REVIEW
Colorimetric protein assay techniques

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There has been an increase in the number of colorimetric assay techniques for the determination of protein concentration over the past 20 years. This has resulted in a perceived increase in sensitivity and accuracy with the advent of new techniques. The present review considers these advances with emphasis on the potential use of such technologies in the assay of biopharmaceuticals. The techniques reviewed include Coomassie Blue G-250 dye binding (the Bradford assay), the Lowry assay, the bicinchoninic acid assay and the biuret assay. It is shown that each assay has advantages and disadvantages relative to sensitivity, ease of performance, acceptance in the literature, accuracy and reproducibility/coefficient of variation/laboratory-to-laboratory variation. A comparison of the use of several assays with the same sample population is presented. It is suggested that the most critical issue in the use of a chromogenic protein assay for the characterization of a biopharmaceutical is the selection of a standard for the calibration of the assay; it is crucial that the standard be representative of the sample. If it is not possible to match the standard with the sample from the perspective of protein composition, then it is preferable to use an assay that is not sensitive to the composition of the protein such as a micro-Kjeldahl technique, quantitative amino acid analysis or the biuret assay. In a complex mixture it might be inappropriate to focus on a general method of protein determination and much more informative to use specific methods relating to the protein(s) of particular interest, using either specific assays or antibody-based methods. The key point is that whatever method is adopted as the 'gold standard' for a given protein, this method needs to be used routinely for calibration.

Introduction

The purpose of this review is to provide a critical evaluation of commonly used colorimetric or chromogenic methods for the determination of protein concentration. Care must be taken in the use of these methods because several of the more frequently used methods depend on protein composition as well as quantity. The composition includes the amino acid content and any covalently bound material, primarily carbohydrate, as well as the protein conformation. It is clear that results obtained with some of the colorimetric assay techniques considered in this review are quite dependent on the protein composition.

The determination of exact protein concentration frequently requires the use of the Kjeldahl procedure, which is both time-consuming and sample-consuming [1–3]. This procedure is therefore infrequently used in research laboratories but has increased application in the biopharmaceutical industry; it is used primarily as a method for the validation of a more facile analytical process for the determination of protein concentration.

This review considers four commonly used methods: the biuret method [4], the Lowry method [5], Coomassie Blue (CB) G-250 dye-binding [6] and the bicinchoninic acid (BCA) assay [7] for the colorimetric determination of protein concentration in solution. Additional sections discuss a collection of procedures that are less commonly used. Emphasis is placed on factors that influence the values obtained for protein concentration, including protein composition issues and solvent composition. These considerations are followed by a section comparing the use of several different assays with the same sample population. The final section briefly discusses some approaches to the validation of a colorimetric protein assay for use under Good Laboratory Practice (GLP) and current Good Manufacturing Practice (cGMP) conditions. It is our intention to provide sufficient information for investigators to be able to select the procedures appropriate to their specific applications. The reader is also recommended to read several review articles that compare protein assay procedures [8–13]. For those readers interested in the earlier development of protein analysis, a reading of Rosenfeld's excellent contribution [14] is recommended.

This review does not consider all colorimetric protein

Abbreviations used: BCA, bicinchoninic acid; CB, Coomassie Blue; GLP, Good Laboratory Practice; cGMP, current Good Manufacturing Practice; PLGA, polylactic-co-glycollic acid; SEC, size-exclusion chromatography.

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assays. Omitted are 3-(4-carboxybenzoyl)quinoline-2-carboxyaldehyde, a newly described fluorogenic reagent [15], reagents such as Bromocresol Green [16] or Albumin Blue [17], which are used specifically to measure albumin, or procedures in which the protein is derivatized with a 'signal' reagent such as biotin, which is subsequently detected by reaction with peroxidase-coupled avidin [18]. Also not considered is the use of far-UV spectroscopy [19], the use of Amido Black [20] or Ponceau S [21] or erythrosin B [22].

Biuret reaction

Although the use of the biuret reaction for the determination of protein concentration dates to 80 years, the current approaches to the use of this procedure can be traced to the work of Gornall et al. [4] in 1949. The biuret reaction is based on the complex formation of cupric ions with proteins. In this reaction, copper sulphate is added to a protein solution in strong alkaline solution. A purplish-violet colour is produced, resulting from complex formation between the cupric ions and the peptide bond. The name of the reaction is derived from biuret, which forms a similar coloured complex with cupric ions [23]. The biuret reaction with proteins is independent of the composition of the protein; therefore protein composition is not a factor [24–27]. However, protein purity and association state could influence the results obtained with the biuret reagent.

The biuret reaction is, however, somewhat insensitive compared with the other methods of colorimetric protein determination. Matsushita et al. [28] have described a recent modification of the biuret reaction, 'a reverse biuret method', which appears to have significantly increased sensitivity. Colour in the 'classic' biuret reaction is produced by the formation of a protein–copper–tartrate complex; in the 'reverse biuret reaction' colour is generated by the reduction of excess cupric ions, not bound in the biuret complex, by ascorbic acid to cuprous ions, which are subsequently measured as a complex with bathocuproine. The amount of Cu^{+}–bathocuproine complex is inversely proportional to the protein concentration. The sensitivity of this reaction is stated to be greater than either the Lowry assay or dye-binding with CB G-250. As with the direct biuret reaction, there does not appears to be a dependence on protein composition.

Solution constituents such as Tris buffer, ammonium ions, sucrose, primary amines and glycerol can interfere with the biuret reaction [4]. Potential interference by dextran has also been described but this appears to be dependent on the exact method used and on instrumentation [29]. Lof et al. [30] demonstrated a good correlation between the biuret reaction (3.6 mg/ml) and the Kjeldahl reaction (3.7 mg/ml) for highly purified human blood coagulation factor IX. The biuret method is unsatisfactory for the direct analysis of urinary protein because of the presence of interfering substances; it is useful after precipitation of the protein before analysis [31]. Another report demonstrated a good correlation between the biuret reaction and the Kjeldahl nitrogen determination in the measurement of protein in the production of yellow-fever vaccine [32]. Reichardt and Eckert [33] reviewed the use of the biuret reaction for the estimation of protein in milk, cheese and meat. For milk, the use of KOH and a detergent with the biuret reagent provided satisfactory results. Interference by lactose could be eliminated by the addition of H_2O_2, as could interference by fat or turbidity. More recently, Cotton et al. [34] have used the biuret reaction to measure protein in cerebrospinal fluid.

It is the experience of one of the present authors that the biuret method is an accurate method for the determination of protein in solution [11]. This is an experience shared by other investigators [10,30,35] but it should be noted that this opinion is not universal [36–38].

The Lowry method

The Lowry method was developed approx. 45 years ago [5]. The name of the method is obtained from the senior author of this study. The technique was adapted from earlier work of Wu [39] suggesting the use of the Folin phenol reagent for the determination of protein concentration. The Lowry reaction is based on the amplification of the biuret reaction by subsequent reaction with the Folin phenol reagent (Folin–Ciocalteu reagent [40]). Factors other than the biuret reaction play a role in the development of colour in the Lowry method, resulting in considerable variation with respect to protein composition [41–43]. This variation is a reflection of the contributions of specific amino acids (tyrosine, tryptophan) on colour development in this reaction [42,43]. The importance of tyrosine and tryptophan in the Lowry reaction is further demonstrated in studies by Viner et al. [44] on the oxidative inactivation by sarcoplasmic reticulum Ca^{2+}-ATPase by peroxynitrite. These investigators observed that oxidation of the protein decreased protein reactivity in the Lowry reaction. A modification in which the detection wavelength is 650 nm instead of 750 nm has been described [45], which reduced protein variability. Other investigators have suggested performing the assay at 600 nm [46]. Various substances interfere with the Lowry reaction, including many nitrogen-containing buffers [8,9]. A modification of the Lowry reaction that minimizes such interference has been reported [47].

As with the biuret reaction, the use of the Lowry reaction has decreased in recent years as more facile and sensitive protein assays have become available. However, there is still substantial use of this technique as evidenced by

the following citations. The Lowry assay has been used by Williams and Halsey [48] to measure protein in latex gloves. Using this technique, protein levels ranged from less than 25 to 1150 μg/g of glove. Other laboratories have also used the Lowry method to measure protein in latex gloves [49–51]. Dieke and Beyer-Mears [52] used the Lowry assay to measure protein concentration in cultured lens epithelial cells. Feldt-Rasmussen et al. [53] used the Lowry assay to determine the protein concentration of a purified human thyroglobulin reference material; this value agreed with the values obtained by Kjeldahl, UV absorbance and amino acid analysis. Wysocki et al. [54] used the Lowry method to determine protein concentration in fluids from acute and chronic wounds and serum samples. Alonso and Martin-Mateo [55] have used the Lowry reaction to measure total protein in oyster extracts. Akai et al. [56] used the Lowry reaction to measure protein in cultured mesangial cells.

CB dye-binding assay (Bradford assay)

The use of the metachromatic response observed on the binding of CB to proteins for the determination of protein concentration was popularized by Bradford [6]. The binding of CB dyes to proteins was first studied by Fazekas de St. Groth et al. [57] in 1963. These investigators presented a systematic analysis of the use of CB R-250 and Procion Brilliant Blue RS to estimate protein concentration on electrophoretic strips. Both of these dyes were originally developed for the textile industry. CB is a triphenylmethane dye belonging to the magenta family. A good correlation was obtained for the value obtained with a micro-Kjeldahl procedure and CB for albumin and rabbit gamma-globulin; a difference was observed with lysozyme (0.75 compared with 1.16 obtained with the Kjeldahl procedure). Reisner et al. [58] extended these observations by the use of CB G-250 [59] in perchloric acid to stain gels. The effective use of CB for the determination of protein concentration in solution required the work of Bradford [6].

The ease and high sensitivity of the CB protein assay have driven its extensive use for the determination of protein concentration in a wide variety of protein samples. The assay is based on the binding of the dye to the protein(s), which results in a dye–protein complex with increased molar absorbance. The assay is performed at acid pH, at which the dye is protonated and absorbs at 465 nm in solution; on binding to the protein, there is a metachromatic response with the development of a species absorbing at approx. 595 nm, where the unprotonated species would absorb. The absorbance maximum of the dye–protein complex is a matter of some debate.

Sedmak and Grossberg [60] have reported that the absorbance maximum of the dye–protein complex varied from 595 to 620 nm depending on the dye source. These investigators recommended that the ratio of absorbances at 620 and 465 nm be used for the quantification of proteins. They also recommended that perchloric acid or hydrochloric acid be used in place of the phosphoric acid recommended in the original procedure. Zor and Selinger [61] have suggested that the use of the ratio of the absorbances at 590 and 450 nm improves the linearity of the CB assay system. The use of this ratio also improves the sensitivity of the assay as well as permitting the use of the CB assay in the presence of SDS. Sedmak and Grossberg also demonstrated that peptides with a molecular mass of less than 3000 Da did not form a complex. It is noted that many important peptide hormones and other bioactive peptides would fall into this category, thus precluding the use of the CB assay for this class of biopharmaceuticals. Similar results on the dependence of colour intensity on the molecular mass of the peptides/proteins has been reported by de Moreno et al. [62]. This lack of reaction of CB with peptides provided the basis for the development of an assay for proteases [58] in which a decrease in the ability of the substrate, casein, to form a complex with CB absorbing at 595 nm is measured as the index of proteolytic activity.

Since the original description of the dye-binding protein assay, there have been a number of studies addressing the mechanism(s) of the CB–protein interaction(s). These have largely been driven by the considerable protein-to-protein variability as described below. Sedmak and Grossberg suggested that the binding of the dye to protein was mediated by interaction with protonated amino groups on the protein [60]. The importance of amino groups on the protein has also been demonstrated by de Moreno et al. [62], suggesting the importance of the sulphonic acid groups on the dye in the formation of a ion pair with lysine and arginine residues. Congdon et al. [63] concluded that only lysine and arginine residues are important in the binding of CB. It is of interest that these investigators did not observe a direct correlation between dye binding and absorption by the bound dye. Chial and Splittgerber [64] also reported the importance of lysine and arginine residues in the binding of CB to proteins and suggested that only the ionized form of the dye (blue form) bound to the protein. The reaction has been carefully modelled by Atherton et al. [65], who also concluded that only the deprotonated (ionized) species of CB bound to the protein, again emphasizing the importance of lysine and arginine.

There are other opinions on the mechanism of the interaction of CB with proteins. Tal et al. [66] have examined the interaction of CB dyes with proteins and suggested that, although positive charges on a protein are important for the dye–protein interaction, hydrophobic interactions are also present. These investigators also reported similarities in the interaction of CB R-250 and CB with proteins. Fountoulakis

et al. [67] have also noted the importance of protein hydrophobicity in the interaction with CB. These investigators also demonstrated that protein glycosylation interfered with the reaction with CB.

The above observations on the apparent importance of basic amino acid residues in the interaction with CB would suggest that basic proteins such as polylysine or lysozyme would be extremely reactive with CB. This is not the situation, as reported by several investigators including Van Kley and Hale [68], Pierce and Suelter [69] and Sedmak and Grossberg [60], who all reported a lower colour yield for lysozyme than for albumin. Experiments reported by Lea et al. [70,71] further confuse the issue with respect to the importance of basic amino acid residues in the dye-binding assay. Carbamoylation of lysine resides in BSA or core histones decreased the absorbance at 595 nm with CB in ethanol/phosphoric acid as described by Bradford [6], whereas the absorbance at 595 nm observed with H1 histones under the same reaction conditions increased. The behaviour of polylysine on reaction with sodium cyanate was unusual because there was initial marked increased in absorbance at 595 nm followed by a subsequent decrease at longer reaction times; the pH of the carbamoylation reaction influenced the subsequent dye–protein reaction, with a greater increase in absorbance at 595 nm with reaction at pH 6 or in the absence of buffer [70]. These experiments are consistent with an increase in the metachromasia on the binding of CB to proteins with a decrease in the positive charge on polylysine. Subsequent studies [71] from the same laboratory showed that using a higher dye/protein ratio decreased the carbamoylation effect and gave better colour yields with the H1 histones. A similar observation has been reported by Chan et al. [72] with the use of CB for the assay of protamines. It has been reported that the CB–protein complexes are insoluble at the point of the measurement of absorbance at 595 nm [73]; this may further contribute to the protein-to-protein variation as described below.

The high dependence of the assay on protein composition presents a major problem to the broad use of CB binding as a quantitative protein assay. It is, however, quite useful as a general, sensitive, semi-quantitative assay for proteins. With the selection of an appropriate standard, the assay can be both accurate and sensitive. Johnson and Lott [74] have used the CB method for the assay of protein concentration in cerebrospinal fluid by using a standard of 70% albumin and 30% globulin. Pollard et al. [75] have used CB binding for the estimation of protein in adrenal tissue extracts. An accurate value for the total homogenate (1.32 mg/ml) (compared with a trichloroacetic acid precipitation followed by Lowry determination, 1.34 mg/ml) was obtained with BSA as the standard, whereas a 2-fold higher value (2.64 mg/ml) was obtained with gamma-globulin as the standard. These two studies emphasize the importance of the selection of an appropriate standard for the reaction.

There have been various attempts to modify the original CB method to decrease the dependence of absorbance on protein composition. Lea et al. [76] substituted perchloric acid for phosphoric acid and increased the sensitivity for H1 histone. Reed and Northcote reported the decrease in variability of the response to protein composition in the CB dye-binding assay by decreasing the phosphoric acid concentration or increasing the dye concentration [77]. Protamines gave a weak reaction with CB under Bradford's original conditions; increased reactivity was obtained by increasing the CB concentration and decreasing the concentration of phosphoric acid [72]. The average number of dye molecules bound to protamine was estimated as described by Congdon [63]. There appears to be a 'threshold' of binding that must be exceeded to obtain a maximal response. Stoschek [78] decreased the acidity in the reaction by the addition of NaOH to decrease the variability in the chromogenic responses of different proteins. Friednauer and Berlet [79] reported that the inclusion of Triton X-100 increased sensitivity and decreased protein-to-protein variability. Lopez et al. [80] included SDS in the dye-binding assay to increase the response of collagen and collagen-like proteins. Previously, Wilsott and Lott [81] had noted an improved response of urinary proteins in the dye-binding assay with the inclusion of SDS.

Despite the above issues regarding the CB assay and protein composition, this assay continues to be used extensively, reflecting the speed and simplicity of this system. Nakamura et al. [82] have used the CB assay to determine protein concentration in culture fluid from rabbit lachrymal gland slices. Hill and MacKessy [83] used the CB assay to determine protein concentration in colubrid snake venoms. Williams et al. [84] developed an automated CB protein assay for cultured skin fibroblasts by using a random-access analyser. Ahmed et al. [85] observed that the interference in the CB assay by vanadyl ribonucleoside and orthovanadate can be eliminated by the inclusion of 0.1% H_2O_2 in the assay prior to the addition of dye reagent. Vanadyl ribonucleoside and orthovandate are frequently used as inhibitors of ribonuclease and protein phosphatase activities in tissue preparations.

BCA assay

A variation of the Lowry assay using BCA was developed by Smith et al. [7]. This assay uses BCA to detect the cuprous ions generated from cupric ions by reaction with protein under alkaline conditions [86]. This assay is sensitive and relatively easy to perform but still is markedly influenced by

protein-to-protein variation [7,11]. In the original study [7], Smith et al. observed a variation in maximal colour development as a function of protein composition similar to that observed with the Lowry reaction with the exception of avidin. The variation as a function of protein composition could be decreased by reaction at 60 °C. In studies in which the BCA reaction was used to measure salivary protein concentration [11], values obtained with the BCA reaction were equal to those observed with the Lowry reaction (1.08 mg/ml) but half those measured with the biuret reaction (2.32 mg/ml) when BSA was used as the standard. The value obtained by quantitative amino acid analysis was 1.95 mg/ml. Wiechelman et al. [87] showed that cysteine, cystine, tryptophan, tyrosine and the peptide bond are capable of reducing cupric ions to cuprous ions in the BCA reaction.

A wide variety of substances can interfere with the BCA protein assay. Materials such as EDTA interfere, presumably by chelating the cupric ions. The presence of glucose results in artifactually high values unless compensated for with a reaction blank [7]. Milton and Mullen [88] emphasized the ability of reducing sugars such as fructose and lactose to interfere in the BCA reaction. It should be noted that the BCA reagent has been used for the direct determination of reducing sugars [89]. Gupta et al. [90] attempted to use the BCA reaction to measure protein (tetanus toxin) in biodegradable microspheres but noted that protein stabilizers (glucose, sucrose) interfered with the assay. Satisfactory results were obtained by the use of acid digestion of the microspheres followed by analysis with the ninhydrin reagent [91]. Yang and Cleland [92] studied the release of recombinant human interferon γ from polylactic-co-glycollic acid (PLGA) microspheres. These investigators used the BCA method and SDS/size-exclusion chromatography (SDS/SEC) to measure protein release from the PLGA microspheres. Lower values were obtained with the BCA reaction than with SDS/SEC. The authors ascribe this difference to protein aggregation. At 100% monomer there was a good correlation between the protein concentration values obtained with the BCA reaction and SDS/SEC; at monomer concentrations of 20% of less, there was a difference in concentration of 50% between those determined by the BCA reaction and SDS/SEC.

H_2O_2 has also been reported to interfere with the BCA assay [93]. Phospholipid interferes with the BCA protein assay, resulting in artifactually high values that reflect the interaction of phospholipid with the BCA reagent to yield a chromophore absorbing close to 562 nm [94]. The addition of 2% (w/v) SDS to the BCA assay eliminated the interference by phospholipid [95]. Various zwitterionic buffers developed by Good et al. [96] interfere with the BCA assay, apparently as a reflection of their ability to bind cupric ions [97,98]. A technique to avoid interference in the BCA reaction by solvent has been described by Gates [99]. This technique utilizes the binding of the protein to a positively charged nylon membrane at pH 8.5. The interfering substances are then removed by washing followed by reaction with the BCA reagent.

Biochemical reducing reagents such as dithiothreitol, glutathione and 2-mercaptoethanol interfere with the BCA reaction by reducing the cupric ions to cuprous ions, resulting in artifactually high values [100,101]. In one study [100] the interference was eliminated by the addition of a 10-fold molar excess (over thiol groups) of iodoacetamide. In another study [101], the protein was precipitated with deoxycholate and trichloroacetic acid prior to assay with the BCA reagent.

Certain detergents can also influence the BCA assay. This is an important consideration as detergents are frequently used for the isolation of proteins and some non-ionic detergents such as Tween (polyoxyethylene sorbitan) derivatives are frequently used in the formulation of some biopharmaceuticals. As discussed above, the inclusion of SDS can eliminate the interference by phospholipids. n-Octanoyl-β-D-glucosylamine has a small effect on the BCA protein assay, which could result in a low value; this could be corrected by the inclusion of the detergent in the reagent blank [102]. Interference of Tween detergents with the BCA protein assay is somewhat more complicated [103]. The interference of Tween 80 in the BCA protein assay is apparently a result of the presence of oxidizing agents. Aged preparations of Tween had a more marked influence on the BCA assay than did fresh preparations. Tween did not interfere with the Lowry assay but a precipitate occurred at concentrations greater than 0.2%; the precipitate could be removed by filtration. Smith et al. [7] reported that Triton X-100, SDS, Brij 35, Lubrol, CHAPS and octyl glucoside did not markedly influence the assay of BSA with the BCA reaction; all of these compounds caused a precipitate in the Lowry reaction.

Other substances that interfere with the BCA reaction with proteins include biogenic amines such as catecholamines [104]. Adrenaline (epinephrine), noradrenaline (norepinephrine) and dopamine demonstrated a linear response (A_{562}) with the BCA reagent. Chlorpromazine, penicillins and paracetamol interfered with the BCA reaction in a similar manner [105]. The mechanism of the interference varied with the substance. Although all resulted in artifactually high absorbance values, chlorpromazine produced turbidity that could not be corrected by a reagent blank, whereas the penicillins and paracetamol produced an immediate increase in absorbance at 562 nm that was linear and could be corrected by inclusion of the material in the reagent blank. Anthracycline derivatives also interfered with the BCA reaction. Kader and Liu [106] reported the reduction of cupric ions to cuprous ions with doxorubicin. As with

adrenaline and noradrenaline as cited above, the interference is linear and the development of absorbance at 562 nm is immediate, without the time dependence demonstrated by proteins. Doxorubicin did not interfere with the CB estimation of protein concentration.

The BCA–cuprous ion complex is a relatively stable chromophore absorbing at 562 nm. The development of colour in the BCA protein assay is dependent on time, temperature and pH. The assay can be performed at room (ambient) temperature; reaction at 37 °C decreases the time required for maximal colour development, whereas reaction at 60 °C further decreases the time required for maximal colour development and increases sensitivity [7]. The authors strongly encourage investigators to record and control, if possible, the actual temperature because room (ambient) temperature can vary considerably with location. For GLP and cGMP studies, recording and validating all experimental conditions, including temperature, is mandatory. Zhang and Halling [107] have suggested that the pH of the BCA reaction be decreased to 10.7 with a defined sodium bicarbonate/carbonate buffer, resulting in more rigorous buffering capacity with only a small effect on colour development.

BCA has been used for the direct determination of cuprous ion concentration [86]. The reduction of cupric ions by proteins has also been used for staining protein blots (Western blots) [108,109]. This staining reaction can be enhanced by silver staining [110]. These investigators have since applied the silver-enhancement reaction to the detection of proteins adsorbed on microtitre plates [111], permitting the estimation of as little as 40 ng of protein. Because the BCA assay detects cuprous ions generated from cupric ions by reaction with protein, as opposed to the formation of a chromophore between the reagent and the protein such as is observed with CB, this assay can be used to measure protein adsorbed or attached on a solid surface [112]. The study by Root and Wang [108] on the silver enhancement of cupric ion protein staining is an example of this application of the BCA reaction. Tuszynski and Murphy [113] have used the BCA reaction to measure cells adherent to a microtitre plate. The BCA reaction has also been used to measure protein adsorbed on polymer films intended for fabrication into intravenous fluid containers [114]. Surface-bound protein determined with the BCA reagent correlated well with the solution protein loss. Potential protein therapeutics such as IgG, albumin and insulin were evaluated in this study.

The ability of the BCA reaction to measure insoluble protein permits the development of procedures for the measurement of very dilute protein solutions [115]. This characteristic of the BCA reaction also permits the determination of protein concentration in solutions complicated by the presence of other materials such as bile [116]. In both of these studies, the protein is precipitated and the subsequent protein analysis with the BCA reagent is performed with the insoluble protein precipitate. In the study with bile, the insoluble precipitate obtained by addition of acetonitrile to reconstituted freeze-dried bile is washed with ethanol to remove bile pigments not removed by the acetonitrile. Wolfe and Hay [117] have developed an on-line protein assay using BCA reagents. This was used with a flow-injection analysis to measure antibodies undergoing periodate oxidation. The effect of temperature was evaluated in this system; a temperature of 80 °C was selected to avoid bubble formation while obtaining high signal production.

Despite the problems cited above with respect to the effect of protein composition on the quantitative use of the BCA reaction to determine protein concentration, this assay system continues to be used for a wide variety of proteins. Keith et al. [118] used the BCA reaction to measure protein extracts from contact lenses. A 50:50 mixture of trifluoroacetic acid and acetonitrile was used to the extract the proteins. Zardeneta et al. [119] used the BCA reaction to determine protein concentration in arthrocentesis fluid. Hall et al. [120] have used the BCA reaction to measure multidrug-resistance efflux *in vitro* in cultured cancer cells. Grealy et al. [121] used the BCA reaction to measure protein in bovine oocytes and pre-implantation embryos; protein content correlated well with embryo surface area. Buija et al. [122] used the BCA reaction to measure protein in tissue extracts from middle-ear cholesteatoma; human skin from the external ear canal was used as a control in this study. Cingle et al. [123] used the BCA reaction to determine total protein in post-nuclear cell homogenate supernatant fractions obtained from retinal pigment epithelial cells.

Results obtained from studies with multiple methods for protein determination

As has been emphasized in the sections above, most of the current colorimetric methods for the determination of protein concentration are dependent on the quality of the sample. This section discusses studies in which several methods were employed for the analysis of the sample.

Schlabach [124] compared the biuret reaction and the Lowry reaction for the post-column determination of proteins. With serum as a sample, both the biuret method and the Lowry method could differentiate protein from other material that absorbed light at 254 or 280 nm; the biuret method was less sensitive than the Lowry method. Gerbaut and Macart [125] compared two turbidimetric methods and two colorimetric methods (biuret and Lowry) for the determination of protein concentration in cerebrospinal fluid by using BSA as the standard. Values obtained

with the Lowry method were higher than those obtained with the CB method in the presence of SDS. Wahl et al. [126] compared the Lowry assay, a modified Lowry (trichloroacetic acid precipitation of protein prior to the Lowry assay) and a CB dye-binding assay for the measurement of protein in allergen extracts. Values obtained with the modified Lowry assay were less than that obtained with the standard assay, most probably reflecting the contribution of non-protein material in the allergen extracts to the value obtained with the standard assay. The dye-binding assay gave a lower value for the allergen extracts with BSA as the standard.

In studies with purified proteins the dye-binding assay gave a higher value than the Lowry assay for BSA but a lower value for ovalbumin. Vik et al. [127] have also studied protein assay methods by using allergen extracts. Again, the Lowry assay was useful after precipitation of protein to remove non-protein material that reacted with the Lowry reagent; the BCA assay also appeared to give excessively high values, reflecting the influence of non-protein material similar to that observed for the direct Lowry assay. The Bradford dye-binding method appeared to underestimate protein (quantitative amino acid analysis was used as the standard).

Keller and Neville [128] compared the biuret method, the BCA method, the Lowry method and a CB dye-binding method for the determination of protein in human milk. In these studies, a value for protein concentration obtained with the micro-Kjeldahl method was used as the primary standard. The biuret, Lowry and BCA methods appeared to overestimate the protein concentration and the CB dye-binding method underestimated protein concentration. Results with purified proteins (α-lactalbumin, lactoferrin, human serum albumin, α-casein and IgA) with the four assay systems were also reported: lactoferrin consistently gave the lowest value in all assay systems, whereas α-lactalbumin gave the highest colour value in the biuret assay, IgA in the Lowry assay and human serum albumin for both the biuret and BCA assays.

Brimer et al. [129] compared the CB dye-binding and BCA methods for the assay of glycated proteins. Incubation of human serum albumin with glucose resulted in an increase in A_{280} but a decrease in A_{595} resulting from CB–protein complex formation. Incubation of glucose with albumin resulted in an increase in apparent protein measured by the BCA reaction. Fountoulakis et al. [67] compared the CB reaction, the BCA reaction and the Lowry method in the assay of non-glycosylated and glycosylated proteins. For non-glycosylated proteins, the three methods yielded values consistent with those obtained from quantitative amino acid analysis. The CB method yielded lower values with glycosylated proteins, whereas both the BCA and Lowry methods gave values higher than that obtained from amino acid analysis.

In studies of salivary protein concencentration [11], the biuret reaction, the Lowry assay, the BCA assay and CB assay in either the original phosphoric acid formulation [6] or in the HCl formulation suggested by Sedmak and Grossberg [60] were evaluated. With BSA as the standard, the biuret assay yielded a value of 2.23 mg/ml, the Lowry assay 1.08 mg/ml, the BCA 1.08 mg/ml, dye-binding in HCl 0.94 mg/ml and dye-binding in phosphoric acid 0.96 mg/ml. The value obtained by amino acid analysis was 1.95 mg/ml. Dawnay et al. [10] reviewed methods used for the determination of serum protein and concluded that the biuret reaction was the only suitable assay for the routine measurement of total serum protein. Kirazov et al. [130] have compared the Lowry and Bradford assays for the determination of protein in membrane-containing fractions. When compared with the Lowry value, none of the modifications of the original dye-binding assay (decreased acid/addition of NaOH or various detergents including SDS, CHAPS and Triton X-100) were able to increase the values obtained with the original Bradford procedure [6] compared with the Lowry assay. Lane et al. [131] compared the BCA assay and protein dye-binding (Bio-Rad). BSA gave the highest colour value in the dye-binding assay (either macroplate or microplate), whereas α-chymotrypsin and gammaglobulin were equivalent and approx. 50–60% of the value obtained with albumin. α-Chymotrypsin gave the highest colour value in the BCA assay, with albumin and gammaglobulin being equivalent and approx. 60–80% of the value obtained for α-chymotrypsin. The BCA, Lowry and CB dye-binding assays have been compared in the assay of protein extracted from latex [132]. With BSA as a standard, the lowest value was obtained with the dye-binding assay with higher values were obtained with either the BCA assay or the Lowry assay. In a study cited above [30], a good correlation was observed between the Kjeldahl reaction and the biuret reaction for purified human blood coagulation factor IX; lower values were obtained with the CB reagent and UV absorbance. As a result, corresponding values for biological specific activity varied from 136 i.u./mg (Kjeldahl) to 200 i.u./mg (CB reagent).

Standards and assay validation

Any assay for protein concentration must be validated to ensure an accurate value for the sample of interest. The selection of an appropriate standard is of critical importance because, with the possible exception of the biuret reaction, amino acid analysis (ninhydrin reaction) or the Kjeldahl method, the protein assays described above (the CB assay, the BCA method and the Lowry reaction) all depend on the quality of the protein for the response. For example, the value measured for the protein concentration of human

saliva varied from 0.94 mg/ml (CB assay) to 2.23 mg/ml (biuret reaction) with BSA as the standard; if polylysine was used as the standard, a value of 65.5 mg/ml was obtained with CB dye-binding, whereas the biuret reaction gave a value of 2.13 mg/ml. Likewise, the study by Lof et al. [29] showed that the specific activity (i.u./mg) of a biopharmaceutical (blood coagulation factor IX) varied significantly with the protein assay used.

The selection of an appropriate standard together with a rigorous validation of the analytical process can, in principle, solve the problems presented by the protein composition. The validation methodology must include measurements to ensure that the sample protein concentration is within the dynamic range of the assay. Furthermore all sample types (including in-process and final product) must be independently validated to assess the effect of protein composition as well as interfering or enhancing substances that may be contained in the sample including diluent, excipients and/or stabilizers. As noted above, these materials have the potential to influence the measured value markedly.

Reference was made earlier in this review to Good Laboratory Practice (GLP) and current Good Manufacturing Practice (cGMP). There are terms that the United States Food and Drug Administration (FDA) and other regulatory agencies such as The European Medicines Agencies and the component Committee or Proprietary Medicinal Products (CPMP) and the Committee on Veterinary Medicinal Products (CVMP) use for regulations that apply to the preclinical development and manufacturing of biopharmaceuticals [131]. Under these regulations, the laboratory assays are performed under the oversight of a Quality Assurance group.

The biopharmaceutical process can be used as a tool to evaluate the best methodology for protein determination. As the purity and sample composition are modified during processing, the selection of the specific protein assay is critical. The intermediate samples may provide answers as to the best assay for even the final product. From initiation to completion, biopharmaceutical processing maintains goals to increase product purity and specifity. These criteria should track throughout a robust process and if not can provide clues to assay problems or assay suitability. In a cGMP environment, all assays must be validated for all sample types. The reader is referred to several articles that address the issue of assay validation in greater detail [133–145].

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Received 16 June 1998; accepted 6 October 1998