

## Comparison of the Results of Serum Total Protein Concentration Measured by 3 Methods: Preliminary Results

Teodora M. GEORGIEVA<sup>1</sup>, Dimitrinka S. ZAPRYANOVA<sup>1</sup>, Evgeniya V. DISHLYANOVA<sup>1</sup>, Stoyan I. TANEV<sup>2</sup>,  
Ivan P. GEORGIEV<sup>1</sup>, Maria I. ANDONOVA<sup>3</sup>, Ivan N. KANELOV<sup>1</sup>, Lazarin V. LAZAROV<sup>4</sup>, Petya I. KOLEVA<sup>1</sup>

<sup>1</sup>Department of Pharmacology, Animal Physiology and Physiological Chemistry, Faculty of Veterinary Medicine,  
Trakia University, Stara Zagora - BULGARIA

<sup>2</sup>Technology Innovation Management Program, Department of Systems and Computer Engineering,  
Faculty Engineering and Design, Carleton University, Ottawa - CANADA

<sup>3</sup>Department of Functional Pathology and Immunology, Faculty of Veterinary Medicine,  
Trakia University, Stara Zagora - BULGARIA

<sup>4</sup>Department of Internal Noninfection Diseases, Faculty of Veterinary Medicine, Trakia University, Stara Zagora - BULGARIA

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**Abstract:** The present study provides the results from a comparative study of the 3 commonly used methods for total protein (TP) measurement. The experiments were carried out with 6 dogs (4-7 year-old, weighing  $12.8 \pm 1.4$  kg). Five blood samples were obtained by *saphena* venepuncture from all dogs, during the time course of the experimentally induced infection with *Staphylococcus intermedius*, administered subcutaneously at a dose rate of 5 ml of  $1.10^9$  CFU/ml within 14 days. TP concentration was measured by 2 macro protein techniques - biuret method (commonly used) and method of Lowry, and a modified version of biuret method (micro protein technique), suggested by Popov. Serum TP concentration determined by the method of Lowry was significantly ( $P < 0.001$ ) higher than the ones obtained by standard biuret and Popov's methods. The mean differences between TP values obtained by standard biuret technique and Lowry's method and, Lowry's and Popov's method were 18.6 g/l and 23.5 g/l, respectively. There was no statistically significant difference between standard biuret method and its modified version suggested by Popov.

**Key Words:** Total protein concentration, dogs, biuret method, method of Lowry, modification biuret technique by Popov

The total protein (TP) concentration is the most frequently examined blood parameter for diagnostic purposes in veterinary clinical laboratory. Changes in serum protein concentration are associated with a number of disease processes (1). Due to the wide range of potential differential diagnoses for protein abnormalities, it is important that a methodical and logical approach is implemented in order to identify the primary disease (2). In some cases the correlation of the values obtained from method to method is quite low.

Although any other method for TP quantification could be and have been used, none is more convenient or practical than the biuret method (3).

Many colorimetric or dye-binding methods are used for the measuring of protein (3). One of the most common is the biuret method. Numerous versions of the biuret method have been reported, such as the methods given in detail later. All the methods are simple and, when used with contemporary equipment, are sufficiently precise for clinical use (3).

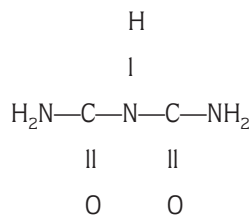
The aim of this study was to determine how the method used for TP measurement may affect the results, and to establish the degree of comparability between 2 macro-techniques and 1 micro-technique that is usually used for cell homogenate samples for protein determination in serum.

\* E-mail: teodora\_g\_2000@yahoo.com

The experiments were carried out with 6 dogs at the age of 4–7 years, weighing  $12.8 \pm 1.4$  kg. The dogs belonged to the Experimental Station of the Faculty of Veterinary Medicine. Blood samples were obtained by saphena venepuncture from all dogs, during the time course of the experimentally induced infection with *Staphylococcus intermedius*, administered subcutaneously at a dose rate of 5 ml of  $1.10^9$  CFU/ml. Sampling period lasted 14 days. During this time, blood sampling was taken 5 times at regular intervals. Blood samples were incubated immediately after collection for 2 h after which it was centrifuged ( $1000 \times g$ , 10 min, 20 °C), the serum supernatant harvested and stored frozen at  $-18$  °C until analyzed within 14 days. After thawing at room temperature, the TP concentration was measured by the biuret method, method of Lowry, and a modified version of biuret technique, designed especially for determination of intracellular proteins, suggested by Popov. A non-automatic spectral colorimeter was used for the analyses. Standard curves for the 3 techniques used were prepared with bovine albumin. The statistical analysis of the data was performed using one way analysis of variance (ANOVA), after estimation for the normality distribution of the data. The significance of the differences of means between the 3 techniques was evaluated by LSD test. All data were expressed as mean  $\pm$  SEM.

Many colorimetric or dye-binding methods are used for the measuring of protein. The biuret assay is one of the most common protein assays.

Most biuret methods can detect between 1 and 15 mg of protein in the aliquot being measured, an amount present in 15 to 200  $\mu$ l of a serum containing protein at 7 g/dL (4). Numerous versions of the biuret assay have been reported, some samples of which are given in detail later. All the methods are simple and, when used with contemporary equipment, are sufficiently precise for clinical use. The biuret reagent contains sodium potassium tartrate to complex cupric ions and maintain their solubility in alkaline solution. KI is included as an antioxidant (4). The biuret method depends on the presence of peptide bonds in all proteins. When a solution of protein is treated with  $\text{Cu}^{2+}$  ions in a moderately alkaline medium, a colored chelate is formed between the  $\text{Cu}^{2+}$  ion and the carbonic oxygen ( $> \text{C} = \text{O}$ ) and amide nitrogen ( $=\text{NH}$ ) atoms of the peptide bond. An analogous reaction occurs between  $\text{Cu}^{2+}$  and the organic compound biuret,



hence the name. Amino acids or dipeptides do not react, but three-, oligo-, and polypeptides do react to give pink to reddish violet products whose absorbance is measured spectrophotometrically at 550 nm. Thus, the biuret reaction with protein is suitable for quantitative determination of TP by spectrophotometry. Either serum or plasma may be used but serum is preferred (3).

The Lowry assay is an often cited general use protein assay (3). Most proteins contain tyrosine or tryptophan or both, but each protein contains a unique proportion of them. Albumin, for instance, has only 0.2% tryptophan by weight, whereas the tryptophan content of individual globulins varies between 2% and 3%. These amino-acids, either free or in an unfolded polypeptide chain, reduce phosphotungstic-phosphomolybdic acid (Folin-Ciocolteu) reagent to produce a blue color. This reaction has been applied to the assay of TP in Lowry's method as an auxiliary to the biuret reaction (3).

In our study we have used the following prescriptions for the reagents:

*Standard biuret method (5):*

- Biuret reagent contains: NaOH, 6.0 mol/l. Dissolve 240 g NaOH in 800 ml of water. Cool and dilute to 1 l. Dissolve 3.0 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in approximately 500 ml of water. Add 9.0 g potassium sodium tartrate [ $\text{K}(\text{OOC}-(\text{CHOH})_2-\text{COONa} \cdot 4\text{H}_2\text{O})$ ] and 5.0 g KJ. When the solution is clear, add 100 ml NaOH, 6 mol/l, and dilute to 1 l with water.
- Biuret blank reagent is prepared exactly as the biuret reagent but no  $\text{CuSO}_4$  is added.
- Protein calibrator, bovine albumin, was used for all techniques, 6 to 7 g/L. Calibration with bovine albumin is less costly than with human albumin.

Blood serum (0.1 ml) was mixed with 5 ml Biuret reagent. Measurements were made after 30 min at wave length 546 nm.

*Method of Lowry (6):*

- Reagent A contains 2%  $\text{Na}_2\text{CO}_3$  in 0.1 N NaOH. Dissolve 2 g  $\text{Na}_2\text{CO}_3$  in 100 ml 0.1 N NaOH.

- Reagent B contains 0.5%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 2% Na-K-tartrate. Dissolve 0.5 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  in 100 ml 2% Na-K-tartrate.
- Reagent C consists of 50 volumes of reagent A and 1 volumes of reagent B.
- Reagent of Folin-Ciocalteu: 50.0 g  $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$  and 12.5 g  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  dissolve in 350 ml bidistilled water. Add 25 ml 85%  $\text{H}_3\text{PO}_4$  and 50 ml concentrate HCl. The mix boils for 10 h, then add 75.0 g  $\text{Li}_2\text{SO}_4$ , 25 ml bidistilled water, and 3 drops of brome. After boiling the solution for 15 min, cool and dilute to 500 ml by bidistilled water and filtrate.

0.1 ml serum was diluted in 50 ml 0.9% NaCl (1:500) before practical assay.

*Modified biuret technique, suggested by Popov (7):*

Reagent contains: 0.25%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.7%  $\text{C}_4\text{H}_4\text{O}_6\text{KNa}$ , 1% NaOH, 0.5% KJ, 24% urea, and 10% NaCl. (2.5 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and 7.0 g  $\text{C}_4\text{H}_4\text{O}_6\text{KNa}$  each dissolve separately in 0.5 l water. Later, after mixing, add 10 g NaOH, 5 g KJ, 240 g urea, and 100 g NaCl).

Blood serum (0.1 ml) was mixed with 5 ml of biuret reagent for the determination of protein. After 30 min, the optical density of the samples was read at 550 nm against blanc (5 ml of reagent and 0.1 ml of water).

Serum TP concentrations (Mean  $\pm$  SEM) by 3 techniques used are presented in Table 1.

Serum TP concentration determined by the method of Lowry was significantly ( $P < 0.001$ ) higher than the ones obtained by the standard biuret and Popov's modification biuret technique. The mean differences between TP values obtained by the method of Lowry versus biuret and Popov's modification technique were 18.6 g/l and 23.5 g/l, respectively. There was no statistically significant difference between standard biuret method and its modified version, suggested by Popov.

In the present study, there was a significant ( $P < 0.001$ ) difference in the concentration of TP, determined

by the method of Lowry and both biuret methods. In the available literature, we found data for the concentration of TP in dogs, measured only by the standard biuret method (8-12).

The results of the present study clearly indicated that the results derived from the original biuret method for serum (macro protein assay) and its modification by Popov for intracellular proteins (micro protein assay) are very close, while the results, determined by the method of Lowry, were significantly higher compared to the others. Therefore, the results differ considerably depending on the method used and based on this big variation in the TP concentration, clinicians could suggest hyperproteinemia.

Our explanation for the higher values of TP concentration, measured by method of Lowry, could be based on 2 reasons: firstly, the dilution errors of the serum before procedure (13), and secondly, its bigger sensitivity, where proteins are allowed first to react with  $\text{Cu}^{2+}$  in alkaline solution to form copper-peptide bond-protein complexes, in which  $\text{Cu}^{2+}$  is reduced to a monovalent ion. When the Folin-Ciocalteu reagent is added, the copper-protein complexes join with tyrosine and tryptophan residues in the reduction process and produce an unstable product that becomes reduced to molybdenum/tungsten. About 75% of the color produced depends on the  $\text{Cu}^{2+}$  complex. The absorbance of the colored complex is measured at a wave length between 650 and 750 nm; less chromogenic complexes are also formed with histidine and cysteine. Lowry's method is sensitive to protein at a lower concentration (10 to 60  $\mu\text{g}/\text{ml}$ ) – i.e., it is 100-fold more sensitive than the biuret reaction alone. The sensitivity is an advantage for measurement of very low concentrations of protein in purified preparations (3). As the Lowry technique is sensitive to tryptophan and tyrosine, differences between the calibrator and test sample might be influenced more with that methodology.

Although TP concentration is one of the constant biochemical parameter, the results of the present study clearly indicated that in, canine sera considerable variations

Table. Total protein concentration (g/L) in dog serum, measured by 3 techniques.

|                  | Standard biuret technique   | Technique of Lowry          | Modification of the biuret technique by Popov |
|------------------|-----------------------------|-----------------------------|---|
| Samples (n = 30) | Mean $\pm$ SEM              | Mean $\pm$ SEM              | Mean $\pm$ SEM                                |
| TP concentration | 65.0 $\pm$ 2.5 <sup>a</sup> | 83.7 $\pm$ 3.4 <sup>b</sup> | 60.2 $\pm$ 1.7 <sup>a</sup>                   |

<sup>a, b</sup> Within a row means of TP concentration with different letters are significantly different ( $P < 0.05$ ).

between reference biuret method and the method of Lowry in the TP concentration were found, depending on the method used. Unquestionably, each of these methods induces an error for determining the total protein concentration. However, the Lowry method had the bigger bias deviation. From this experiment it was established that the biuret technique for determining the serum protein (macro method) and the Popov's modification for determination of cellular proteins (micro protein technique) agree well, which was expected. The results were very

close; there was no significant difference in the obtained data and we could recommend the application of the modification technique of Popov for the determination not only for the intracellular proteins, but also for serum protein concentration. Moreover, we propose to compare the obtained results and the reference range for the method used (biuret or Lowry), as even a small difference between measured and the normal range value could result in confusion in the clinical diagnosis of a disease in an animal.

## References

1. Megrotty, Y., Knottenbelt, C.: Significance of plasma protein abnormalities in dogs and cats. In Practice, 2002; 24: 512-517.
2. Baudner, S.: Neglected aspects in the standardization of protein measurements. A standardized comparison method. Pure Appl. Chem., 1996; 68, 1857-1861.
3. Burtis, C.A.: Methods for the determination of proteins in serum and plasma. In: Burtis, C.A., Edward, R., Ashwood, M.D., Tietz, N.W. Eds. Tietz Textbook of Clinical Chemistry. W.B. Saunders Company, USA, 2<sup>nd</sup> edn., 1994; 692-698.
4. Martínez-Subiela, S., Tecles, F., Eckersall, P.D., Cerón, J.J.: Serum concentrations of acute phase proteins in dogs with leishmaniasis. Vet. Rec., 2002; 23: 241-244.
5. Popov, Ch., Dishlyanova, E.: Determination of a total protein in blood serum. Practical Book in Biochemistry. Zemizdat, Sofia, 1993; 42-43. (article in Bulgarian)
6. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem., 1951; 193: 265-275.
7. Popov, Ch.S.: Effect of chlorpromazine on the stability of rat liver mitochondria, lysosomes and peroxisomes. Comp. Rend. Acad. Bulg. Sci., 1972; 25: 1293-1296.
8. Cerón, J.J., Martínez-Subiela, S., Tecles, F.: Total protein measurement in canine blood samples: answers to some common questions. 3<sup>rd</sup> Annual Meeting, Abstracts, European Society of Veterinary Clinical Pathology (ESVCP), Edinburgh, Scotland, 26 June 2001; 164.
9. Martínez-Subiela, S., Bernal, L.J., Cerón, J.J.: Serum concentrations of acute-phase proteins in dogs with leishmaniasis during short-term treatment. Am. J. Vet. Res., 2003; 64: 1021-1026.
10. Ulutas, B., Bayramli, G., Ulutas, P.A., Karagenc, T.: Serum concentration of some acute phase proteins in naturally occurring canine babesiosis: a preliminary study. Vet. Clin. Pathol., 2005; 34: 144-147.
11. ANONYMOUS: Normal values for dog and cat health parameters. Available at: <http://www.thepetcenter.com/exa/nv.html>. Accessed September 19, 2006.
12. Hamill, A.: Blood profiles for iditarod dogs. Available at: <http://www.iditarod.com/2-6-08.html>. Accessed September 19, 2006.
13. Nishi, H.H., Kestner, J., Elin, R.J.: Four methods for determining total protein compared by using purified protein fractions from human serum. Clin. Chem., 1985; 31: 95-98.