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Serum albumin assay – easy or problematic analysis?

Jaroslav Filípek, Josef Illek

University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Veterinary Medicine,
Large Animal Clinical Laboratory, Brno, Czech Republic

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Abstract

Serum albumin determination is an important biochemical investigation in clinical laboratories. Photometric methods using albumin binding to organic dyes – bromocresol green (BCG) or bromocresol purple (BCP) – are most commonly used. These determinations are quick, simple, and inexpensive. They are, however, associated with a number of problems. Discussions on the methodological unification and use of BCP are ongoing in human laboratory medicine. The reaction of human albumin with this dye is more specific. On the other hand, its affinity for animal albumin is significantly lower, for which reason BCG is used in veterinary medicine. However, due to the lower reaction specificity of this dye, the results are slightly overestimated as the dye reacts to a lesser extent with alpha and beta globulins. This disadvantage can be largely eliminated by reducing the incubation time to about 30 s. Another problem is the method calibration. Some laboratories use species-specific albumin as calibrators, but this is technically challenging for a laboratory that analyzes albumin of many species. We therefore recommend using a commercially available calibrator that is traceable to the European Reference Material for Specific Proteins. We consider these following principles – using BCG, shortening the incubation time to 30 s and using the mentioned calibrator – as a basic condition to obtain clinically correct and inter-laboratory comparable results.

Bromocresol green, bromocresol purple, electrophoresis

Albumin is a protein involved in a number of important functions in the body. Albumin is the main source of oncotic plasma pressure, performs important transport functions (free fatty acids, hormones, minerals, bile acids, metals, drugs, etc.), and, last but not least, serves as a protein reserve of the organism. The monitoring of its concentration is one of the most important and frequent biochemical examinations, especially in liver and kidney diseases and nutritional status assessment (e.g. Podhorský et al. 2007).

A variety of methods is used in the laboratory to determine albumin. Photometric methods are the most common, with albumin reacting with an organic dye to form an albumin-dye complex with a subsequent dye change. These methods are simple to design, easy to automate, fast and cheap. Almost every clinical biochemical laboratory, human or veterinary, performs these examinations. Of the many dyes used in the past, bromocresol green (BCG) and bromocresol purple (BCP) are used almost exclusively today. Other methods to be mentioned include electrophoresis and immunochemical methods. They are usually not used routinely; they are technically more demanding and expensive. ELISA, RIA and other methods are used very little, and generally for special purposes (Kumar and Banerjee 2017).

At present, a large number of diagnostic kits from various manufacturers based on the BCG or BCP photometric principle are available on the market. There are also increasing discussions about the lack of harmonization of these methods. Kits based on the same principle also show unacceptably large differences between results due to different methodologies (up to 13%). Some studies point to the use of BCP rather than BCG to

Address for correspondence:
RNDr. Jaroslav Filípek, CSc.
Large Animal Clinical Laboratory
Faculty of Veterinary Medicine
University of Veterinary and Pharmaceutical Sciences Brno
Palackeho tr. 1946/1, 612 42 Brno, Czech Republic

Phone: +420 541 562 412
E-mail: filipekj@vfu.cz
http://actavet.vfu.cz/

improve the level of harmonization, and discussions on the subject are far from having come to a conclusion (Korbin et al. 2014; Bachman et al. 2017). Calibration is also a problem. The European Specific Protein Reference Material (ERM DA 470 k/IFCC) has been available for many years, but its use has not yet produced the desired effect (Infusino et al. 2011). In addition to BCG and BCP methods, some diagnostic kit manufacturers also offer an immunochemical method and let the laboratories decide which method to choose. This situation is certainly not satisfactory and efforts are being made to resolve it (Friedecký and Kratochvíla 2017).

The same analytical techniques as in human laboratories are also used in veterinary laboratories, and it goes without saying that all of the above-mentioned shortcomings also apply here. In addition, another extremely important factor is that albumin of different species needs to be analysed. Its structure is different and so is its ability to bind different dyes. For this reason, the BCG method is used in veterinary laboratories because its binding to albumin is less dependent on the animal origin of albumin than it is with BCP. This is an important factor in the use of BCG in veterinary medicine.

Another big problem is calibration. Ideally, the albumin calibrator of a given species would be used, but it is technically impossible for each animal to have its own calibrator. This is achievable, for example, in human laboratories where only one type of albumin is analyzed. We have, therefore, decided to use a commercially available calibrator that is traceable to ERM DA 470 k/IFCC. While this calibration method is not ideal, setting the method to this calibration material should provide a basic level of comparability between the results produced by all laboratories using this calibrator. It is obvious that even such a simple method as the determination of albumin has a number of pitfalls.

The aim of our study was to verify the use of both BCG and BCP dyes in serum determination in large animals. We used common commercially available diagnostic kits and followed the manufacturers' instructions. We then compared these results with electrophoresis (ELFO), a generally recognized method providing correct results independent of the animal origin of albumin. Subsequently, we evaluated the results and, on their basis, made recommendations for laboratories that analyse albumin in the serum of large animals.

Materials and Methods

Bromocresol green method

The BioVendor (Brno, Czech Republic) Albumin Diagnostic Kit (citrate buffer pH 4.2, 30 mmol/l, BCG0.26 mmol/l) was used. The procedure followed the manufacturer's instructions.

Bromocresol purple method

The Beckman Coulter (CA 92821, USA) Albumin Reagent Kit (BCP 0.35 mmol/l) was used. The manufacturer's instructions were also followed.

Calibrator

Biocal (BioVendor, Brno, Czech Republic) was used – a universal calibrator for *in vitro* determination of different analytes photometrically. This is a lyophilized calibrator, based on a human matrix, enriched with other substances of biological origin (bovine plasma and others). It is, therefore, a mixture of human and bovine albumin. For the determination of albumin, it is traceable to the European Reference Material DA 470.

Electrophoresis

Separation was performed with a commercial Hydragel Protein K 20 kit (Sebia, France).

A 0.8% agarose gel, barbital buffer pH 8.5, amidoblack stain was used. Densitometric evaluation was performed using a DVSE manual densitometer. Electrophoresis is a ratio method – for the quantification of individual fractions it is necessary to know the concentration of total protein in the analysed sample.

Determination of total protein

BioVendor (Brno, Czech Republic) diagnostic kit – total protein (biuret), photometric determination using the biuret method was used. The procedure followed the manufacturer's instructions.

Machines

Measurements were performed on a Cobas Mira Plus automatic analyser (Roche, Switzerland).

Animals and sampling

Samples were taken at the Clinic of Ruminant Diseases and the Horse Clinic of the University of Veterinary and Pharmaceutical Sciences Brno in a standard way – puncture from the jugular vein (sheep, goat and horse) and from the vena coccygea media. Samples were taken from clinically healthy animals.

Statistical analysis

Common statistical calculations – mean, standard deviation and paired *t*-test – were performed using standard Excel 2010.

Results

The results are summarized in Table 1. The BCG method slightly overestimates the results in cows; this can be fixed by reducing the measuring time from 10 min (according to the manufacturer's instructions) to 30 s (the last column in Table 1). In this case, the results are already in good agreement with the ELFO method. The BCP method fails and is not applicable for this type of analysis. For goats and sheep, BCG gives good results for both long and short measurement times. Good agreement with BCP was achieved only when analysing sera in horses. The BCG method overestimates the results in this case. Shortening the incubation time to 30 s (versus the 10 min recommended by the manufacturer) improves the results.

Table 1. Statistical evaluation of serum albumin results.

Animal species	Albumin (g/l) BCG	Albumin (g/l) BCP	Albumin (g/l) ELFO	Albumin (g/l) BCG 30 s
Cows	36.03 ± 4.01	19.03 ± 2.87	34.19 ± 3.92	34.06 ± 3.62
n = 23	$P < 0.01$	$P < 0.0001$		NS
Goats	30.41 ± 3.30	20.4 ± 3.30	29.98 ± 3.61	28.6 ± 3.93
n = 12	NS	$P < 0.001$		NS
Sheep	25.7 ± 1.68	15.01 ± 1.86	26.20 ± 2.22	25.1 ± 1.75
n = 7	NS	$P < 0.001$		NS
Horses	33.11 ± 5.03	29.32 ± 4.36	29.35 ± 2.32	31.0 ± 4.71
n = 13	$P < 0.001$	NS		$P < 0.01$

BCG – method using bromocresol green (BCG 30 s – incubation time 30 s); BCP – method using bromocresol purple; ELFO – results obtained by electrophoresis; NS – not significant. The BCG or BCP results were always compared to the ELFO paired *t*-test method.

The electrophoreogram is shown in Fig. 1 (Plate I) – the albumin fraction is seen to be pronounced, sharply delimited and well quantifiable. This is because it is made up of only one type of protein, unlike the other fractions, which are always protein groups. Electrophoresis is therefore a suitable method for the quantitative determination of albumin of different animal origins.

Figure 2 (Plate I) presents the time course of colour development for BCG. The reaction of the dye with albumin is rapid and the colour change occurs almost immediately. In the case of bovine albumin, this can be seen very well – the absorbance value no longer changes after several seconds of colour development. In other cases where serum was used, the increase in coloration is seen to continue, which is due to the interaction of the dye with other proteins and hence the overestimation of the result. In a similar way, the calibrator responds to the sample, which could compensate for the error, but the calibrator used is composed of partly human and partly bovine materials which do not react identically to the dye.

Discussion

Albumin behaves as a cation in a weakly acidic environment and readily binds organic dyes usually containing anionic -SO_3H groups. The first work of this type suitable for quantitative determination of serum albumin, in which the authors used methyl orange, was published in 1953 (Bracken and Klotz). Other dyes were later used, including BCG (Doumas and Biggs 1972) and BCP (Pinell and Northam 1978), and these two dyes have been successfully used to this day. Upon binding to albumin, the absorption maximum is altered and used for quantitative measurements. The colour transition for BCG lies in the pH range of 3.8–5.4 and for BCP in the pH range of 5.2–6.8. This is related to the degree of bromination of the molecule (Fig. 3). Electronegative bromine reduces the electron density on the aromatic ring, thereby increasing the acidity of the -OH group. The higher pH value of the colour transition for BCP means that the weak electrostatic interaction of the dye-protein is partially eliminated and there is a reduction in non-specific reactions with other proteins such as alpha and beta globulins. Therefore, BCP provides more accurate results for human albumin which is why there is a trend to switch to this method. In the case of BCG, non-specific electrostatic interactions are more pronounced and, as a result, the BCG method is slightly overestimated. Therefore, it is recommended to shorten the measurement time (to 30 or even 15 s after mixing the serum with the reagent) to minimize these adverse interactions (Doumas and Peters 2009).

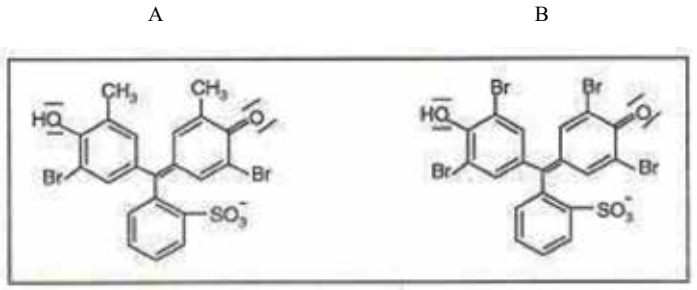

Fig. 3. Chemical structure of bromocresol green (BCG) and bromocresol purple (BCP).

A - bromcresol purple, BCP (5,5'- dibrom-o-cresolsulphophthalein)
B - bromcresol green, BCG (3,3',5,5'- tetrabrom-m-cresolsulphophthalein)

The results in Table 1 essentially confirm the above findings. The BCG method tends to be slightly overestimated, the BCP method fails in most cases. For example, in cows which are analysed extremely often, the values recorded by this method were only 55–60% of the values measured by electrophoresis. Such differences are unacceptable. It is also evident from Table 1 that the use of shorter times (up to 30 s) leads to improved results (except for horses). The BCP method provides comparable results with the ELFO method only in horses, as confirmed by other authors (Blackmore and Henley 1983). Keay and Doxey (1983) also reported a good agreement with ELFO in cattle, sheep, and horses when using short times. However, they used species-specific calibrators, demonstrating that the use of non-species-specific calibrators does not give correct results. A number of other studies have discussed the issue of calibrators, reaction times or even the preanalytic phase (serum

or plasma) (Walsh 1983; Pekcan and Fidanci 2008). Their conclusions are generally to use serum instead of plasma, and to use the BCG method and short measurement times. This is also consistent with our results. Our final recommendations are, therefore to

1) Use the bromcresol green (BCG) method in veterinary laboratories. Veterinarians in the field who have blood examined in human laboratories are advised to make sure the laboratory uses this method;
2) Shorten the measurement time to 30 s or less. Vast majority of modern instrumentation makes this easy to achieve.
3) Use only ERM DA 470 k/IFCC follow-up serum as a calibrator.

Following these recommendations, clinically applicable results that are comparable within Europe can be expected.

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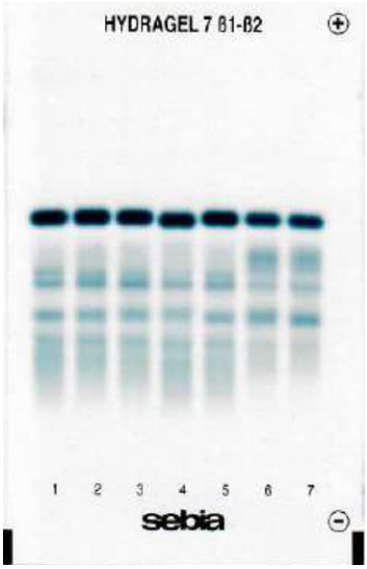

Fig. 1. Electropherogram of bovine sera on agarose. The albumin zone (the fastest travelling) is distinct, sharply delimited and well evaluable.

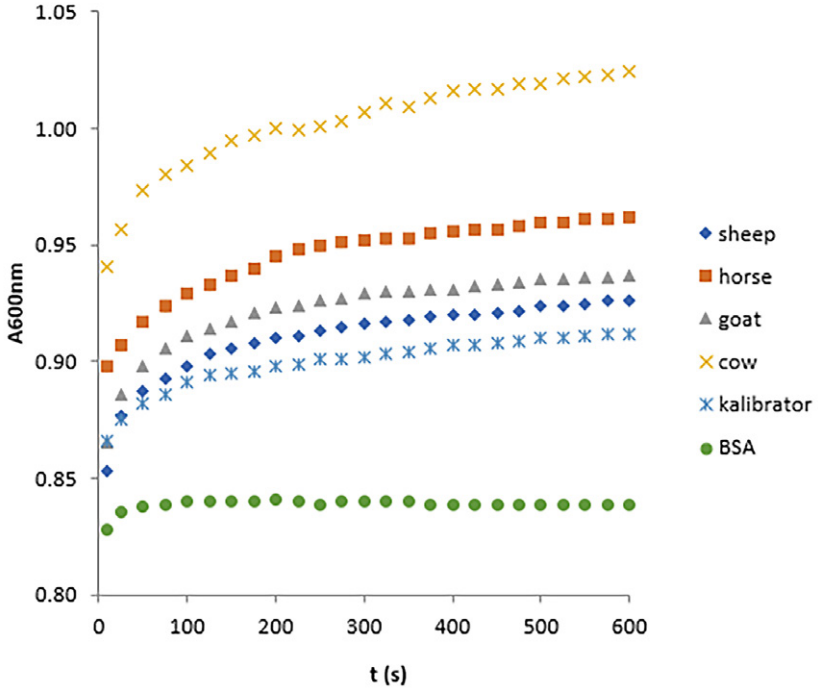

Fig. 2. Time dependence of the increase in absorbance A (600 nm) by the bromocresol green method. It can be seen that the reaction with albumin (BSA – bovine serum albumin) is fast, and there is a steady growth in the case of real samples.