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Direct capture of factor IX from unclarified human plasma by IMEBAC

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Abstract

The method development for direct capture of clotting factor IX (FIX) from unclarified human plasma using immobilized metal expanded bed affinity chromatography (IMEBAC) has been described. Cu(II), Ni(II), Zn(II) and Co(II) metal ions were evaluated in immobilized metal affinity chromatography for the recovery of FIX. Immobilized Cu(II) chelating adsorbent was found to be the most effective for capture of general protein and FIX. The adsorption characteristic of Cu(II)-STREAMLINE chelating adsorbent used in this work for general protein and FIX in unclarified plasma has been assessed by the measurement of equilibrium isotherm. The influence of liquid velocities between 100 and 300 cm/h on the selective adsorption of FIX from clarified plasma was investigated in small packed bed experiments. Optimal conditions for the elution of FIX were also investigated in packed bed experiments conducted with clarified plasma. On the basis of the results, IMEBAC for direct capture of FIX from unclarified plasma was established. The purification results showed that FIX was directly recovered using 20 mM glycine buffer (pH 7.0) from unclarified plasma with a purification factor of 83 and yield of 16% in a single step. Other contaminants and FIX were completely eluted by using 25 mM EDTA (pH 7.0).

Keywords: Immobilized metal affinity chromatography; Expanded bed adsorption; Clotting factor IX; Unclarified human plasma; Direct capture; Purification

1. Introduction

Human plasma is a highly complex mixture of more than 100 known proteins of biological material and the total protein content of approximately 60 mg/ml [1,2]. Clotting factor IX (FIX) is one of the proteins in the human plasma, which plays an important role for hemostasis [3]. The concentration of FIX in human plasma is very low (approximately 5.0 μg/ml) [4]. Moreover, it is a glycoprotein with an apparent molecular mass between 55 and 65 kD, depending on the method used for determination and displays microheterogeneity with isoelectric point in the range of 4.0–4.6 [4]. Due to the low abundance of FIX and the presence of a large number of other proteins in plasma, a multi-step purification process would be necessary to achieve a high purity of FIX [3]. Industrial-scale chromatographic fractionation and purification methods have been used increasingly in the last few years for plasma FIX [1]. When the frozen plasma was thawed and centrifuged, the cryoprecipitate (factor VIII rich fraction) was separated. The cyrosupernatant called cyropoor plasma was used for the production of FIX. The plasma-derived FIX concentrates are routinely obtained by the combination of chromatographic purification processes from cyropoor plasma [5]. These purification processes involved ion exchange, size exclusion, hydrophobic interaction, immuno-affinity and/or metal chelate affinity chromatographic techniques [2–4,6–11]. In the case of immobilized metal chelate affinity chromatography (IMAC), it has become a widespread analytical and preparative method for therapeutic proteins [5,12–13]. The key advantages of IMAC include the stability of metal chelates, ease of product elution and regeneration of the matrix, and the relative high specificity of protein–metal interactions [14–17].

There has been considerable interest in recent years in application of expanded bed adsorption (EBA) to direct

Abbreviations: BSA, bovine serum albumin; E1–E4, elution steps 1–4 for FIX; FIX, clotting factor IX; F + W, flow through and wash phases; IDA, iminodiacetic acid; IMAC, immobilized metal affinity chromatography; IMEBAC, immobilized metal expanded bed affinity chromatography

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Nomenclature

A	activity of clotting factor (FIX) in the out stream (units/ml)
A_o	activity of clotting factor (FIX) in the feed stream (units/ml)
C	general protein concentration in the outlet stream (mg/ml)
C_A	equilibrium activity of FIX in the liquid phase (mg/ml)
C_o	general protein concentration in the feed stream (mg/ml)
C_p	equilibrium concentration of general protein in the liquid phase (mg/ml)
H_{exp}	expanded bed height (cm)
H_s	settled bed height (cm)
K	Henry's constant or distribution constant between liquid and adsorbent phases
K_F	Freundlich constant
n	Freundlich constant
q_A	equilibrium activity of FIX in the adsorbent phase (units/ml)
q_p	equilibrium concentration of general protein in the adsorbent phase (units/ml)
q_{max}	maximum binding activity of Cu chelating adsorbent (units/ml)
V_a	applied feed stream volume at $A/A_o = 0$
V_c	applied feed stream volume at $A/A_o = 1$
V_o	column void volume (ml)
V_s	volume of adsorbent phase (ml)
X	relation of concentration of A/A_o

recovery of proteins from unclarified feedstocks [15–22]. The reason is that EBA technique eliminates the needs for filtration, centrifugation, and concentration steps and has advantage over the contacting methods of near plug flow of liquid. It results in adsorption performance similar to packed beds [16,23]. The use of IMAC with expanded bed adsorption (EBA) technique has been evaluated for direct recovery of proteins from unclarified feedstocks [15–20].

Recently, immobilized Cu(II) chelate affinity chromatography as a final chromatographic step has been used to purify a therapeutic FIX concentrate under clinical evaluation [9,10]. The results showed that IMAC is an effective method of virus reduction for both non-enveloped viruses and enveloped viruses. In this paper, the purpose of the work was to evaluate different transition metals in IMAC for direct recovery of FIX from unclarified human plasma as an initial capture step. In order to develop a simple and fast method for direct recovery of FIX, the method development of Cu(II)-STREAMLINE chelating adsorbent in expanded bed modes for the direct capture of FIX from unclarified human plasma was described.

2. Materials and methods

2.1. Materials and apparatus

2.1.1. Materials

STREAMLINE chelating adsorbent (100–300 μm, 1.2 g/ml) and STREAMLINE 25 column (i.d. 2.5 cm) are obtained from GE Healthcare Amersham Biosciences (Uppasala, Sweden) and are specially designed for use in immobilized metal expanded bed affinity chromatography (IMEBAC). Coomassie® protein assay reagent was obtained from Pierce Chemical Co., (Rockford, IL, USA). Fresh frozen citrate stabilized human plasma was obtained from blood bank at the Chung-Gung Hospital in Taipei (Taiwan). FIX deficient plasma and all other reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) and were of analytical grade. All the solutions were prepared with double distilled water. The stock solutions of all metal ions were standardized by EDTA titration.

2.1.2. Apparatus

The pH of the adsorption buffer was monitored with a pH meter (Mode SP-2200, Suntex Ins. Co., Taiwan). A vortex mixture (Vortex Genie 2) was obtained from Scientific Industries Inc. (Bohemia, NY, USA) and was used to mix the treated samples while assaying for protein concentration and FIX activity. Test tube rotator was obtained from Labinco Ltd. (Model LD-79, Netherlands). Centrifugation was carried out using a mirocentrifuge (Model 5410, Eppendorf). A UV–VIS spectrophotometer (Model Ultrospec 3100 pro, GE Healthcare Amersham Biosciences, Uppsala, Sweden) was used to measure the absorbance of protein samples. ÄKTAprime chromatographic system was obtained from GE Healthcare Amersham Biosciences (Uppsala, Sweden). A Watson–Marlow peristaltic pump (Model 302S) was obtained from Waterson–Marlow Ltd. (Falmouth, UK). Coagulation tests were carried out with a Fibrometer (BBL, FibroSystem, Becton Dickinson Microbiology Systems, Cockeysville, Maryland, USA). Atomic absorption spectrophotometer (Varian Model AA55, Australia) was used for all metal determinations.

2.2. Protein determination

The total protein concentration in the samples was determined using the Pierce Coomassie reagent with BSA used as a standard. The soluble protein concentration in the unclarified samples was evaluated using series dilution of clarified samples prepared by centrifugation (Eppendorf 5410, 8800 g, 10 min).

2.3. Factor IX activity assay

A one-stage coagulation assay for FIX was carried out by mixing the corresponding deficient plasma (0.1 ml) with the diluted reference plasma (0.1 ml) and APTT reagent (0.1 ml) and incubating for exactly 3 min at 37 °C in a Fibrometer