

PREPARATION OF ENRICHED IMMUNOGLOBULIN M AND IMMUNOGLOBULIN A FROM HUMAN PLASMA

KAMRAN MOUSAVI HOSSEINI,* Ph.D., MOJGAN
POURMOKHTAR,** Pharm.D., RASSOUL DINARVAND,** Ph.D.,
HOURI REZVAN,* Ph.D., AND MOHAMMAD ALI JALILI,* Ph.D.

From the *Iranian Blood Transfusion Organization Research Center and Iranian Blood Research and Fractionation Co., Tehran, and the ** Department of Pharmaceutics, School of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

ABSTRACT

As IgM and IgA-enriched preparations are needed to complete the immunotherapeutic spectrum, a simple procedure is described for the preparation of IgM and IgA-enriched immunoglobulins. Fraction III which was prepared by cold ethanol fractionation was treated by octanoic acid followed by ethanol precipitation and ion-exchange chromatography using Sephadex DEAE A-50 and 0.1 M tris-0.35M NaCl buffer, pH 8.1, resulting in recovery of 85% IgM, 84% IgA and 33% IgG. The comparison of our results with immunoglobulins' percentage in plasma indicates that IgM and IgA-enrichment was obtained by three times.

MJIRI, Vol. 17, No. 4, 315-318, 2004.

Keywords: Immunoglobulin; IgM; IgA; Enrichment; Ion exchange chromatography.

INTRODUCTION

Evidence has indicated that antibodies against viruses and bacteria are not equally distributed among the main classes of immunoglobulins such as IgG, IgA and IgM.¹ It has been found that IgM is mostly concerned with host defence mechanisms in Gram-negative septicemia (*Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella* and *Klebsiella pneumoniae*), and IgA with high antibody titers for Poliomyelitis virus I whereas antibody activities against *Haemophilus influenza* and Measles virus occur preferentially in IgG preparations.²⁻⁴

Furthermore, it has been demonstrated that IgM and IgA-enriched preparations are needed to complete the immunotherapeutic potential of immunoglobulins whereas purified IgM has just been used for antiserum production and diagnostic purposes.⁵ So preparation of pure isolated IgM for clinical use is not the main objective.⁶

Methods for the preparation of IgM have been improved through the introduction of ammonium sulphate, ion exchange chromatography, affinity chromatography, polyethylene glycol and caprylic acid fractionation.⁷⁻⁹ Fractionation of human plasma into therapeutic plasma-derived products is an exciting area in the bioseparation field. Plasma is a unique biological mixture of over one hundred proteins which are important for therapy or prophylaxis of human diseases.¹⁰⁻¹² Large scale batchwise fractionation of human plasma was developed by Cohn et al. and in their study plasma proteins were separated by exploiting the low dielectric constant of ethanol and the differential solubilities exhibited by proteins under precise conditions of temperature, pH, ionic strength and protein concentration.^{13,14} Fractions I to V can be derived from human plasma by cold ethanol plasma fractionation.¹⁵ Distribution of plasma proteins in the individual fractions obtained by Cohn's method are as follows: fibrinogen in fraction I; IgG, IgA and IgM in fraction II+III; Antithrombin III, α_1 -anti-trypsin, ceruloplasmin, haptoglobin and transferrin in fraction IV and albumin in fraction V.¹⁶ Fraction III obtained in the large scale alcohol fractionation of pooled human plasma is

Correspondence: K. Mousavi Hosseini, Ph.D., Department of research and Development, Iranian Blood Research and Fractionation Co., Tehran, Iran, P.O.Box:14665-369, Fax: +98-21-8266117.

Preparation of Enriched IgM and IgA from Human Plasma

normally considered as a waste product. Most of the IgM is however concentrated in this fraction and it may therefore be considered an ideal starting material for isolation of polyclonal IgM.^{7,17}

MATERIAL AND METHODS

Fraction III paste which was prepared by cold ethanol plasma fractionation was stored deep frozen (-30°C).

Reagents

Sodium acetate trihydrate (extra-pure food grade), glacial acetic acid, octanoic acid for biochemistry, tri-calcium phosphate (dry, extra-pure), tris (hydroxymethyl)-aminomethane GR, sodium chloride (extra-pure) and hydrochloric acid fuming 37% GR were obtained from Merck. The purity of ethanol was 96% (v/v).

Extraction

Frozen Cohn fraction III paste (200 g) was dissolved in 2000 mL of 0.05 M sodium acetate buffer, pH 4.8, by stirring for 1 h at room temperature. Solubilized fraction III was treated with 1.5% (w/w) octanoic acid at 22°C for 1 h. Tri-calcium phosphate 0.4% (w/w) was added and the mixture was stirred for 45 min and was kept overnight at 4°C. The resultant yellow suspension was centrifuged at 16000 rpm (Hermle BHG model ZK 401 centrifuge, A8.24 rotor) for 30 minutes at 20°C. Ethanol was added to a final concentration of 30% at 0°C and the solution was stirred for 30 minutes at -10°C, followed by centrifugation at 16000 rpm for 30 minutes at 0°C which leads to obtaining 9g precipitate of immunoglobulins. Then 3g of the resultant precipitate was dissolved in 100 ml of 0.1 M Tris - 0.05 M NaCl buffer, pH 8.1 (solution A), for applying to the column.

Column chromatography

A 26±200 mm glass column (XK26, Pharmacia Biotech) was packed with Sephadex DEAE A-50 (Pharmacia Biotech). The column was equilibrated with 0.1 M Tris-0.05 M NaCl buffer, pH 8.1. Forty mL of 0.45µm filtered solution A containing about 1g protein was applied to the column and eluted at room temperature with 0.1 M Tris - 0.2 M NaCl, pH 8.1 and 0.1 M Tris-0.35 M NaCl, pH 8.1 buffers in two steps, at 250 mL/h. The UV absorption was monitored by Uvicord SII, Pharmacia LKB, 5 mL fractions were collected using the Fraction Collector (2211 Superrac, Pharmacia LKB) and the resultant peaks were recorded by 2210 Recorder, Pharmacia LKB.

Determination of protein concentration

A Philips PU 8750 UV/Vis Scanning Spectrophotometer was used for determination of protein concentration using the following empirical equation:¹⁸
“protein concentration (mg/mL) = 1.55A280 - 0.76A260”

Determination of IgG, IgA, and IgM concentrations

The immunoturbidimetric test was used for measuring IgG, IgA and IgM concentrations by Cobas Mira Photometer.

Identification of proteins

Immunoglobulins were identified by electrophoresis technique using a Vario 6, Elphor instrument.

RESULTS

The purification procedure of IgM-enriched immunoglobulin can be divided into three steps:

1) Cold ethanol fractionation was included for removal of cryoprecipitate at -4°C, precipitation and removal of fraction I by addition of 8% ethanol and treatment at -2.5°C (pH 7.2) for 2h, precipitation of fraction II/III by addition of about 18% ethanol and treatment at -5°C (pH 5.85) for 8h, precipitation and removal of fraction III by addition of 10% ethanol to the solution of fraction II+III and treatment at -2.5°C (pH 5.15) for 2h.

2) Octanoic acid treatment as described above was used for the precipitation of proteins other than the immunoglobulins present in fraction III. This was demonstrated by immunoturbidimetric measurement which showed that the amount of immunoglobulins was increased from 17% of the total protein to 80% by octanoic acid treatment.

3) Column chromatography of the 40 mL of filtered solution (0.45 µm) containing 178mg IgG, 110mg IgA and 142mg IgM, on Sephadex DEAE A-50 typically yielded three peaks as shown in Fig 1. Most of the immunoglo-

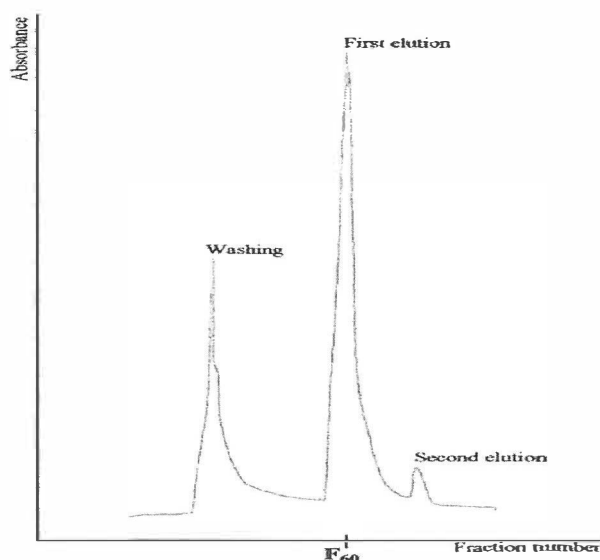


Fig. 1. The resultant ion-exchange chromatography spectrum of immunoglobulin solution (solution A) using Sephadex DEAE A-50.

bulins were obtained by fraction 60 (after 300 min). The immunoturbidimetry results indicated that the eluted solution contained 18% IgG, 47% IgA and 35% IgM.

In the first peak which is related to washing (using 0.1 M tris-0.05 M NaCl buffer, pH 8.1), 64.7% of loaded IgG, 17.4% of loaded IgA and 15% of loaded IgM were found. The second peak (first elution using 0.1 M tris-0.2M NaCl buffer, pH 8.1) contained 32.4% of loaded IgG, 79.3% of loaded IgA and 79.4% of loaded IgM. The third peak (second elution using 0.1 M tris-0.35 M NaCl buffer, pH 8.1) was made up of 3.1% loaded IgG, 2.5% loaded IgA and 7.9% loaded IgM. The amounts of IgG, IgA and IgM obtained in different steps of ion-exchange chromatography are shown in Table I.

The electrophoretic analysis (Fig. 2) reveals that the major portion of immunoglobulins was obtained in the first elution.

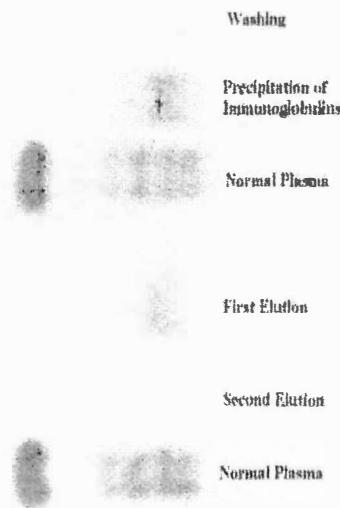


Fig. 2. Electrophoresis diagram of immunoglobulin solution ion-exchange chromatography.

DISCUSSION

Since approximately three-quarters of the lipoproteins of plasma are concentrated in fraction II+III obtained by cold ethanol fractionation, the separation of these lipoproteins from intended macroglobulins became of utmost importance. In this study, macroglobulins were precipitated simply by removal of fraction III and addition of ethanol after octanoic acid treatment.

Sephadex DEAE A-50, even after swelling to a gel in aqueous media, does not admit large molecules, while there is little or no restriction of the diffusion of smaller molecules. Besides it rapidly swells in water, and can be regenerated and used over again for new experiments without any detectable difference in its properties. Therefore Sephadex DEAE A-50 was employed in this study.

A comparison of immunoglobulins' percentage in human plasma¹⁹ (IgG 72%, IgA 16%, IgM 12%) with our results (IgG 18%, IgA 47%, IgM 35%) indicates that in our work, a three-fold enrichment was obtained for IgM and IgA.

Therefore we suggest the single step ion-exchange chromatographic method using Sephadex DEAE A-50 gel and 0.1 M tris-0.35M NaCl buffer, pH 8.1, giving recovery of 36% IgG, 82% IgA and 87% IgM.

REFERENCES

1. Audran R, Pejaudier L, Steinbuch M: Obtention d' une preparation d' immunoglobulines G, A, M (IgGAM): a usage therapeutique. Rev Franc Transf Immunol Hematol 18(2): 119-135, 1975.
2. Sisti AM, Vitali MS, Manfredi MJ, Zarzur JA: Preparation of lyophilized and liquid intravenous immunoglobulin G: development and scale-up. Vox Sang 80:216-224, 2001.
3. Mould GP, Sutton JA, Matejtschuk P, Gascoigne EW, Dash CH: Solvent / detergent treatment does not alter the tolerance or uptake of human normal immunoglobulin for intramuscular injection. Vox Sang 80: 151-158, 2001 .
4. Ballou M: Intravenous immunoglobulins: Clinical experience and viral safety. J Am Pharm Assoc 42(3): 449-459,

Table I. Comparison of immunoglobulins', percentage obtained in different steps of ion- exchange chromatography by Sephadex DEAE A-50.

Step	Obtained Ig / Loaded Ig	IgG (%)	IgA (%)	IgM (%)
	Washing		64.7	17.4
First elution		32.4	79.3	79.4
Second elution		3.1	2.5	7.9

Preparation of Enriched IgM and IgA from Human Plasma

- 2002.
- Rieben R, Ross A, Muizert Y, Tinguely C, Geritsen AF, Daha MR: Immunoglobulin M-enriched human intravenous immunoglobulin prevents complement activation *in vitro* and *in vivo* in a rat model of acute inflammation. *Blood* 93(3): 942-951, 1999.
 - Wichman A, Borg H: Purification of human immunoglobulin M by affinity chromatography on protamine-sepharose. *Biochim Biophys Acta* 490: 363-369, 1977.
 - Peterfy F, Miholics E, Boros B: Improved method for isolation of human IgM as a by-product. *Ann Immunol Hung* 18:123-129, 1975.
 - Wiederkehr F, Bueller MR, Vonderschmitt DJ: Chromatographic and electrophoretic studies of circulating immune complexes in plasma. *J Chromatogr* 566: 77-87, 1994.
 - Palombo G, Verdoliva A, Fassina G: Affinity purification of immunoglobulin M using a novel synthetic ligand. *J Chromatogr B* 715: 137-145, 1998.
 - Nowak T, Gregersen JP, Klockmann U, Cummins LB, Hilfenhaus J: Virus safety of human Immunoglobulins: efficient inactivation of Hepatitis C and other human pathogenic viruses by the manufacturing procedure. *J Med Virol* 36(3): 209-216, 1992.
 - Dichtelmuller H, Rudnick D, Breuer B, Ganshirt KH: Validation of virus inactivation and removal for the manufacturing procedure of two immunoglobulins and a 5% serum protein solution treated with γ -propiolactone. *Biologicals* 21: 259-268, 1993.
 - Burnouf-Rodosevich M, Appourchaux P, Huart JJ, Burnouf T: Nanofiltration, a new specific virus elimination method applied to high-purity factor IX and factor XI concentrates. *Vox Sang* 67: 132-138, 1994.
 - Cohn EJ, Strong LE, Huges WL, Mulford DJ, Ashworth JN, Melin M, Taylor HL: Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J Am Chem Soc* 68: 459-475, 1946.
 - Burnouf T: Integration of chromatography with traditional plasma protein fractionation methods. *Bioseparation* 1: 383-396, 1991.
 - Chandra S, Cavanaugh JE, Lin CM, Pierre-Jerome C, Yerram N, Weeks R, Flanigan E, Feldman F: Virus reduction in the preparation of intravenous immunoglobulin: *in vitro* experiments. *Transfusion* 39: 249-257, 1999.
 - Kistler P, Friedli H: Ethanol precipitation. In: Curling JM (ed), *Methods of plasma protein fractionation*. London: Academic Press, pp. 3-15, 1980.
 - Van Der Hoven A, Conradie JD, Bubb M: The isolation of immunogenically pure IgM from Cohn fraction III of pooled normal human plasma. *Immunochemistry* 10: 107-114, 1973.
 - Boyer RF: General laboratory procedures. In: *Modern Experimental Biochemistry*, 2nd ed., Redwood City, California: Benjamin/Cummings Publishing Company, pp. 29-58, 1993.
 - Hellstern P, Sachse H, Schwinn H, Oberfrank K: Manufacture and *in vitro* characterization of a solvent/detergent-treated human plasma. *Vox Sang* 63: 178-185, 1992.