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A N I N T E R N A T I O N A L J O U R N A L

Spectrophotometric Determination of Total Proteins in Blood Plasma: A Comparative Study Among Dye-Binding Methods

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ABSTRACT

A comparative study between the biuret method (standard method for total proteins) and spectrophotometric methods using dyes (Bradford, 3',3'',5',5''-tetrabromophenolphthalein ethyl ester-TBPEE, and erythrosin-B) was carried out for the determination of total proteins in blood plasma from rats. Bradford method showed the highest sensitivity for proteins and biuret method showed the lowest. For all the methods, the absorbance for different proteins (BSA, casein, and egg albumin) was measured and Bradford method showed the lowest variation of absorbance. The concentration of total protein obtained by using Bradford method was not statistically different ($p>0.05$) from concentration of total protein obtained by the biuret method. But in regard to erythrosin-B and TBPEE methods the concentrations of total protein were statistically different ($p<0.05$). Thus, Bradford method could be used instead of the biuret method for determination of total proteins in blood plasma.

Key words: Total proteins, spectrophotometry, blood plasma, dye-binding methods

INTRODUCTION

Determination of total proteins is widely used in several areas such as: clinical analysis, food science, food technology, biochemistry, physiology, protein chemistry, medical research, ecology as well as in many other areas (Zaia et al., 1998). In spite of several methods, using different analytical tools (spectrophotometry, chromatography, polarography, etc.), developed for the determination of total proteins, the UV-Vis spectrophotometric methods are widely used (Zaia et al., 1998). There are many studies of interfering substances using the spectrophotometric methods, however there are few comparative studies among these methods. The dye methods (TBPEE, Bradford, and erythrosin-B) were used for the determination of total protein in blood plasma and results were compared to the biuret method as a standard method (Gornall et al., 1949; Bradford, 1976; Soedjak, 1994). In the present paper, the dye 3',3'',5',5''-tetrabromophenolphthalein ethyl ester (TBPEE) was tested for determination of total proteins and a comparative study among spectrophotometric methods using dyes was carried out.

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MATERIALS AND METHODS

Materials and reagents

Bovine serum albumin (BSA-Sigma) solutions were prepared with distilled water and used as standard in all assays in the following concentrations: 60.0 g/l for the biuret, 1.00 g/l for the Bradford, 1.50 g/l for the erythrosin-B, and 1.50 g/l for the 3',3'',5',5''-tetrabromophenolphthalein ethyl ester methods.
Ultraviolet and visible spectrophotometries were carried out with a spectrophotometer Shimadzu UV-1203. All reagents were of analytical grade.

Methods

Spectrophotometric methods

The spectrophotometric methods of biuret, Bradford, and erythrosin-B were used as described by Gornall et al., *(1949);* Bradford, (1976) and Soedjak, (1994) respectively.

3',3'',5',5''-tetrabromophenolphthalein ethyl ester-TBPEE method

A 50 µl aliquot of blood plasma was transferred to a test tube and the volume was made up to 2.0 ml with distilled water. A 50 µl aliquot of this solution was transferred to a second test tube and the volume was adjusted to 2.0 ml with 0.2 mol L^{-1} of acetic acid. Standard curve was prepared by taking 0.0, 20.0, 40.0, 60.0, 80.0, 100.0 and 120.0 µl of standard solution of BSA (1.5 g L^{-1}), a calibration curve with the concentrations from 0.0 to 84.0 µg mL^{-1} was obtained, the volumes were adjusted to 2.0 ml with acetic acid (0.2 mol L^{-1}). After, in all tubes, 100 µl of TBPEE (0.005% m/v) was added, shaken and incubated at 37ºC for 10 minutes. The tubes were then cooled to room temperature and, after 30 minutes the absorbances at 610 nm were read against the blank (0.0 µg mL^{-1}).

Statistical analysis

Comparisons between means were assessed by using Student t-test at a significance level of $p<0.05$.

RESULTS AND DISCUSSION

The reaction between proteins and TBPEE was described by Feigl (Feigl, 1975) as proteic error in volumetric titulation acid/base. However, there is not a method using TBPEE for determination of total proteins. A study of the reaction between TBPEE and bovine serum albumin (BSA) was undertaken in order to establish the conditions to use this dye for protein analysis. The following acids and buffer were studied: sulfuric acid (0.2 mol L^{-1}), nitric acid (0.2 mol L^{-1}), hydrochloric acid (0.2 mol L^{-1}), acetic acid (0.2 mol L^{-1}) and buffer acetic acid/acetate 0.2 mol L^{-1} (pH 3.0 and pH 5.0). The reaction between TBPEE and BSA did not occur in strong acids media. In the acetic acid and buffer (pH 3.0 and 5.0) media, the product of reaction BSA/TBPEE showed a band at 610 nm (spectra not shown) and the best specific absorbance was obtained with the acetic acid medium. The effect of different concentrations of TBPEE on the absorbance of the product of the reaction BSA/TBPEE was studied. Using 75 µg mL^{-1} of BSA and concentrations from 0.001 to 0.005% of TBPEE in acetic acid (0.2 mol L^{-1}) medium, 0.005% of TBPEE showed the best specific absorbance for the product of reaction BSA/TBPEE (data not shown). The stability of the absorbance of the products of reaction between TBPEE and BSA, at room temperature, after heating for 10 minutes at 37ºC, was also studied (data not shown). After the heating, the absorbance of the product of reaction BSA/TBPEE showed a small decrease in the first 30 minutes, and was stable for the next 60 minutes. Here the reading of the absorbance of products of reaction BSA/TBPEE should be done during 30-90 minutes after the heating. When log (Abs) was used versus log ([BSA]), the Beer-Lambert law was followed in the range of concentration of BSA from 14 to 89 µg mL^{-1}.
Table 1 shows straight-line equation, number of straight-line equations (N), relative specific absorbance [RSA = (specific absorbance of ***X*** method) / (specific absorbance of biuret method)], absorbance of the BSA (bovine serum albumin), casein, and EA (egg albumin) proteins, range of work of protein concentration and concentration of total proteins in rat plasma.

Table 1 - Straight-line equation, number of straight-line equations (N), relative specific absorbance (RSA), absorbance of proteins, range of work of protein concentration and total proteins in rat plasma using the methods: biuret (Gornall et al., 1949), Bradford (Bradford, 1976), erythrosin-B (Soedjak, 1994) and TBPEE.

Method	Straight-line equation Y (Abs)=mX (g L^{-1})+b	N	RSA	Absorbance			Range of concentration (μg mL^{-1})	Rat plasma[#] (g L^{-1})
				BSA	Casein	EA		
Biuret	Y=0.0823X+0.040	7	1.0	0.843	0.650	0.870	1,500-7,500	63.5±4.0 (7)
Bradford	Y=55.69X+0.038	17	676.7	0.340	0.293	0.320	1.0-5.0	56.2±4.6 (10)
Erythrosin-B	Y=18.32X+0.005	9	222.6	0.204	0.252	0.159	3.8-12	50.5±3.3* (10)
TBPEE[Φ]	Y=0.8398X+0.599[Ψ]	11	84.0	0.420	0.120	0.119	14-89	21.7±2.5* (10)

[Φ]3',3'',5',5''-tetrabromophenolphthalein ethyl ester-TBPEE, [Ψ]Y=log(Abs) and X=log([BSA]/g L^{-1}) The specific absorbance from straight line was used to calculate RSA, using the following equation: RSA = [specific absorbance of ***X*** method]/[specific absorbance of biuret method]. Bovine serum albumin (BSA) egg albumin (EA) For each method the absorbances were obtained using the following concentrations of protein: biuret method [BSA]=[Casein]=[EA]=4,500 μg mL^{-1}; Bradford method [BSA]=[Casein]=[EA]=3.0 μg mL^{-1}; erythrosin-B method [BSA]=[Casein]=[EA]=7.5 μg mL^{-1} and TBPEE method [BSA]=[Casein]=[EA]=54 μg mL^{-1}. [#]The results are presented as mean±S.E.M and number of assays are given in parentheses. *For comparison between biuret and other methods ($p<0.05$).

The correlation coefficients for all the straight-lines showed in Table 1 were at least 0.98. Bradford method showed the highest sensitivity for proteins (RSA=676.7 and range of concentration of protein 1.0-5.0 μg mL^{-1}) and biuret method showed the lowest sensitivity for protein (RSA=1.0 and range of concentration of protein 1,500-7,500 μg mL^{-1}) (Table 1). For all the methods, the absorbance of different proteins was also measured. The absorbance of product of reaction with casein was lower for the biuret method than for the product of reaction with BSA and EA.

For all the methods, the absorbance of different proteins was also measured. The absorbance of product of reaction with casein was lower for the biuret method than for the product of reaction with BSA and EA. Gornall et al. (1949) and Keller and Neville (1986) also observed that the specific absorbance of casein was lower than the specific absorbance of other proteins. As the specific absorbance should not depend on amino acids composition of proteins, this was not expected because the principle involved in this method (planar square complex between the cooper and the peptide bond). Bradford method showed the lowest variation of absorbance for the proteins studied herein. This was expected because the absorbance of product of reaction between Coomassie brilliant blue G-250 and proteins depends on the molecular weight of proteins (Goren and Li, 1986; Marshall and Williams, 2000), and the proteins BSA, casein, and EA have high molecular weight. Erythrosin-B method showed a variation of absorbance with the proteins that it was bigger than observed for Bradford method (Table 1). Soedjak (1994) tested the response of erythrosin-B for several proteins and also observed a variation of absorbencies. The TBPEE method showed no variation for the absorbances of casein and EA, but the absorbance of BSA was 3.5 times bigger than them. The biuret method can be recommended for determination of total proteins in blood plasma (Doumas et al., 1981), thus, it was used as standard method. The concentration of total protein obtained by using the erythrosin-B and TBPEE methods were statistically different ($p<0.05$) from concentration of total protein obtained by the biuret method (Table 1). Hence, these methods could not be used for the determination of total protein in blood plasma samples. The concentration of total proteins for biuret and Bradford method were not statistically different ($p>0.05$) from each other (Table 1). Bradford method showed the highest sensitivity for proteins and it could be used instead of the biuret method.

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RESUMO

A determinação de proteínas totais em plasma sangüíneo é importante em diversas áreas de pesquisa. Um estudo comparativo entre o método de biureto (método padrão para proteínas totais) e diversos métodos que utilizam corantes (Bradford, tetrabromofenolftaleína etil éster-TBPEE, e eritrosina-B) foi realizado para a determinação de proteínas totais em plasma sangüíneo de ratos. O método de Bradford mostrou a maior sensibilidade para proteínas e o de biureto a menor. Para todos os métodos, as absorbâncias para diferentes proteínas (BSA, caseína, e ovoalbumina) foram medidas e o método de Bradford mostrou a menor variação da absorbância. Utilizando o método de Bradford a concentração de proteínas totais obtida não foi estatisticamente diferente ($p>0.05$) daquela obtida pelo método do biureto. Porém, para os métodos da eritrosina-B e TBPEE as concentrações de proteínas totais foram estatisticamente diferentes ($p<0.05$) da obtida pelo método de biureto. Portanto o método de Bradford pode ser utilizado no lugar do método de biureto para a determinação de proteínas totais em plasma sangüíneo.

REFERENCES

Bradford, M. M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72,** 248-254.

Doumas, B. T.; Bayse, D. D.; Carter, R. J.; Peters Jr, T. and Schaffer, R. (1981), A candidate reference method for determination of total protein in serum. I. Development and validation. *Clin. Chem.*, **27**, 1642-1650.

Feigl, F. (1975), *Spot test in organic analysis*. 7th ed. New York : Elsevier Scientific Publishing Company.

Goren, M. P. and Li, J. T. L (1986), The Coomassie brilliant blue method underestimates drug-induced tubular proteinuria. *Clin. Chem.*, **32**, 386-388.

Gornall, A. G.; Bardawill, C. J. and David, M. M. (1949), Determination of serum proteins by means of the biureto reaction. *J. Biol. Chem.*, **177**, 751-766.

Keller, R. P. and Neville, M. C. (1986), Determination of total protein in human milk: comparison of methods. *Clin. Chem.*, **32**, 120-123.

Marshall, T. and Williams, K. M. (2000), Total protein determination in urine: elimination of a differential response between the Coomassie blue and pyrogallol red protein dye-binding assays. *Clin. Chem.*, **46**, 392-398.

Soedjak, H. S. (1994), Colorimetric micromethod for protein determination with erythrosin-B. *Anal. Biochem.*, **220**, 142-148.

Zaia, D. A. M.; Zaia, C. T. B. V. and Lichtig., J (1998), Determination of total protein by spectrophotometry: advantages and disadvantages of proposed methods. *Química Nova*, **21**, 787-793.

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