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RESEARCH ARTICLE

INTERFERENCE OF COMPONENTS OF PEGYLATION REACTION MIXTURE IN COLORIMETRIC PROTEIN ASSAYS

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ABSTRACT

Suitability of protein quantification method for intended application largely depends on tolerance due to interfering substances in the assay. PEGylation is commonly used procedure to enhance the bioavailability of biotherapeutic proteins. In a PEGylation reaction methoxy-polyethylene glycol aldehyde (mPEG-ALD) is conjugated to protein of interest in presence of cyanoborohydride (CBH) and conjugation reaction is stopped by addition of glycine. Therefore, in the present study components of PEGylation reaction mixture namely mPEG-ALD, CBH and glycine were tested for interference in Lowry, Bradford and Bicinchoninic acid (BCA) assays. The differences in sensitivity of these assays were observed for interfering substances. The mPEG-ALD and glycine interfered in Lowry and BCA assays whereas CBH interfered in all the three assays. Overall Bradford assay was superior to Lowry and BCA assays in quantification of proteins in presence of mPEG-ALD and glycine.

INTRODUCTION

Conjugation of polyethylene glycol (PEG) to biotherapeutic protein is a well established procedure in biopharmaceutical industry to increase the bioavailability of recombinant proteins [1]. The enhanced bioavailability of PEGylated protein is due to increased hydrodynamic radius of molecule that is otherwise cleared rapidly from the body. Use of PEGylated proteins has resulted in increased *in vivo* circulation time and thereby reducing the frequency of dosing to target patients considerably [2]. Administration of correct dose and frequency of PEGylated proteins is essential to the anticipated outcome of the treatment [3]. Therefore, correct quantification of biotherapeutic protein is crucial and challenging due to the fact that different methods of estimation of protein give different results [4]. The colorimetric assays are commonly used to quantify protein in solution due to higher sensitivity, robustness and reproducibility associated with these tests [5-7]. Although these colorimetric methods have been commonly used for measuring PEGylated and non-PEGylated proteins they also suffer from interference from various chemical entities [8-11]. Therefore, it is necessary to test the suitability of these assays for a given protein in presence of possible contaminants before making a final decision. The mPEG-ALD is commonly used as substrate for conjugation to protein. The mPEG-ALD preferably reacts with the N-terminal amine in proteins to produce imine linkage and in the presence of reducing agent such as cyanoborohydride a stable PEG-protein conjugate is formed [12]. The reaction is allowed to carry for 1 hr to 20 hrs at room temperature or 4□C before being stopped by addition of glycine [13-15]. Recently, interference of *N*-hydroxysuccinimide (NHS) a component of conjugation mixture has been shown to interfere in Lowry [16] and in BCA [10] assays. It is anticipated that the mPEG-ALD and CBH might interfere in colorimetric assays that are carried out in presence of these chemicals. Since there are no comprehensive reports on the possible interference of selected components of PEGylation conjugation mixture in protein assays present study was undertaken to evaluate interference due to mPEG-ALD, CBH and glycine in Lowry, Bradford and BCA assays.

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MATERIAL AND METHODS

Preparation of samples

mPEG-ALD (JenKem Technonolgy, China) was weighed and dissolved to concentration of 50 mg/ml in water for injection (WFI) and was used as stock solution for obtaining desired lower concentrations when needed. The stock solution of Granulocyte Colony Stimulating Factor (GCSF, Gennova Biopharmaceuticals Ltd, India) was adjusted to 1mg/ml using UV 280nm absorbance and extinction coefficient of GCSF. In spiking studies final concentrations of GCSF and mPEG were obtained by mixing equal parts of double concentrations of individual solutions. The assays were carried out in triplicates.

Lowry method

Method was performed based on Peterson's modification of Lowry's method [5]. The 96 well plates containing the sample

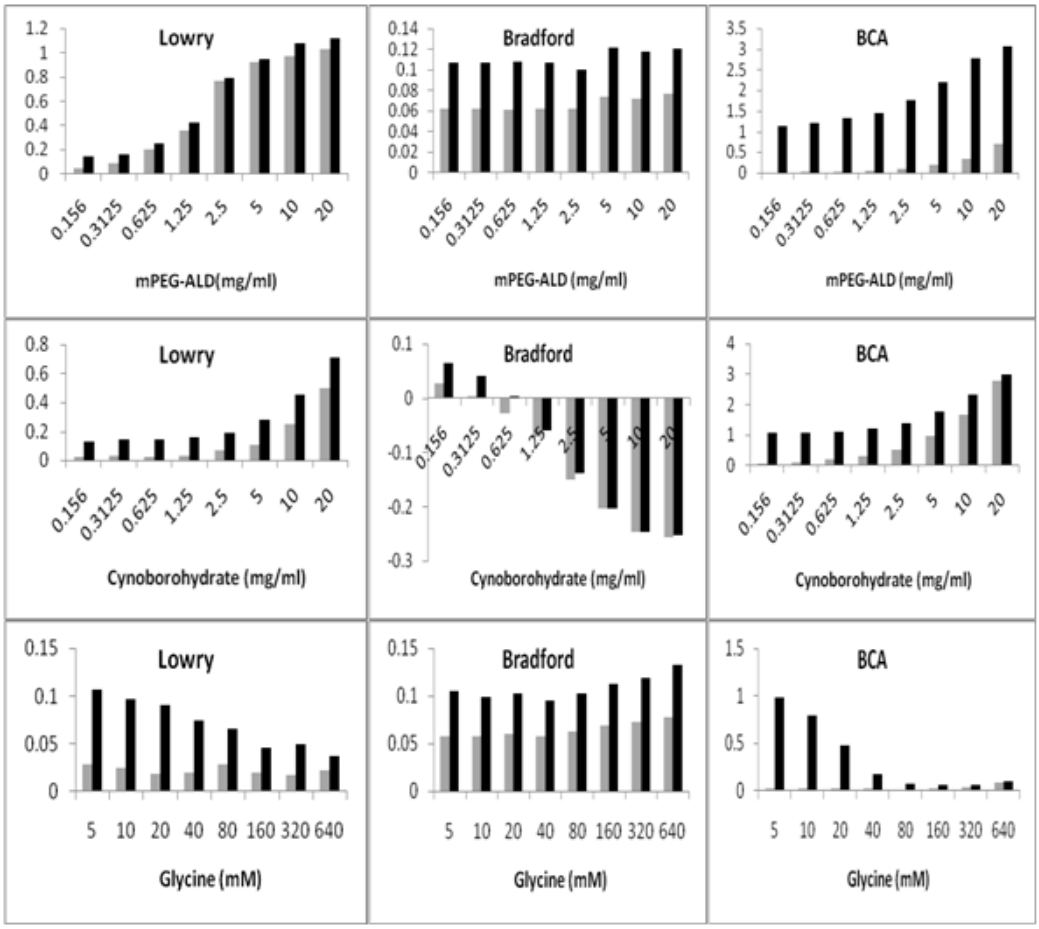

Fig.1 Protein assays with mPEG-ALD, cyanoborohydride & glycine either alone or in presence of 0.1mg/ml GCSF. Bars in light shade depict absorbance of chemical alone and bars with dark shade depict absorbance of GCSF spiked samples. The x-axis denotes concentration of chemical in mg/ml or mM and y-axis denotes absorbance values in a particular assay (Lowry: 750nm, Bradford: 595nm & BCA: 562nm).

and assay reagents were read at wavelength of 750 nm with blank correction in a Micro plate reader (Molecular Devices).

Bradford method

Method was performed as per method described in Ref [6]. The plate containing 10µl of sample/ standard and 200 µl of Bradford reagent was incubated at room temperature for 7 mins. The plate was read at wavelength 595 nm with blank correction in a Micro plate reader.

Bicinchoninic acid method

The method was performed using BCA Protein estimation kit (Thermo scientific Cat# 23225) as per manufacturer's recommendations. The plates were read at wavelength of 562 nm with blank corrections in micro plate reader.

RESULTS AND DISCUSSION

Due to greater implications that would arise due to incorrect dosing of biotherapeutic in patients correct quantification of drug is necessary. Although colorimetric assays are commonly used for the purpose however they are susceptible to interfering substances. In the present study, mPEG-ALD, CBH and glycine were tested for interference in Lowry, Bradford and BCA assays. Various concentrations of these chemicals were prepared in WFI and were tested alone or in presence of known concentration of GCSF (0.1mg/ml) in the protein assays.

Concentration dependent increase in OD values was observed in mPEG-ALD samples and GCSF spiked mPEG-ALD samples in Lowry and BCA assays (Fig.1). The mPEG-ALD contributed maximally to the total absorbance of spiked samples in Lowry assay. However, small increase in concentration of mPEG-ALD had greater contribution (amplification) to total absorbance of GCSF spiked samples. In contrast, mPEG-ALD gave more or less similar absorbance across various concentrations in Bradford assay suggesting interference of mPEG-ALD in Lowry and BCA but not in Bradford assay. The absorbance of GCSF can be obtained by subtraction of total absorbance with blank containing corresponding concentration of mPEG-ALD in Bradford assay (Fig.2). In Lowry and BCA assays CBH showed increase in absorbance with increase in concentration of CBH in samples. However, CBH gave concentration dependent decrease in OD values in Bradford assay (Fig.1). The OD values contributed by GCSF in GCSF spiked samples were decreased by presence of CBH in sample in Bradford assay. Presence of glycine in samples as assayed by Lowry and BCA gave concentration dependent decrease in OD values for GCSF

spiked samples (Fig.1). However, in Bradford assay the OD contributed by GCSF spiked glycine samples were not much perturbed by presence of glycine upto 80mM (Fig.2). Moreover, uniform absorbance values were obtained by subtraction of blank containing corresponding concentration of glycine in Bradford assay (Fig.2).

The colorimetric assays are susceptible to interference from various chemical entities [8-11]. The interference could be due to reactivity of chemicals at any of the steps involved in the protein assay reaction or could be due to modification of protein to react with protein assay reagents. The three assays used in the study differ in mechanism of quantitation of protein. The Lowry method is based on reaction of peptide bond of protein with copper in alkaline condition and then copper treated protein reduces the phosphomolybdic-phosphotungstic present in Folin-Ciocalteu's reagent. The resulting blue color solution is read at absorbance of 750nm [5]. In Bradford assay binding of Coomassie Blue G250 dye to proteins shifts the absorption maximum of dye from 465 nm to 595 nm [6]. The BCA assay is similar to that of Lowry assay in that the Cu^{2+} is reduced to Cu^{1+} by peptide bond of protein. Then copper peptide complex reacts with BCA molecules to give purple color to solution which is read at absorbance of 562 nm [7]. Since mPEG-ALD has reactive aldehyde group and CBH is a reducing agent thereby interfering in oxidation and reduction reactions involved in protein assays. This might explain inherent high OD values contributed by mPEG-ALD and CBH in Lowry and BCA assays. While glycine might consume copper ions in Lowry and BCA assays thereby reducing the OD values contributed by protein in dose dependent manner in Lowry and Bradford assays. Further, it is suggested that estimation of protein concentration in presence of mPEG-ALD, CBH and glycine by Lowry, Bradford and BCA should be considered with caution as these chemicals interfere with colorimetric protein assays.

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