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Review

Determination of residual dextran sulfate in protein products by SEC–HPLC

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ABSTRACT

Dextran sulfate is a polyanionic derivative of dextran, produced by esterification of dextran with chlorosulphonic acid. Dextran sulfate with an average molecular weight of 8000 Da can be added to the cell culture to inhibit binding of proteins to cells, increasing cellular growth and productivity. Residual dextran sulfate levels must be monitored during the purification process development to insure clearance. A size-exclusion chromatography based HPLC assay has been developed for the separation and quantitation of dextran sulfate in a highly concentrated purified protein drug substance sample. Trichloroacetic acid (TCA) was used to precipitate the protein and separate the dextran sulfate. Detection and quantitation of dextran sulfate was achieved by post column reaction with dimethylene blue to form a metachromatic complex that absorbs visible light at 530 nm. The quantitation limit (LOQ) was determined to be 1.5 μg/mL dextran sulfate in high concentration protein samples.

Contents

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1. Introduction

Dextran sulfate is a white to off-white powder that is freely soluble in water and supplied as the sodium salt form in the range of 3 to 2000 k Da. Dextran sulfate has been tested for its activity as an anticoagulant, antiviral, as well as an adjuvant [1,2,3]. During these studies, the toxicity of dextran sulfate has also been evaluated and was found to be dependent on the dose and molecular weight of the substance as well as the route of administration. Studies showed that daily administration of 20 mg/kg of dextran sulfate for two weeks to a group of rabbits causes systemic toxic effects which are not related to its anticoagulant activity [4,5]. Therefore, adequate removal of dextran sulfate during the purification process should be demonstrated using appropriate analytical methods.

In this study, dextran sulfate with a mean molecular weight of 8000 was added to the cell culture media to inhibit protein binding to the cell walls and thus increase the cell growth and productivity. Due to its toxicity, dextran sulfate is removed during the purification process and its levels are monitored to insure clearance in the final purified protein drug substance formulated at 80 g/L.

Currently, there are no methods available that describe dextran sulfate quantitation in the presence of highly concentrated protein product. Dextran sulfate binds to the protein and separation by a chromatography technique alone is not feasible. In addition, injection of highly concentrated protein sample into the column cannot be made and large dilutions of the protein sample is not suitable for a clearance assay that requires an extremely low LOQ.

The detection of dextran sulfate, even in the absence of protein is not trivial. ELISA based assays offer high sensitivity but lacks selectivity [6] and fluorometric labeling of the dextran sulfate involves a long sample preparation procedure [7]. The method presented here offers a simple and quick sample treatment procedure using Trichloroacetic acid (TCA) precipitation and a single HPLC system with a binary pump to perform post column derivatization with dimethylene blue. The dextran sulfate-DMB dye complex has a strong absorbance at 530 nm which can easily be monitored by a UV–vis detector.

2. Experimental

2.1. Materials and reagents

Dextran sulfate sodium salt, PharmaGrade and ethanol, ACS reagent, ≥99.5% (200 proof) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dimethylene blue zinc chloride double salt was purchased from Santa Cruz biotechnology (Dallas, TX, USA). Potassium hydroxide solution, 10 N was purchased from Ricca Chemical Company (Arlington, TX, USA). HPLC grade water, Trichloroacetic Acid (TCA), ACS grade, potassium phosphate monobasic, ACS grade, potassium phosphate dibasic, anhydrous, USP, potassium chloride, USP, and tris–hydrochloride, 1 M, pH 7.5 were all purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Equipment

The work presented here was performed on an Agilent 1100HPLC system with a binary pump (model # G1312A), a degasser (model # G1379A), a temperature controlled autosampler (G1329A and G1330B), a column compartment (model # G1316A), and a DAD detector (model # G1315B). Pump A was used to deliver the buffer while pump B supplied the DMB dye. The separation was performed on a Sepax, Zenix, SEC-150, 3 μm, 7.8 × 300 mm column (Sepax Technologies, Newark, DL, USA). The eluate passed through a 10 μL biocompatible PEEK static mixer from analytical scientific instruments US (Richmond, CA, USA) where it was mixed with the dye delivered directly by pump B. Peek tubing 20 cm long and 0.4 mm ID was used to connect the online mixer to the DAD detector. All the data was collected using empower 2 chromatography data software (Waters Corporation, Milford, MA, USA). Protein samples were centrifuged using an eppendorf centrifuge (model # 5417R).

2.3. Method parameters

The mobile phase was composed of 25 mM potassium phosphate monobasic, 25 mM potassium phosphate dibasic, 50 mM potassium chloride, and 10% ethanol. The dye solution was composed of 10 μg/mL DMB. The total flow rate was set at 1.4 mL/min with 72% mobile phase and 28% dye solution which reflects that the buffer solution was delivered at 1.0 mL/min while the DMB dye was delivered at 0.4 mL/min. The column temperature was kept at 25 °C while the autosampler temperature was set at 5 °C. The detector was set at 530 nm, Bw 64, reference 520, Bw 100.

2.4. Standard solutions

Dextran sulfate stock solution was 0.10 mg/mL in HPLC grade water. A five point standard curve ranging from 1.0 μg/mL to 10.0 μg/mL dextran sulfate was prepared in mobile phase A. A standard plot was generated with every run.

2.5. Sample preparation

In a 1.5 mL eppendorf tube, 475 μL of protein sample was mixed with 125 μL of trichloroacetic acid. The solution was centrifuged at 14000 rpm for 15 min, and then 400 μL of the supernatant was transferred to an HPLC vial. The sample was then mixed with 40 μL of 10 N KOH and 60 μL of 1.0 M Tris–HCl buffer pH 7.5 before being placed in the autosampler for SEC–HPLC analysis. An injection volume of 50 μL was used.

3. Results and discussion

3.1. Dextran sulfate-dye complex absorbance

Dimethylene blue is a potent cationic dye with maximum absorption around 670 nm. However, when it interacts with dextran sulfate, a polyanionic molecule; a metachromatic complex is formed. The dye molecules are close enough to form dimeric and polymeric aggregates that have different absorption properties than the non-aggregated dye molecules [8]. The formation of the dextran sulfate-dye complex depends on the concentration of both dextran sulfate and the DMB dye. It was previously demonstrated that the reaction occurs rapidly and is stable between formation and measurement [9]. Optimal tubing length and diameter (20 cm and 0.4 mm I.D.) between the mixing tee and detector were used to provide sufficient time for the interaction between the dye and dextran sulfate to occur while limiting diffusion. Using the DAD detector, a spectrum of the dextran sulfate-dye complex was obtained from 190 nm to 700 nm. The dextran sulfate-dye complex eluting at approximately 8.1 min has a maximum absorbance at 530 nm as shown in Fig. 1.

3.2. Protein precipitation by TCA

The initial protein precipitation attempts using organic solvent such as acetonitrile or acetone [10] resulted in very low recovery of dextran sulfate from the sample. Other approaches for sample treatment, such as using formic acid, acetonitrile, and water, are more complex and require larger sample dilutions [11]. Protein precipitation by TCA proved to be simple and efficient. The three chloro