Bias between two methods of albumin measurement in the white rhinoceros, *Ceratotherium simum*

Running header: Albumin measurement in the white rhinoceros

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Abstract

Background: The bromocresol green (BCG) method has been reported to overestimate serum albumin concentration in several species due to non-specific binding to globulins. As the white rhinoceros has high concentrations of serum globulins, significant differences in albumin measured by the BCG method, and the field method of agarose gel serum protein electrophoresis (SPE) are expected.

Objectives: We aimed to compare the BCG and SPE methods for albumin determination in the serum of white rhinoceroses.

Methods: SPE and BCG albumin were measured in 82 white rhinoceros serum samples. Results were compared using Bland-Altman difference plots and Passing-Bablok regression analysis.

Results: BCG albumin showed a significant mean constant positive bias of 7 g/L, or 36%, which was more than the total allowable error of 15% and was clinically significant. Methods were not comparable within the inherent imprecision of each method.

Conclusions: The BCG method overestimates albumin concentrations in this species compared to agarose gel SPE, and method-specific reference intervals should be used.

Keywords

Bromocresol green

Ceratotherium simum

Method comparison

Serum protein electrophoresis

The conventional automated method for measurement of total serum protein (TSP) is the biuret reaction, with the bromocresol green (BCG) dye-binding method used for albumin measurements in most veterinary laboratories. Although the BCG method is simple and inexpensive, with sufficient analytical sensitivity, it is known to overestimate albumin concentrations in both humans and several domestic species due to non-specific binding to globulins.[1-5] The BCG dye-binding affinity for albumin appears to be species-specific; it has been shown, for example, that the peak absorbance using this method differs between canine, murine, and human samples.[3] Furthermore, the inaccuracy of the BCG method increases in samples with high globulin and low albumin concentrations.[6] An alternate method of albumin determination, serum protein electrophoresis, is considered by many to be the preferred reference method for albumin measurements in animal species.[7]

The white rhinoceros is a species with unusually high TSP concentrations, with relatively low albumin and high globulins. We have previously reported TSP reference intervals of 76-111 g/L and SPE reference intervals of 10-27 g/L for albumin, 60-87 g/L for globulins, and 0.12-0.39 for the albumin-to-globulin ratio in this species.[8] We hypothesized that albumin measurements using the BCG method would be significantly affected by high globulin concentrations in this species. The objective of this study was to assess the accuracy of the BCG method for measuring albumin concentrations in white rhinoceros, compared with SPE albumin concentrations.

Stored serum samples from both healthy white rhinoceros and animals with tissue trauma were used. Details concerning these two populations, as well as sample

collection and handling, have been reported in another publication.[8] Ethics approval for this study was granted by the University of Pretoria Animal Ethics Committee (V042-15, V011-17).

TSP was measured by the biuret reaction, and albumin by the BCG method on an automated wet chemistry analyzer, the Cobas Integra 400 Plus (Roche Products (Pty) Ltd, Basel, Switzerland). Analyzer maintenance was carried out according to the manufacturer's guidelines, and assay performance was monitored by daily internal quality control (QC) and monthly external QC according to laboratory protocols. Both assays were calibrated using a standard derived from human serum (C.f.a.s., Roche Products (Pty) Ltd, Basel, Switzerland). The reaction time for the endpoint measurement of the BCG method was 35 seconds.

SPE was performed on split beta agarose gels using the automated Interlab Pretty platform (Interlab S.r.L., Rome, Italy) according to the manufacturer's instructions and as described previously.[8] Albumin was identified as the peak closest to the anodal side of the tracing. The cathodal gate for albumin was placed at the deepest point of the trough between albumin and the first α-globulin peak. The concentration of albumin was calculated by multiplying the relative albumin percentage derived from the electrophoretogram by the TSP concentration obtained by the biuret method. SPE analysis took place within 24 hours of the biuret and BCG measurements; samples were kept at 4°C in closed tubes, then allowed to warm to room temperature and gently mixed between the 2 sets of analyses.

Paired results were used to generate a comparison plot. Linearity was assessed visually and by means of a CUSUM test. Correlation was assessed using Spearman's correlation coefficient. Passing-Bablok regression analysis was performed; statistically significant proportional or constant bias was considered to be present if the 95% confidence intervals (CI) of the slope and *y*-intercept from the regression equation did not include 1.0 and 0.0, respectively.[9] A Bland-Altman plot with percentage mean differences and limits of agreement was constructed. As the Bland-Altman plot does not take imprecision of the two methods into account, acceptance limits derived from the coefficient of variation (CV) calculated from internal QC data for the BCG method and inter-gel imprecision for each method were calculated as follows:[8,9]

$$Upper\ or\ lower\ limit\ (\%)\ = 0\ \pm 1.96\ \times \sqrt{CV_{SPE}^2 + CV_{BCG}^2}$$

Absolute lower and upper acceptance limits were then calculated for each mean value of the two methods, using the percentage limits. The absolute difference between the methods was calculated, and the number of times that the absolute difference was outside of the upper and lower limits was counted. Agreement between methods was acceptable if ≥95% of differences between the two measurements were between the acceptance limits.[9] The clinically allowable bias between the two albumin methods was set at 15%.[10] Statistical analysis was performed using MedCalc for Windows, version 15.0 (MedCalc Software, Ostend, Belgium).

Fifty serum samples originating from 50 healthy white rhinoceros and 32 serum samples originating from 30 white rhinoceros with tissue trauma were used.[8]

The performance of the TSP and BCG albumin assays was acceptable over the course of the study. Estimates of imprecision (CV) derived from internal QC results were 2.1% for TSP and 2.6% for BCG albumin. The SPE method had an intra-gel imprecision of 2.8 % and inter-gel imprecision of 6.5% for albumin.[8]

Albumin concentrations measured using the BCG assay ranged from 11 to 40 g/L, with a mean concentration of 23 g/L and a median concentration of 24 g/L. Albumin concentrations measured with SPE ranged from 6.0 to 30.2 g/L with a mean concentration of 16.0 g/L and median concentration of 17.0 g/L (Figure 1). The correlation between the two methods was significant and high (Spearman's r = 0.76, P<0.0001).

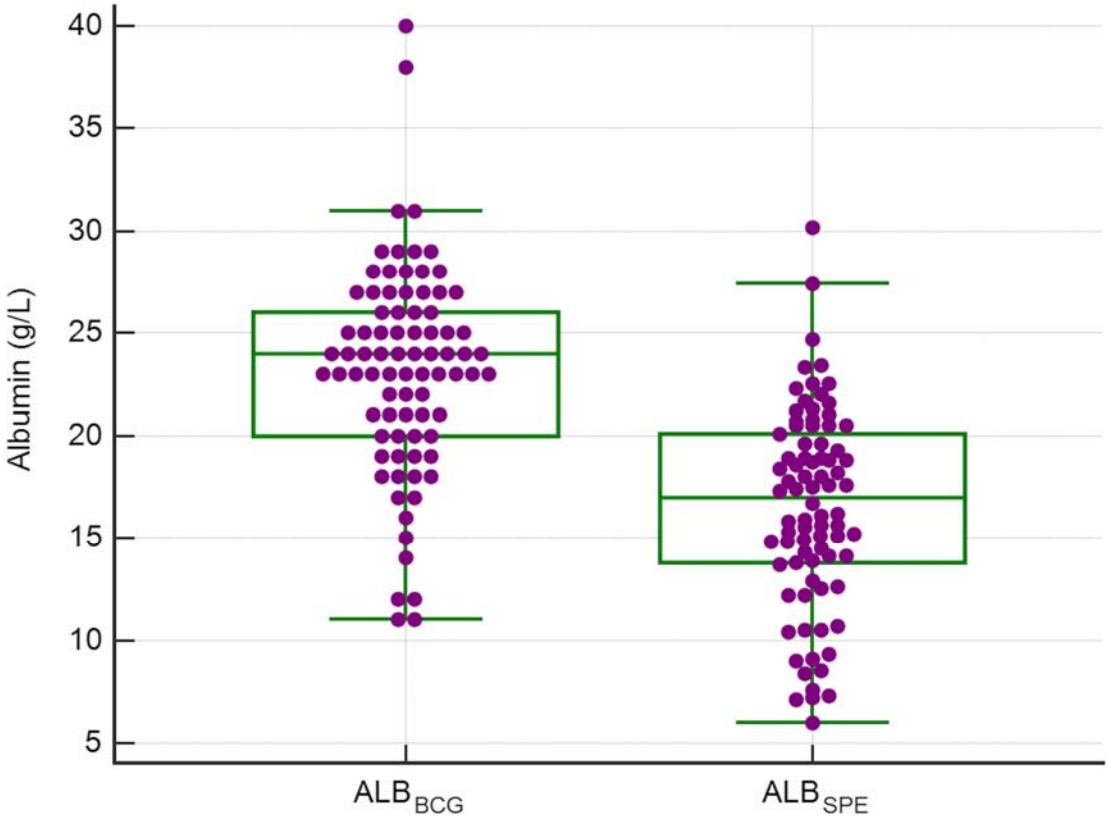

Figure 1.Box and whisker plots showing the distribution of results for white rhinoceros serum albumin measured using the bromocresol green (BCG) and serum protein electrophoresis (SPE) methods

The Passing-Bablok regression and Bland-Altman difference plots are shown in Figure 2. The equation of the regression line (95% CI in parentheses) was

$$y = 6.45\ (3.64 - 9.31) + 1.00x\ (0.84 - 1.17)$$

revealing a significant constant bias of 6.5 g/L (rounded up to 7 g/L) for the BCG vs the SPE method.

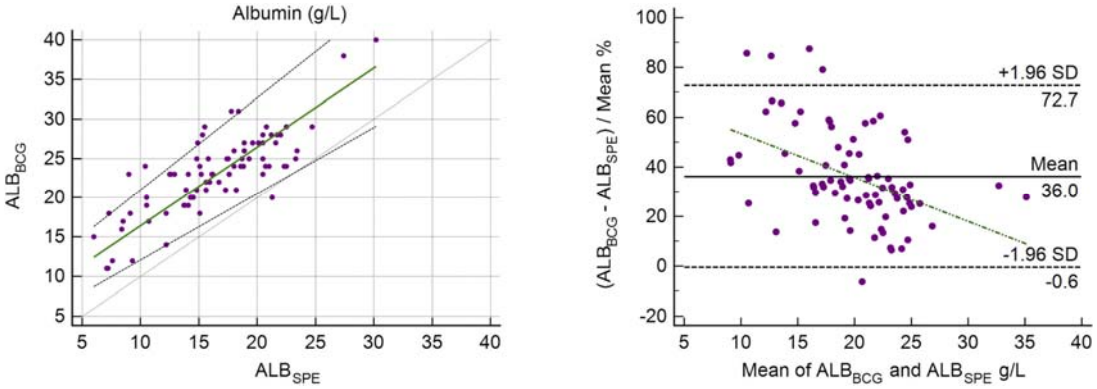

Figure 2. A Passing-Bablok regression analysis (left) and Bland-Altman difference plot (right) for the comparison of albumin measured using the bromocresol green (BCG) and serum protein electrophoresis (SPE) methods. On the regression plot, the solid green line indicates the regression line, the grey line represents the line of identity ($y = x$), and the two dashed lines show the 95% limits of agreement. On the difference plot, the solid black line represents the mean percentage difference, the two horizontal dashed lines on either side indicate the limits of agreement (±1.96 SD from the mean difference), and the green dash-dotted line is the regression line of the differences

Using this equation and substituting an SPE albumin concentration of 10 g/L, 20 g/L or 30g/L for *x*, the following values were obtained for the BCG method (*y*): 16 g/L, 26 g/L, and 36 g/L, respectively. This equates to a positive bias of 65%, 33%, and 22% for SPE albumin measurements of 10 g/L, 20 g/L, and 30 g/L, respectively.

There was a mean positive bias of 36% for the BCG albumin method compared with the SPE method on the Bland-Altman difference plot. Acceptance limits based on the imprecision of each method were determined to be ± 13.7% of the mean of both methods. Only 12.5% of the differences between the two methods fell into these limits; thus, the methods were not comparable within the inherent imprecision of

each method. In summary, the BCG albumin method shows a statistically and clinically-relevant positive bias compared with the SPE method.

A significant positive bias, with a mean of 7 g/L or 36%, was found for the albumin measurements in white rhinoceros serum using the BCG method compared with the SPE method. The bias is clinically significant, as it is higher than the allowable error goal of 15% at concentrations ranging from low to high (10-30 g/L). This positive bias is well described for albumin measurements in human serum, which has been reported to vary from 1.5 to 11.0 g/L.[1,6] This bias results from the binding of the BCG dye to globulins, in particular, to the α- globulins, like α2-macroglobulin and haptoglobin.[6] The error is directly proportional to the concentrations of α-globulins, and the error is greater when albumin is low, and globulins are high.[6] The bias is also time-dependent and increases when reaction times are greater than one minute.[1] Shorter assay times have been found to minimize the error.[6] The accuracy of the BCG method has also been investigated in various domestic animal species. Non-specific BCG dye binding to α- and β-globulins, but not γ-globulins, was demonstrated in equine, bovine, and ovine serum, and was significantly greater with a ten-minute reaction time compared with an immediate (<20 s) reaction time.[2] This same study also demonstrated that error was not present when species-specific (horse, cow, sheep), rather than human standards were used to calibrate the BCG assay and that as the species-specific standard curves differed, the degree of BCG-globulin binding was species-dependent. The use of a caprine, rather than a bovine standard, improved the accuracy of BCG albumin measurement in goat serum.[7] Other studies have demonstrated a similar error in canine, murine, and bovine serum.[5] In rats, in particular, non-specific binding to transferrin, a β-globulin, has

been demonstrated.[11] It was found that 37% of transferrin present was bound by the BCG dye, leading to an overestimation of albumin by 20%. Transferrin binding occurred within the first minute and increased with reaction time.[11] Inaccurate albumin measurements using the BCG method have also been demonstrated in various avian and reptilian species, with a positive bias particularly evident in ill animals or those with a decreased A/G.[4,12] The BCG assay used in our study had a short reaction time of 35 seconds, which would have minimized, but not eliminated, globulin binding. However, as white rhinoceroses have a lower A/G ratio than other species, and a human standard was used to calibrate this assay, the significant inaccuracy demonstrated here is not unexpected.[8]

Although SPE has been described as the preferred reference method for the measurement of albumin and globulin [1,7], the true accuracy of this method is also questionable. Albumin was found to be present in the α1-fraction of cats and dogs and the α1- and α2-fractions of the white rhinoceros, indicating that albumin concentrations determined by calculation from the SPE albumin fraction are an underestimation of this protein.[8,13,14] In addition, SPE albumin determination could be inaccurate due to disproportional staining of the albumin band because of the high affinity of albumin for the dye, or non-linearity of light absorbance during densitometric measurements.[15] The relatively high serum albumin concentrations compared with various globulins might also be outside of the linear dynamic range of densitometric dyes.[16]

Immunonephelometric or immunoturbidometric methods are considered to be the gold standard for albumin determination in human laboratory medicine, along with

the use of certified reference material.[17] For immunological methods to be used in veterinary species, for example, the white rhinoceros, both validated anti-albumin antibodies, as well as a reference standard containing white rhinoceros albumin, need to be available. The first step towards the development of an accurate albumin assay would entail an investigation of the molecular structure of albumin in this species, using proteomic techniques.

As there is no gold-standard method available, and biases between available methods have been demonstrated, the use of method-specific (ie, for both BCG and SPE) reference intervals when considering albumin and A/G results in the white rhinoceros and other species is essential to avoid misinterpretation.

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References

1. Brackeen GL, Dover JS, Long CL. Serum albumin. Differences in assay specificity. *Nutr Clin Pract.* 1989;4:203-205.
2. Keay G, Doxey DL. A study of the interaction between bromocresol green dye and bovine, ovine and equine serum globulins. *Vet Res Commun.* 1984;8:25-32.
3. Evans GO, Parsons CE. A comparison of two dye-binding methods for the determination of dog, rat and human plasma albumins. *J Comp Pathol.* 1988;98:453-460.
4. Cray C, Wack A, Arheart KL. Invalid measurement of plasma albumin using bromcresol green methodology in penguins (Spheniscus species). *J Avian Med Surg.* 2011;25:14-22.
5. Chorfi Y, Lanevschi-Pietersma A, Girard V, Tremblay A. Evaluation of variation in serum globulin concentrations in dairy cattle. *Vet Clin Pathol.* 2004;33:122-127.
6. Duly EB, Grimason S, Grimason P, Barnes G, Trinick TR. Measurement of serum albumin by capillary zone electrophoresis, bromocresol green, bromocresol purple, and immunoassay methods. *J Clin Pathol.* 2003;56:780-781.
7. Bain MS. Determination of albumin in caprine serum. *Res Vet Sci.* 1986;41:82-84.
8. Hooijberg EH, Miller M, Cray C, Buss P, Steenkamp G, Goddard A. Serum protein electrophoresis in healthy and injured southern white rhinoceros (Ceratotherium simum simum). *PLOS ONE.* 2018;13:e0200347.

9. Jensen AL, Kjelgaard-Hansen M. Method comparison in the clinical laboratory. *Vet Clin Pathol.* 2006;35:276-286.
10. Harr KE, Flatland B, Nabity M, Freeman KP. ASVCP guidelines: allowable total error guidelines for biochemistry. *Vet Clin Pathol.* 2013;42:424-436.
11. Barber BJ, Stanhope VL. Bromcresol green assay is nonspecific for rat plasma albumin. *Am J Physiol.* 1992;262:H299-302.
12. Muller K, Brunnberg L. Determination of plasma albumin concentration in healthy and diseased turtles: a comparison of protein electrophoresis and the bromcresol green dye-binding method. *Vet Clin Pathol.* 2010;39:79-82.
13. Atherton MJ, Braceland M, Harvie J, et al. Characterisation of the normal canine serum proteome using a novel electrophoretic technique combined with mass spectrometry. *Vet J.* 2013;196:315-319.
14. Gerou-Ferriani M, McBrearty AR, Burchmore RJ, Jayawardena KGI, Eckersall PD, Morris JS. Agarose gel serum protein electrophoresis in cats with and without lymphoma and preliminary results of tandem mass fingerprinting analysis. *Vet Clin Pathol.* 2011;40:159-173.
15. Howerton DA, Check IJ, Hunter RL. Densitometric Quantitation of High Resolution Agarose Gel Protein Electrophoresis. *Am J Clin Pathol.* 1986;85:213-218.
16. Gauci VJ, Wright EP, Coorssen JR. Quantitative proteomics: assessing the spectrum of in-gel protein detection methods. *J Chem Biol.* 2011;4:3-29.
17. Infusino I, Panteghini M. Serum albumin: accuracy and clinical use. *Clin Chim Acta.* 2013;419:15-18.