International Journal of General
Medicine and Pharmacy (IJGMP)
ISSN(P): 2319-3999; ISSN(E): 2319-4006
Vol. 2, Issue 5, Nov 2013, 1-4
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International Academy of Science, Engineering and Technology
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CONJUGATION OF ANTI-IMMUNOGLOBULIN (M), ANTI IGMFLUOROCHROME DERIVATIVE (FITC, ISMOER 1)

ADIL MOHAMMED HASHIM, M. K. ABDUL-MAJEED, S. M. ALSOUAIDY & ZEINA H. HABIB

Ministry of Science and Technology, Baghdad, Iraq

ABSTRACT

Immunochemical methods, based on antigen-antibody interaction, have become the most important-tool in qualitative and quantitative plasma proteins determinations due to these high specifically and sensitivity. Immunoglobuline can be divided into five classes IgG, IgA, IgM, IgE and IgD. The normal human serum IgM is about 0.5-2.0 mg/ml. IgM is the dominant antibody formed early in the primary immune response to most antigens (immunogens). Its concentration in the circulation is high enough for it to be purified from normal serum.However,the preparation is facilitated by the use of serum from patients with waldenstromsmacroglobulinaemia .Because of the largesize (Mwt. 900,000) for IgM, gel filtaration is an important fractionation step in the preparation. Thechromatograghically purified IgM fraction antiserum is conjugated with Fluorescein-Isothiocyanate (FITC, Isomer 1). After conjugation, removing of unreacted (unconjugated) and reacted (conjugated) fluorochrome is carried out using gel filtration. The conjugation of anti-IgM-FITC proceededby nucleophile attack of the unprotonated €- amino group of lysine to the fluorochroe, resulting in a thiourea bond in alkaline medium.

KEYWORDS: Immunoglobulin (M), Anti-IgM, Fluorochrome Derivative, IgA Immunofluoresene

INTRODUCTION

Several approaches for investigation of possible abnormalities in the plasma globulin were indicated previously. More than thirty different components have been identified and characterized. In as much as many if not allof these are functionally and structurally distinct, independent variations may occur in single components, or in small groups of related components, in response to various types of stimuli or asa result of disease. Different globulin fractions may vary in opposite directions, with no significant change in the total globulin concentration. This is due to (a) the relative quantitative insignificance of decreased component, and (b) to the fact that other globulins may be increased simultaneously [1].

IgM is a pentamer of five IgM subunits. A deficiency of IgM is manifested by a marked tendency to septicemia. It can be secreted by intestinal epithelial cells and ay act on their luminal surfaces as a second line of defense to (IGA). It seems that the major function of (IgM) is to protect the blood circulation [2].

In addition to traditional immunology and pathology applications involving particles such as lymphocytes, macrophages, monocytes and tumor cells, flow cytometers are widely used in conjunction with fluorescence-based protein reporters, such as green fluorescent protein (GFP). In this arena, flow cytometers can monitor both transfection efficiency and protein expression levels [3,4].They also can detect fluorescence resonance energy transfer (FRET), which provides information about molecular interactions; protein structure and DNA sequence [5].

MATERIALS AND METHODS

SepharoseCL-6B and Sepharose G-25 medium were obtained from pharmacia fine chemicals Co., Upsalla, Sweden. Sodium chloride, sodium dihydrogen orthophosphate, anhydrous calcium chloride and fluorescein isothiocyanate

(FITC, Isomer I) were purchased from Fluka AG., W. Germany, whereas potassium dihydrogen orthophosphate, disodium hydrogen orthophosphate (12 hydrates): tri-basic, sodium orthophosphate (12 hydrates) were from BDM chemicals Co. England.

Tris-(Hydroxy methyl)- amino ethan-HCl, potassium chloride were obtained from Ferak, W. Germany. Polyethylene glycol-6000 (PEG-6000) was purchased from merk, W. Germany. Agarose was obtained from Difco, USA. Finally, sodium azide was obtained from Riedel-Dehaen AG, W. Germany.

PLASMA SAMPLES

There were obtained fro National Centre of Blood Transfusion, Baghdad- Iraq.

Buffers

- 0.010 M Tris-HCl Buffered Saline, PH=7.3 containing (0.06%) sodium azide.
- Phosphate Buffered Saline (PBS),PH=7.2.
- 0.2 M disodium hydrogen orthophosphate: dibasic.
- 0.1 M sodium phosphate- 12 hydrate: tribasic.
- 0.01 M disodium hydrogen orthophosphate: dibasic, Ph=7.5
- 0.01 M phosphate Buffered Saline (PBS), Ph=7.5.

METHODS

- **Isolation of IgM**

The isolation of Igm carried out using 100 ml of donor normal serum. This serum was dialysed against PEG-6000, until became at least 25 ml. the dialysed serum was applied to the column of sepharose CL-6B (2.6x100cm) at room temperature using the (TBS) buffer as eluent, then fractions of (8ml) each were collected and the absorbance of each fraction was measured at 280 nm. The first peak, which was the IgM peak, was selected and also dialsed against 20% PEG.

- **Preparation of Anti-IgM Antiserum**

Four rabbits were immunized with IgMimmunogenic following a method mentioned below:

Anti-IgM antisera were obtained using an emulsion of immunogenic containing 250 ug purified IgM in 2 ml complete Freunds adjuvant. For first immunization, each rabbit received 2 ml of emulsion distributed at six injection sites subcutaneously and intramuscularly. After 15 days, the animals received boosting injection and 15 days later, the same procedure was repeated. The rabbits were bled later after 5 days and sera stored at- 20C^{o}.

- **Preparation of Anti-IgM Conjugated with Fluorescein Isothiocyanate (Anti-IgM-FITC)**

The first step was determination of protein content in the antiserum. Purification of the conjugate carried out either by dialysis or by applying chromatographic technique that separated the free unreacted and conjugated dye [7].

RESULTS AND DISCUSSIONS

IgM, which has a molecular weight about (900,000), can be split by reduction of disulphide bonds into five subunitsof IgG. I as a membrane receptor molecule ismonomeric, but most of this immunoglobulin in the serum is pentameric. In isolation of IgM from the serum, serum can be dialed against 2 mM phosphate buffer Ph=6.0 4 C^{o}[8].

In our experiment, 20% PEG-6000 were used facilitate the dialysis, so it is preferably usedin this operation.it tends to precipitate up on long term storage, and advisable to store at 4 C^o, but not frozen. In the preparation of anti-IgM antiserum, the sites of injection were distributed (2x0.5 ml i.m., 4x0.25ml subcutaneously). Booster immunization using incomplete Freunds adjuvant with the same dose of Ig was administered at (15) days intervals [6].

The specificity and potency of each bleeding were assessed by immunodiffusion to evaluate the suitable and best one for conjugation procedure[9].

As might be expected from knowledge of structure for IgM molecule, its five combining sites make it avery efficient agglutinating antibody molecule. Rabbit IgM antibacterial antibody is known to be 22 times as active as IgM antibody (mol/mol) in bringing bacterial agglutination [10].

The chromatographically purified IgM fraction of antiserum is conjugated with (FITC, IsomeI) then gel filtration also applied to separate "to remove" the unreacted dye. This can be accomplished by sephadex G-25 medium [11].

The mechanism of this removal, consist of eluting the conjugate by the effect of molecular weight and size, happened either by adsorption or penetration on the gel mentioned. Another factor affecting this process was the elution of the buffered saline entered the column and had been driven through several times, leaved the column regenerated and ready to use [12, 13, and 14].

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