Colourimetric Assay of Serum Protein

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Abstract

Total protein measurement is key for detecting diseases associated with albumin and immunoglobulins. Piotrowski's test, also called the biuret test, is the main test used for this task. The results of this test are measured by using spectrophotometry and the output is the absorbance. The Beer-Lambert law associates the concentration of the solution with the absorbance measured. A standard curve was created from the results obtained and it showed the expected output. The appropriate specific absorption coefficient was calculated using the slope of the standard curve. Using a spectrophotometer, the unknown solution was identified as sample B. Altogether, the aims of this experiment were met, and the results obtained agreed with the theory previously established.

Introduction

Many proteins are found dissolved in plasma. The main proteins in serum are the albumin (up to 40%) and the immunoglobulins, such as IgM and Ig. After the plasma has been clotted and the fibrinogen has been removed, the remained fluid is called serum (Busher, 1990). Total protein measurement provides an indication of the concentration of all proteins in the serum. Protein measurement can be useful for disease detection such as liver function tests, humoral immunodeficiency, or a paraprotein in the blood, as it calculates the individual immunoglobulins.

Piotrowski's test (biuret test) is a chemical test used for determining peptide bonds. This is the most popular technique to detect and measure total protein using mechanical chemistry analysers. In the biuret method, peptides stimulate the activity of copper (II) ions to create purple coordination complexes if the solution is under alkaline conditions. The complex presents a blue colour since it absorbs light at a wavelength of 540 nm. Although it changes from blue to violet if the protein is present (Vedantu, 2022).

In Piotrowski's test, copper ions produce a chelate complex by using the electron pairs of peptide nitrogen and oxygen from water. Sodium potassium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) is also included in the biuret reagent to stabilize CU^{2+} and avoid their reduction to Cu^{+} (Sánchez Rojas, Bosch Ojeda and Cano Pavón, 2013). Modifications such as the Lowry and Folin-Ciocalteu have been done to improve the sensitivity. They facilitate the detection of small amounts of protein present in separation procedures. Lowry protein assay includes the Folin-Ciocalteu reagent, phosphotungstic and phosphomolybdic acid that reacts with the cuprous ions and amino acids such as cysteine and tyrosine. Consequently, a blue-green colour can be detected at a wavelength of 650 and 750 nm (Shen, 2019).

Spectrophotometry represents the quantitative analysis and measurement of the interaction between light and materials. It links the amount of concentration of the solution with the proportion of absorbed light by using the Beer-Lambert law. Since the spectrophotometry displays the absorbance (A), it is possible to find the solution concentration using Beer-Lambert equation ($A=\varepsilon l\ c$) (Edinburgh Instruments, 2022). In addition, the spectrophotometer used in the experiment has a length of the light path through the solution of 1 cm.

It is common to dilute a solution to decrease its concentration by adding extra solvent. In this case, the moles of solute from the stock solution and the final diluted solution are the same. This fact can be calculated mathematically with the following equation: $C_1*V_1=C_2*V_2$, C_1 denotes the concentration of the stock solution, V_1 the volume of stock solution taken for dilution, C_2 concentration of the diluted solution in the same units, and V_2 volume the diluted solution. Serial dilutions dilute a standard solution multiple times and the dilution factor has to be considered.

One of the aims of the experiment was to calculate the molar absorption (ε) for this compound by creating a standard curve. The slope of the line of the standard curve when concentration is plotted against absorbance represents the relationship between absorbance and concentration, which is the value ε (ε=A/C) if l=1cm. The concentration of any solution with this compound can be calculated once the ε value has been found.

All data are estimates, including the gradient and intercept analysed by software. For instance, when the experiment is repeated, the values are usually different to a small extent. Despite this, standard curves are still used to calculate the concentration. Therefore, it is important to be aware of the prediction error (Savage, 2022). Another aim of the experiment is to understand the associated error from the result calculated.

Pipettes are usually used to transfer aliquots of liquids to accurately measure the volume from one container to another. It is fundamental to use the appropriate pipette in the correct way to avoid increasing the errors associated with the experiment. This experiment was an opportunity to use micropipettes.

Methods

Firstly, using the 10 mg/ml Bovine Serum Albumin (BSA) stock, solutions with 0 mg/ml, 1 mg/ml, 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml, and 10 mg/ml concentrations of BSA were prepared in test tubes. They were diluted with saline solution (0.9%NaCl) and thoroughly mixed.

Secondly, 1 ml was taken of the solution sample assigned (A, B or C) and it was diluted 1 in 5 to produce a final volume of 10 ml. 1ml was pipetted into 3 tubes.

Finally, 4ml of biuret reagent was added to all 10 tubes and after 30 minutes, the absorbance of each tube at 550 nm was measured.

Results and calculations

Standard calibrations BSA

Several calculations were carried out to find out how much BSA volume was needed to make the dilutions of BSA concentrations from the 10 mg/ml BSA stock. They were made using the equation $C_1*V_1=C_2*V_2$

Table 1. Volumes used to prepare the different BSA concentration solutions

Solution concentration (mg/ml)	Volume BSA stock solution (ml)	Volume saline solution (ml)	Total volume (ml)
0	0	1	1
1	0.1	0.9	1
2	0.2	0.8	1
4	0.4	0.6	1
6	0.6	0.4	1
8	0.8	0.2	1
10	1	0	1

The absorbance of the standard calibrations BSA (mg/ml) were calculated using the spectrophotometer.

Table 2: Spectrophotometer absorbance results

Standard calibration BSA (mg/ml)	Abs @550 nm
0	0
1	0.082
2	0.153
4	0.269
6	0.375
8	0.478
10	0.612

A graph was plotted using the absorbance results obtained by measuring each tube containing the BSA dilutions at 550nm.

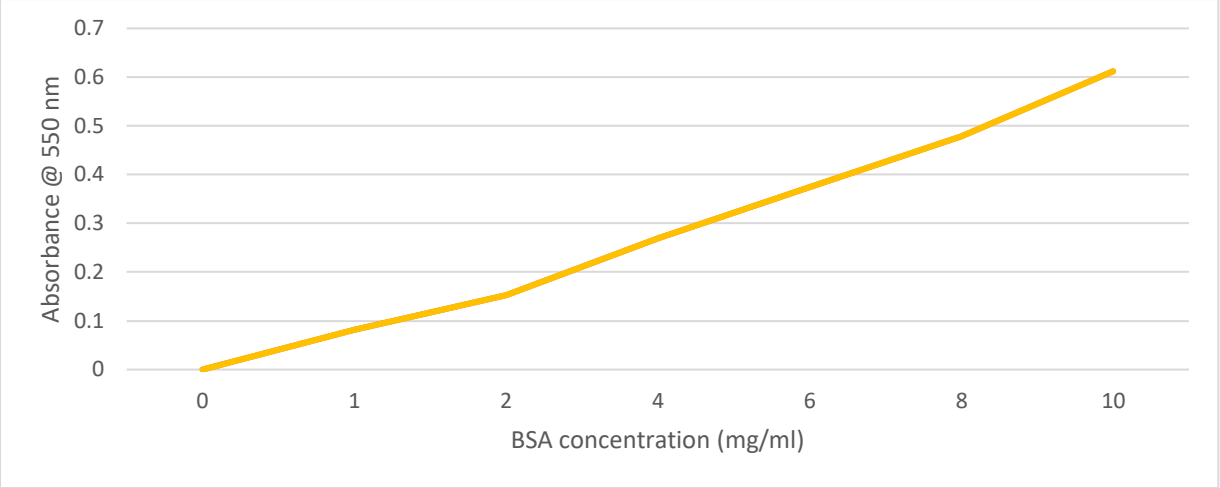

Figure 1: Graph showing the absorbance readings of BSA concentration in each reaction.

The gradient (m) was determined by two points from the graph: M= (y2-y1)/(x2-x1)= (0.590-0.100)/(9-1.5)=0.49/7.5= 0.06533

In this case, the intercept is 0 because the calibration curve passes by the (0,0) origin. So, y=mx+c -> y=mx where x is the concentration and y the absorbance. Applying the Beer-Lambert equation (A=ξlc): A=y, L=1 cm, c=x (mg/ml), so A=ξc, y=mc, m=ξ. If m=ξ then ξ =0.06533 ml^{-1}*mg*cm

Unknown solution

The absorbances of the unknown concentration solution were calculated using the spectrophotometer: 0.254 A, 0.241 A, 0.258 A. The overall is 0.251 A. This result was used to calculate the concentration by applying the equation Y=mc. Same as before, the intercept is 0 because the calibration curve passes by the (0,0) origin. The dilution factor is 5, so c=y*dilution factor/m= (0.251*5)/0.06533= 19.21 mg/ml.

To calculate the prediction error associated with the results, the prediction interval was calculated using the software Jamovi: xE + tdfSXE, where Xe is 3.914, tdf is 2.571, and Sxe is 0.991.

Lower limit 3.914-(2.571*0.991)= 1.366
Upper limit 3.914-(2.571*0.991)= 6.462

Measurement by interpolation estimated a value of 19.21 mg/ml. 95% prediction interval (1.366, 6.462) for the sample.

Discussion

The standard calibrations needed to create the standard curve were made by mixing specific volumes from the BSA stock solution and the saline solution. For this task, the equation

$C_1*V_1=C_2*V_2$ was used, and the appropriate volumes were calculated to make a total volume of q ml of the solution concentration (see table 1).

The standard curve that establishes the relationship between absorbance and concentration (see figure 1) showed the expected and usual output. It grows gradually displaying a linear output. As stated, when the BSA concentration had a bigger absorbance (0.478 A), the larger the concentration was (8 mg/ml). This relationship can be seen in Figure 1. Due to having a usual standard curve, it was possible to calculate the specific absorption coefficient for this protein serum by using the standard curve and the Beer-Lambert law, as it was mentioned in the introduction. This protein had a value of 0.06533 $ml^{-1}*mg*cm$. Using this value, the concentration of any solution with this compound can be calculated.

The expected value for the unknown sample B is 20 mg/ml. The result obtained in the calculations was 19.21 mg/ml, so it can be concluded that the unknown sample was solution B. As mentioned in the introduction, all the measurements are estimates and they come with a prediction error. This was calculated with Jamovi and the prediction interval output reports the 95% prediction interval for the unknown concentration. It can be said with 95% of confidence that the unknown concentration is sample B with a margin error of 1.366 to 6.462.

Uncertainties in measurements are an important factor that directly affects the results of the experiment. In this case, micropipettes were used to measure the volumes of prepared solutions. An increase in the error associated with the experiment is assured without accurate pipetting.

Conclusion

The colourimetric assay of serum protein was carried out to detect the presence of peptide bonds and estimate the specific absorption coefficient for the protein serum. The specific absorption coefficient of the protein was obtained so the analysis of the concentration of the same compound in any solution can be made. It was discovered that the unknown sample was taken from solution B. In conclusion, the results were coherent with the theory discussed in the introduction, and the aims of the experiment were met.

References

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