

Selection of Protein Standards in Estimation of Total Protein Content in Commercially Available Intravenous Immunoglobulin (IVIG) Preparations by Biuret Method and its Comparison with Kjeldahl Method: Guidance to IVIG Manufacturers

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Abstract: *Immunoglobulins (IgG) derived from the human plasma are used as bio therapeutics primarily in the form of polyclonal IgG or as hyper immune sera. Intravenous Immunoglobulin (IVIG) has been the driving force of the growth of plasma products industry. The manufacturing process of IVIG begins with collection & testing of single donation plasma, pooling of thousands of these donations, confirmation of plasma pool for freedom from viral markers, followed by very extensive process of separation and purification of the plasma pool with ethanol or chromatography. To assure the quality each batch of the product has to go through various quality control tests as mentioned in the Indian Pharmacopoeia monograph. The total protein content is directly linked to the efficacy of the treatment of patients having Primary Immune Deficiencies or other immunological disorders. The gold standard method of Kjeldahl for total protein estimation has been specified in the pharmacopoeia but most of the time the manufacturers prefer to use biuret method for total protein estimation due to its ease of performance and comparative less time taken for obtaining the results. National Institute of Biologicals (NIB) being the notified Central Drug Laboratory for Blood products has been considered as the nodal organization in India to assess and verify test methods applicable for assuring the quality of blood products. The present study aims at assessing the suitability of biuret method without Trichloroacetic acid precipitation of proteins, for total protein estimation in the commercially available IVIG preparations and also to highlight various critical parameters to be considered to ensure reliable results which will be useful for the IVIG manufacturers for reliable assessment of the protein content of their product.*

Keywords: Intravenous Immunoglobulin, total protein, biuret, kjeldahl, Protein standard

1. Introduction

Immunoglobulins (IgG) derived from the human plasma are used as bio therapeutics primarily in the form of polyclonal IgG or as hyper immune sera. Intravenous Immunoglobulin (IVIG) has been the driving force of the growth of plasma products industry. (IgG) derived from the human plasma are used as bio therapeutics primarily in the form of polyclonal IgG or as hyper immune sera. The demand for IVIG has been growing on account of increased usage of IVIG in the treatment of primary immunodeficiency as well as newer neurological indications. The global immunoglobulin market is also expected to grow markedly in the near future. This growth is fueled by exciting therapeutic discoveries such as the recent announcement from the American Academy of Neurology that immunoglobulin agents may ease the symptoms of Alzheimer's disease [1]. The manufacturing process of IVIG begins with collection & testing of single donation plasma, confirmation of plasma pool for freedom from viral markers, followed by very extensive process of separation and purification of the plasma pool with ethanol or chromatography. Though the basic method of fractionation remains the same, by incorporation of various modifications in the process of purification, stabilization and

virus inactivation / removal, have yielded products clearly different from one another.

Even with stringent monitoring of the production process and various virus validation studies, adverse reactions like hypotension, hemolysis etc. have been documented globally. Any therapeutic product for human use has to fulfill two basic qualities – safety, and efficacy. [1]. Therefore quality control testing of such products are of highest significance to assure its safety and efficacy. The products are tested as per the recommendations of pharmacopoeia and for marketing approval ideally it should comply the Pharmacopoeial standard of the country where the products are going to be used. The Indian pharmacopoeia 2014 recommends that the IVIG preparation contains not less than 30 g /L of protein and not less than 90 % and not more than 110 % of the quantity of protein stated on the label. The method as per the pharmacopoeia is the Kjeldahl method for total nitrogen estimation.[3] but most of the time the manufacturers prefer to use biuret method for total protein estimation due to its ease of performance and comparative less time taken for obtaining the results.

The potency of the IVIG product is directly linked to the concentration of IgG in the product. Blood viscosity after IVIG infusion increases proportionally to the IgG concentration (directly proportional to total protein content) in the preparation used and may still be elevated 3 to 5 days after IVIG infusion. The authors of this finding postulated that changes in viscosity occurring after IVIG therapy could impair blood flow and increase the risk of myocardial infarction or stroke in patients at risk from cardiovascular and thromboembolic events and also other side. Therefore increased plasma viscosity or higher protein concentration could represent an additional risk for elderly patients and for HIV-infected patients with effects such as acute renal failure hypergammaglobulinemia who receive large amount of IVIG. It has been found that increase in the viscosity of blood could also result from increase in the red cell aggregation, mediated by higher level of IVIG and change in the red cell properties. Hence, a higher concentration of the protein above the recommended value may contribute to significant adverse events such as renal complications or thromboembolic episodes. Contrarily, a lower concentration below the recommended value will result in ineffective therapy. Hence the determination of total protein content is essential [2].

There are several methods for estimation of Total Protein such as Lowry Assay, Bradford Assay, Bicinchoninic Acid (BCA) Assay, Biuret Assay, Kjeldahl Assay & Ultra violet absorption Assay etc. The various protein analysis techniques available are all uniquely dependent on protein composition – amino acid content and any covalently bound material – and protein quantity. Different methods will yield slight variations when quantifying proteins. On the contrary, the Kjeldahl method measures only total organic nitrogen content and is not affected by amino acid content. Thus, the Kjeldahl method measures absolute concentration and is considered the ‘gold standard’ in protein quantification [4]. Andrew Wong, Andre C. Siegel, Justin Manuel, Gary A. Levy et al [5] in a study observed that the use of human serum IgG in Kjeldahl had ambiguity due to the multiple isotypes that exist for IgG. Constant nitrogen content could not be determined and therefore, the protein concentration determined was only approximate. When confronted with the need to determine the total protein concentration of a sample, one of the first issues to consider is selection of a protein assay method. The choice among the available protein assays usually is made based upon consideration of the sensitivity of the method related to the concentration of protein in the sample. Because the working range for the Biuret assay is from 2 to 160 mg/ml, the Biuret reagent has found utility in the clinical laboratories for the quantitation of total protein in serum [4].

National Institute of Biologicals (NIB) being notified vide Gazette notification No. 684, Extraordinary, Part-II, Section-3-subsection (i) G.S.R. 908E dated 22nd December 2014 as Central Drug Laboratory for Blood products, is the nodal organization in India to assess and verify test methods applicable for assuring the quality of blood products. The present study aims at assessing the suitability of biuret method without Trichloroacetic acid precipitation of proteins, for total protein estimation in the commercially available IVIG preparations and also to highlight various critical parameters to be considered to ensure reliable results. .

2. Materials and Methods

The selection of a protein standard is potentially the greatest source of error in any protein assay. In the study the choice of standards were assessed for estimating total protein in intravenous immunoglobulin preparations. Here in the study two different immunoglobulin preparations and Bovine Serum Albumin were used for standard curve preparation for estimation of total protein in different commercially available intravenous immunoglobulin preparations with label claim of 5% total protein content. These products designated as protein standards are:
Standard 1: Immunoglobulin preparation Containing IgG and enriched with IgM, glucose used as stabilizer
Standard-2 Immunoglobulin preparation containing more than 99% IgG and Glycine stabilizer
Standard 3: BSA (20% solution)

The biuret method followed is as given in British Pharmacopoeia 2009 which is also the same as in the 2015 Volume V V-298 VIII P. However Trichloroacetic acid precipitation of protein was omitted as the dissolution of the precipitate in alkali after centrifugation was a tedious job as the protein precipitate tends to remain as a hard insoluble mass because of the long centrifugation process which binds them very closely which gave rise to inconsistent results . Hence the method as in British Pharmacopoeia without Trichloroacetic acid precipitation was studied for different incubation periods to get optimal reading for the color development which gives consistent and reliable estimation of total protein. The total protein of the samples and standard were also correlated to the results obtained kjeldahl method which was validated in-house.

3. Results & Discussion

The estimated protein concentration in different IVIG preparations using two immunoglobulin protein standards and Bovine Serum Albumin standard is depicted in Fig 1,

Figure 1: Total protein estimation of IVIG 5% (>96% IgG, Maltose Stabilizer) using different protein standards, without trichloroacetic acid precipitation) and 30 minutes incubation after addition of biuret reagent in comparison to Kjeldahl method

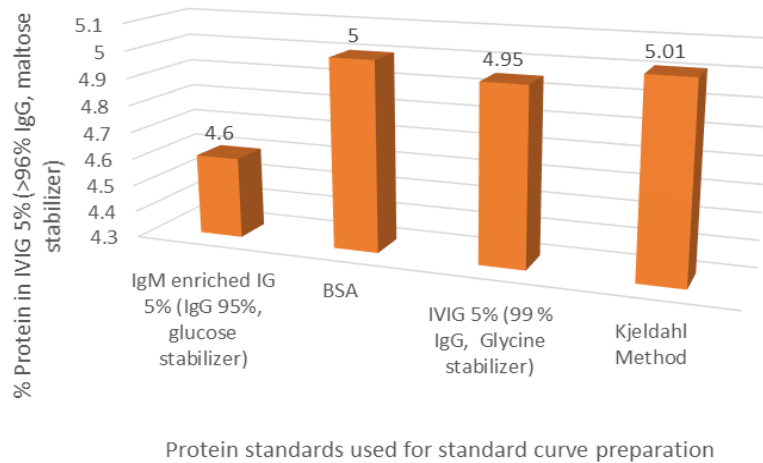


Figure 2: Total protein estimation of two different commercially available IVIG preparations by biuret method at various incubation periods (Without TCA precipitation) using IVIG 5% (>99% IgG, Glycine stabilizer)

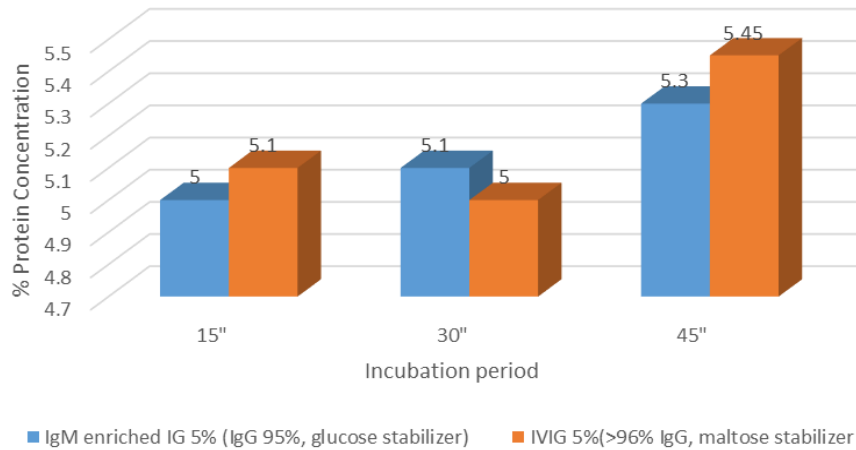
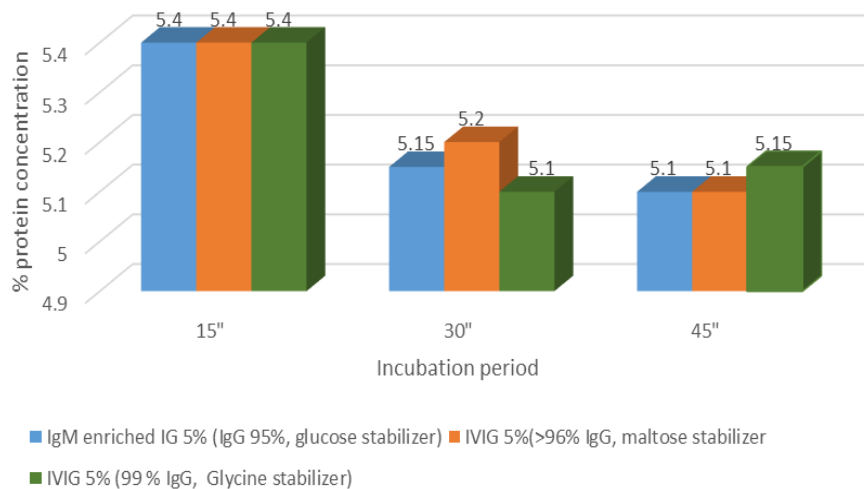


Figure 3: Total protein estimation of three different commercially available IVIG preparations by biuret method at various incubation periods (Without TCA precipitation) using BSA for standard curve



4. Discussion

The total protein content of the plasma derived products like human albumin and human normal immunoglobulin is directly linked to the efficacy of the treatment using these products. Blood viscosity after IVIG infusion increases proportionally to the IgG concentration (directly proportional to total protein content) in the preparation used and may still be elevated 3 to 5 days after IVIG infusion [6]. The authors of this finding postulated that changes in viscosity occurring after IVIG therapy could impair blood flow and increase the risk of myocardial infarction or stroke in patients at risk from cardiovascular and thromboembolic events and also other side effects such as acute renal failure [7]. Therefore increased plasma viscosity or higher protein concentration could represent an additional risk for elderly patients and for HIV-infected patients with hypergammaglobulinemia who receive large amount of IVIG. It has been found that increase in the viscosity of blood could also result from increase in the red cell aggregation, mediated by higher level of IVIG and change in the red cell properties. Hence, a higher concentration of the protein above the recommended value may contribute to significant adverse events such as renal complications or thromboembolic episodes. Contrarily, a lower concentration below the recommended value will result in ineffective therapy [8].

The gold standard method of Kjeldahl for total nitrogen estimation has been specified in the pharmacopoeia but many a times biuret method has also been used for total protein estimation due to its comparative less time taken and easy performance. Unlike human albumin protein estimation it has been observed that the composition of the immunoglobulin preparation and the differences in the stabilizers, do affect the estimation of protein content in IVIG products by biuret method. The biuret reaction was chosen because of the relative specificity of the biuret reagent for proteins, the reproducibility of absorbance values when the reaction (color development) is allowed to go to completion, the close similarity in the absorptivity values (color yield) of the main serum protein fractions, and the relatively few substances that interfere [4].

In the present study samples of immunoglobulin preparations having different composition with respect to immunoglobulin content and the stabilizer added were analyzed against different protein standards to find out what kind of protein standard will be preferable to get a comparable result with kjeldahl method. The procedure followed by the global document as which is also mentioned in the British Pharmacopoeia was the choice of method. The interference of the stabilizers were also analyzed for recommending a suitable protein standard for the performance of biuret assay for total protein determination in intravenous immunoglobulin.

The selection of a protein standard is potentially the greatest source of error in any protein assay. Of course, the best choice for a standard is a highly purified version of the predominate protein found in the samples. This is not always possible nor always necessary. In some cases, all that is needed is a rough estimate of the total protein concentration

in the sample. For example, in the early stages of purifying a protein, identifying which fractions contain the most protein may be all that is required. If a highly purified version of the protein of interest is not available or it is too expensive to use as the standard, the alternative is to choose a protein that will produce a very similar color response curve with the selected protein assay method.

For general protein assay work, bovine serum albumin (BSA) works well as the choice for a protein standard, because it is widely available in high purity and relatively inexpensive. Although it is a mixture containing several immunoglobulin, bovine gamma globulin (BGG) is also a good choice for a standard when determining the concentration of antibodies, since BGG produces a color response curve that is very similar to that of immunoglobulin G (IgG) [9].

For greatest accuracy of the estimates of the total protein concentration in unknown samples, it is essential to include a standard curve in each run. This is particularly true for the protein assay methods that produce nonlinear standard curves (e.g., Lowry method, Coomassie dye-binding method). The decision about the number of standards used to define the standard curve and the number of replicates to be done on each standard depends upon the degree of nonlinearity in the standard curve and the degree of accuracy required of the results. In general, fewer points are needed to construct a standard curve if the color response curve is linear.

In the current study using the method of biuret without TCA precipitation, standards of different composition were compared to the gold standard Kjeldahl method. It was observed that the protein standard of liquid nature containing a major amount of immunoglobulin IgM shows a lower especially when the sample contains maltose as a stabilizer. The other products did not show considerable variation.

Another Immunoglobulin preparation which has $\geq 99\%$ IgG content and glycine as stabilizer was analyzed for its suitability as protein standard for standard curve preparation. This standard also gave protein concentration of different types of immunoglobulin preparations which were comparable to the gold standard Kjeldahl method results.

The protein standard solution BSA (Bovine serum Albumin) P5369, 200 mg/ml, procured from Sigma Aldrich, gave a very consistent and accurate result irrespective of the composition of the immunoglobulin samples with glucose, maltose or glycine stabilizers. This standard also gave a good correlation to the results obtained with the Kjeldahl method.

The caution which needs to be exercised while using any protein standard is that each lot of the standard should be evaluated before use either by kjeldahl method or by biuret. If using biuret method the new bottle should be tested against an already evaluated lot of the protein standard having an assigned value.

The method done routinely for total protein estimation of intravenous immunoglobulin in the laboratory is by the TCA

precipitation method for biuret. Even though this is a sturdy method it has been found that the procedure requires adherence to strict timings for addition of NaOH and biuret reagent the delay in which may affect the results by giving a lower correlation co-efficient. Hence the method was modified and assessed without the step of adding TCA or subsequent dissolving the precipitate in NaOH. The validation of the method was done to assess the accuracy, repeatability and robustness of the procedure. Various time periods for incubation of the standard dilutions and the sample dilutions after the addition of biuret reagent was critically analyzed, as the samples containing maltose started giving brown precipitate if left at room temperature when these samples are not TCA precipitated. It is observed that the and the Immunoglobulin solution containing $\geq 99\%$ IgG and glycine stabilizer gave a good correlation of total protein content with the Kjeldahl method result and an incubation time not less than 15 minutes and maximum 30 minutes as IVIG product with maltose as stabilizer started precipitating after 30 minutes and hence gave a higher reading. and BSA protein standard solution 200 mg/ml M/s Sigma also was found to be acceptable to be used as protein standard for determination of total protein content of different types IVIG preparations when the incubation period after adding the biuret reagent was limited to a period of 30-45 minutes.

5. Conclusion

The Biuret Method as given in British pharmacopoeia 2015, Volume V V-298 Appendix VIII P, is based on the interaction of cupric (Cu^{2+}) ion with protein in alkaline solution and resultant development of absorbance at 545nm. This test shows minimal difference between equivalent IgG and albumin samples. Addition of sodium hydroxide and biuret reagent as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and addition of the biuret reagent will give IgG samples a higher response than albumin samples. The Omission of precipitation of proteins with Trichloroacetic acid was studied as the dissolution of the precipitate in alkali after centrifugation was a tedious job as the protein precipitate tends to remain as a hard insoluble mass because of the long centrifugation process which binds them very closely.

The present study, clearly shows that biuret method even without precipitation with TCA can be introduced for routine total protein estimation of plasma derived intravenous immunoglobulin preparations since it shows good correlation with the Kjeldahl method. The choice of the composition of the protein standard and the incubation time after addition of biuret reagent for color development are two factors that are to be strictly adhered to. On general principles, the best standard for an analytical method is a primary standard, i.e., a pure sample of the substance to be measured. However, IVIG products contains a mixture of proteins of somewhat different biuret values. It was observed in this study that such preparations containing higher amount IgM may be avoided to be used as protein standard for total protein estimation of IVIG samples as this gives a very low reading compared to the result obtained with Kjeldahl method. Even though the study does not include the use of Immunoglobulin preparations stabilized with glucose or

maltose for its suitability as standard for preparation of standard curve, it was observed in the study that such products tend to precipitate and hence the method should include TCA precipitation for yielding a comparable result to Kjeldahl.

The liquid immunoglobulin preparation containing more than 99% IgG with glycine stabilizer is found to be suitable for use as protein standard for preparation of standard curve but those that are IgM enriched needs to be avoided. The optimum incubation period using this standard is a maximum of 30 minutes but not less than 15 minutes. It was observed that the BSA as already published earlier, is the choice of standard even for estimating protein concentration in various types of Intravenous Immunoglobulin preparations. The incubation time within 30- 45 minutes gave a good correlation to the gold standard Kjeldahl method.

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