containing 0 g/L, 8.8 g/L, 12.5 g/L, 26.3 g/L, and 35 g/L albumin and 35 g/L globulin. Lines show the average levels of the bias with each assay and the discrepancy between assays.

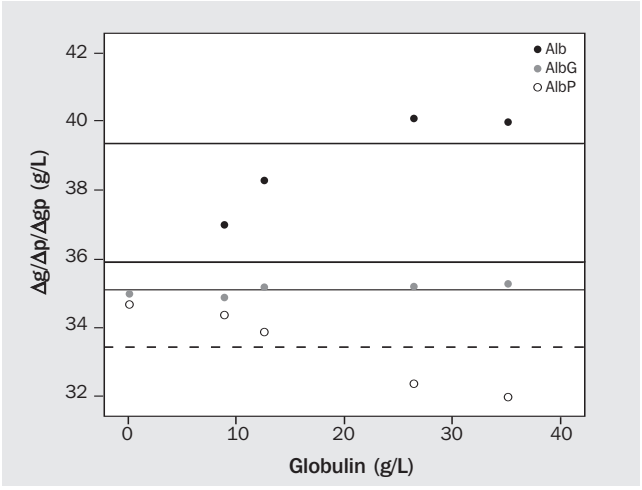

Fig. 3. Relationship between the measured concentration of albumin and the concentration of globulins in mixtures with a constant albumin concentration (35 g/L). The concentrations of the globulins were 0 g/L, 8.8 g/L, 12.5 g/L, 26.3 g/L and 35 g/L. Alb represents the concentration of albumin measured by electrophoresis; AlbG and AlbP are the concentrations of albumin measured by the BCG and BCP assays, respectively. The line shows the average level of albumin measured by the protein electrophoresis method

Table 2. Levels of serum globulins and albumin by clinical condition.

	Post-operative trauma	Pregnancy	Cirrhosis	Acute hepatitis	Nephrotic syndrome	Chronic hepatitis	Chronic renal failure	Healthy individuals
	(*n*=15)	(*n*=26)	(*n*=31)	(*n*=15)	(*n*=18)	(*n*=13)	(*n*=18)	(*n*=61)
Alb	47.3±10.6	36.8±4.9*	40.7±7.8	42.2±11.3	36.4±7.9*	57.8±14.1*†‡§#	35.6±7.4*§	49.4±7.5†§¥
AlbG	50.1±10.9	40.4±5.2*	42.8±7.2	43.5±11.9	40.2±8.2*	58.1±14.2†‡§#	39.5±6.8*§	51.8±7.4†‡§#¥
AlbP	39.4±11.5	28.7±5.7*	31.9±7.6	32.9±12.0	27.9±8.5*	48.3±14.6†‡§#	28.5±7.3*§	41.4±7.6†‡§#¥
α1-globulin	2.2±0.5	2.4±0.5	2.2±1.1	1.9±0.7	2.2±0.8	1.7±0.6	2.2±1.0	1.8±0.6†
α2-globulin	6.5±1.5	6.6±1.0	6.3±1.5	6.4±1.0	8.0±1.0*†‡§	4.7±0.9*†‡§#	6.2±1.0§#	6.9±1.3§#
β-globulin	6.3±2.4	6.7±1.4	6.8±1.6	5.7±1.6	5.7±1.3	6.4±1.9	6.3±1.4	6.8±1.0
γ-globulin	9.3±2.7	8.3±1.8	10.6±2.7	11.3±1.7	8.9±2.7	10.6±2.3	4.7±1.1*†‡§#	9.2±2.3§§

*$P<0.05$ compared to post-operative trauma
†$P<0.05$ compared to pregnancy
‡$P<0.05$ compared to cirrhosis
§$P<0.05$ compared to acute hepatitis
#$P<0.05$ compared to nephrotic syndrome
§$P<0.05$ compared to chronic hepatitis
¥$P<0.05$ compared to chronic renal failure
(all after Bonferroni adjustment)

correlations between albumin and globulin concentrations in patients' specimens are shown in Figure 3. The apparent albumin concentration measured by the BCG assay correlated positively with globulin concentration ($r^2 = 0.943$, $P = 0.016$), while for the BCP assay they were inversely related ($r^2 = –0.985$, $P = 0.002$). Albumin concentration determined by protein electrophoresis proved to be almost constant over the globulin concentration range ($r^2 = 0.816$, $P = 0.096$).

Globulin subtype and assay variation

The concentrations of the various globulin subtypes among patients with different clinical conditions were determined by electrophoresis, together with the albumin concentrations determined by electrophoresis, BCG and BCP are shown in Table 2. Results with the BCP assay were consistently lower than those obtain with BCG and electrophoresis. Significant differences were seen in albumin concentration determined by the different methods for all categories except patients with chronic hepatitis (for whom the difference between Alb and AlbG was not significant). Patients with nephrotic syndrome showed a significantly higher α2-globulin level compared to those in the other categories and the healthy controls.

Discussion

This study evaluated the relationships between different concentrations of albumin and the globulin subtypes (α1-globulin, α2-globulin, β-globulin and γ-globulin) on the biases observed when serum albumin concentration was determined with the BCG and BCP assays. Although the presence of albumin did introduce a bias in both assays, it did not change over the range of albumin concentrations tested. In contrast, the bias of the BCG assay was directly proportional to α1-globulin and α2-globulin concentration, and inversely correlated with the β-globulin and γ-globulin concentration.

According to the standardised coefficients of each equation, it is concluded that the contributions of the tested globulins to colour development in the BCG test are in the following order: α1-globulin>α2-globulin>β-globulin>γ-globulin. The BCP assay was less influenced by the α1-globulin and α2-globulin subtypes and showed a relatively smaller and inverse relationship with the globulin concentration. Furthermore, there was no significant relationship between the bias of the BCP assay and β-globulin and γ-globulin concentration.

With respect to the α1-globulin and α2-globulin subtypes, only patients with nephrotic syndrome had significantly elevated α2-globulin levels, which might alter the results of the BCG serum albumin assay. Indeed, the result of the BCG assay was significantly higher than that obtained using electrophoresis in this group, and also gave higher results in all the other categories.

The results of this study extend and confirm others that have investigated the effects of globulins on the BCG and BCP assays. The discrepancy between the two assays is believed to be caused by differences in chemical equilibrium between the dissociated dye anions and the positively charged residues of albumin molecules.[16] Although both dyes react with albumin, the serum globulins also consist of positively charged residues, and the BCG assay, which is considered less specific for albumin, has previously been shown to be influenced by some of the serum globulins.[12,13] In contrast, the BCP assay is reported to be less affected by globulins,[17] although Tel *et al*. reported that the α-globulin, β-globulin and γ- globulin fractions also react with BCP.[14] The present study, which is the first to analyse the relationship between the globulins and the results of the BCG and BCP assays, has shown that α1-globulin and α2-globulin have a strong positive effect on the BCG assay and a weaker, negative effect on the BCP assay. β-globulin and γ-globulin

had a weaker, negative effect on the results of BCG assay and no effect on the BCP results.

In this study, the BCP assay gave consistently lower values than the BCG assay for the same sample, and this agrees with previous studies.[1,7,8,14] It also showed that the results of the BCG assay were closer to those obtained with electrophoresis, whereas the values obtained with the BCP assay have been reported to be closer to those obtained by nephelometry.[18]

The BCG and BCP assays can produce biased results when used to measure serum albumin concentrations in various patient categories,[1–4] and in plasma specimens treated with heparin,[19] and Clase *et al*. proposed an equation to convert the results of one assay to another.[8] However, it has since been shown that the biases and difference in values between the two assays are not constant. For example, Parikh *et al*. reported that the difference in values between the BCG and BCP assays was greater in serum from patients undergoing peritoneal dialysis than from those undergoing haemodialysis.[18]

Although the reasons for the discrepancies remain unclear, some evidence has begun to emerge. Watanabe *et al*. reported that albumin is a heterogeneous mixture of oxidised and reduces forms of albumin and glycoalbumin, the ratios of which can be influenced by the progression of liver disease as well as oxidative stress due to ageing, nephritic syndrome, haemodialysis and diabetes.[11] The authors showed that increases in the ratio of oxidised albumin led to increasing bias from the BCP assay. The present study has shown that various compositions of globulin subtype can also affect the results of the BCP and BCG assays. Thus, it is clear that a simple conversion of the values of one assay to another will lead to errors in the assessment of albumin level in different patient groups, as several established equations are based on the statistical association between the BCG and BCP assays[1,3,4,8,20] and do not take into account the influence of serum globulins, albumin microheterogeneity, oxidation ratios, or any of the other variables that might affect biases between the assays.

Regarding the clinical significance of the present results, it was found that α2-globulin concentration was significantly higher in patients with nephrotic syndrome than in other patient groups or the controls. The corresponding value for albumin concentration was higher when determined by BCG assay and lower by BCP assay compared to the results of electrophoresis. Results obtained in patients with other clinical conditions (except for those with chronic hepatitis) showed significant differences in albumin concentration when comparing the results of the BCG and BCP assays and electrophoresis. However, none could be attributed to differences in globulin subtype concentration.

This study has several limitations. First, the reference assay (protein electrophoresis) is also subject to bias (e.g., in sera from patients with multiple myeloma,[21] and as a result of discrepancies in amido black staining). Other comparative methods such as albumin immunoassay and high-performance liquid chromatography (HPLC) also have drawbacks that may limit their use in a clinical laboratory: they are relatively expensive, serum protein samples must be diluted prior to albumin immunoassay (immunoassays have their own sources of bias[22]) and HPLC may also produce biased results depending on the proportions of tyrosine and tryptophan in different proteins.[23]

Techniques such as capillary electrophoresis have been used for better separation of serum proteins,[24] and might further refine the results obtained. The present study tested only a single albumin concentration (35 g/L) at close to the upper limit of the reference interval. It would be of interest to test the influence of globulins in other clinically significant ranges of albumin concentration (e.g., 15–25 g/L) in future studies.

Furthermore, only a relatively small number of patients were included in the various clinical categories studied. A larger study with more patients in each category might reveal differences in BCG and BCP assay results that could be attributed to differences in globulin content.

In summary, the authors conclude that serum globulins influence BCG and BCP assay biases in the determination of serum albumin concentration. However, further evaluation of these effects in a larger patient population is necessary in order to confirm the superior performance of one assay over another in patients with specific clinical conditions. □

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