Evidence of human hemoglobin interaction with chorionic gonadotropin hormone: Prospects for the use of hemoglobin as ligand in affinity chromatography for the purification of the hormone

Reza Khodarahmi*[1], Shabnam Maghsoodi[1], Hamid Rahi[1], Koorosh Hamzehee[1], Sara Zamaheni[2], Elahe Davarpanah[3]

[1]Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran
[2]School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[3]Proteomics Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

*Corresponding author: e-mail addresses: rkhodarahmi@mbrc.ac.ir; rkhodarahmi@kums.ac.ir (R. Khodarahmi)

ABSTRACT

The purification of biomolecules is a necessary step in many biochemical researches. In this regard, developments of convenient, specific and low cost methods of purification are of particular interest. Given the human hemoglobin (Hb) affinity toward some charged carbohydrates, interaction of this molecule with human chorionic gonadotropin (hCG) which is a glycoprotein hormone containing sialic acid, was examined. In the current study, we gathered evidence of free hCG and free Hb interaction using spectroscopic and radiometric techniques. Then, based on the affinity of hemoglobin (Hb) toward charged carbohydrates on human chorionic gonadotropin (hCG), a known sialic acid containing glycoprotein hormone, Hb-sepharose as well as native and denatured globin columns for isolation of the hormone were prepared. Sepharose-6B was activated by cyanogens bromide. Native Hb, normal globin and denatured globin were bound to cyanogen bromide–activated sepharose. Then, uptake of hCG by these gels were compared. Among the columns only native hemoglobin-sepharose column was able to catch a limited number of serum proteins such as hCG. Using the above column hCG hormone was purified with fold purification of 34 and efficiency of 80%. The chromatographic behavior of growth hormone (GH) and hCG in binding to the DEAE-Cellulose column were identical but GH showed no binding to Hb-sepharose column, indicating that the retention mechanism of hCG to Hb-sepharose column is not a simple ion exchange mode. Since globin had no property to attach to hCG but native Hb-sepharose was able to catch hCG, the BPG cavity of Hb is suggested as the possible binding site for hCG to Hb.

Keywords: Human chorionic gonadotropin; Hemoglobin; Affinity chromatography; Chorionic gonadotropin purification

INTRODUCTION

Several methods for the purification of biomolecules, especially proteins are used. One of the most common methods in this regards is chromatography in which has numerous types. Among different types of chromatography, affinity type is preferred in terms of high capability and specificity. Affinity chromatography is based on the complementary interaction of structures, charges and hydrophobicity between the target proteins and the stationary phase.

Affinity supports are often designed based on structure of their natural ligands so that possibility of non-specific interactions in this method is very low. Using substrate or its analogue to purify the enzymes, using antibodies to purify antigens or vice versa, and the use of lectins for glycoprotein purification and vice versa is common [1, 2].

Lectin affinity chromatography is a highly specific method for the purification of glycoproteins. Each type of lectins has unique properties in diagnosis and specific binding to the glycoproteins. This property is caused due to affinity of lectins to sugar moieties. But purification of lectins due to their low concentration and polymorphism is relatively expensive. For this reason, the use of other proteins which have affinity to glycoproteins and are also found abundantly in nature much is worthwhile [3-5].

Human hemoglobin is a hetero-tetrameric protein that has been formed by two types of subunits ($\alpha_2\beta_2$). Four subunits of human hemoglobin are located next to each other so that create a small cavity in the center of the molecule. The center cavity is surrounded by positively charged residues. Molecule, 2,3 Bis-phospho glycerate (BPG), with a negative charge is placed in this position. BPG is natural regulating of hemoglobin affinity for oxygen. Other anions other than BPG can bind to BPG central cavity and decrease the affinity of hemoglobin to oxygen. Such anions are notable as effectors of hemoglobin [6]. Gorner et al [7] considered the natural affinity of hemoglobin to poly-anions to design and synthesis of a new affinity supports. They found that at a pH close to the Hb pI (total protein charge is zero), hemoglobin interacts with poly-anions through its allosteric BPG sit.

Each adult person naturally in his/her erythrocytes has different types of Hb (HbA, 96%, HbA2, 2.5%, HbF, 1%). HbA that is approximately included 96% of the total hemoglobin has higher clinical importance than other types of hemoglobin [8, 9]. Haptoglobin and hemopexin are two serum proteins that with high affinity interact with the α-chain and heme group of hemoglobin, respectively. Due to this property, immobilized hemoglobin on solid phase is used in the purification of the two mentioned proteins. Recently, a number of hetero polysaccharides, such as heparin, dermatan sulfate, keratan sulfate have been purified using hemoglobin-Sepharose. Regarding the negative charge of these hetero-polysaccharides, BPG site has been suggested as their possible binding site. Reaction of hemoglobin with glucose (Glc) and glucose-6-phosphate (G6P) and hemoglobin glycation, especially in diabetic conditions, are also extensively studied [10, 11].

Human chorionic gonadotropin (hCG) hormone is a glycoprotein with molecular weight of 37,900 Daltons and has two α/β subunits with 92 and 145 amino acids, respectively. This glycoprotein contains 35% w/w oligosaccharides in its structure and more importantly ~10% w/w of the hormone mass belongs to the negative charged sialic acids. The plasma level of this hormone in the initial months (8^{th} week) of pregnancy reaches its maximum [12].

In this study, due to the ease of purification, the availability and affinity for some charged carbohydrates, we decided that examine the affinity of human hemoglobin to glycoproteins such as hCG and after hemoglobin immobilization on the solid phase, we used it for purification of this hormone.

MATERIALS AND METHODS

Materials

Sephacryl S-200, sepharose 4-B and sepharose 6-B, were purchased from Pharmacia; glucose, galactose, mannose, fructose, ribose, silver nitrate, coomassie brilliant blue R-250, diethylaminoethyl cellulose (DEAE-Cellulose), cyanogen bromide and para-Nitrophenyl Phosphate (PNPP) were purchased from Merck; β-hCG kit was prepared from Diasorin. Other chemicals were analytical grade.

Purification of hemoglobin A

Citrated human blood prepared from Blood Transfusion Center of Kermanshah was washed several times in normal saline and centrifuged in 2700g at 4˚C. Then, red blood cells were lysed with distilled water and centrifuged in 11000g. Upper phase (supernatant) was then dialyzed against 50 mM Tris-HCl buffer, pH 7.5 and loaded on the DEAE-Cellulose column which previously equilibrated with the same buffer. Bound HbA was eluted with a buffer containing NaCl (0.1 to 1 M).

Fractions containing HbA were identified by measuring absorption at a wavelength of 415 nm, collected and then dialyzed against distilled water and dried using Freeze dryer machine and were stored at -40 ˚C for later use. To determine the purity of HbA, Disc electrophoresis method (7.5% polyacrylamide gel) was used. To identify the Hb types, Helena electrophoresis system (Helena) was used [13, 14].

Activated Sepharose Preparation

Gel (sepharose) activation was performed by using cyanogen bromide (CNBr) and buffer titration methods (due to high efficiency, simplicity and safety) [15]. CNBr at alkaline conditions reacts with amino groups of matrix and forms the cyanamide group. This reactive group then reacts with a primary amine of the ligand to form guanidine. CNBr at alkaline conditions with hydroxyl (OH) yields chemically active cyclic imidocarbonate ester (Figure 1).

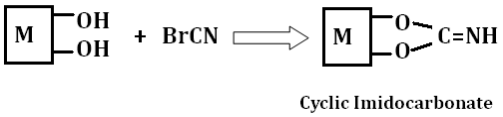

Figure 1. Activation of the solid phase (sepharose).

Preparation of heme-less globin

To separate heme from the protein, acidic-acetone method (16) was used. For this purpose, 4.8 mL of acetone with 0.2 mL HCl 37% were mixed and then incubated for 10 minutes at -20 ˚C. Then 0.5 ml of pure HbA was added drop by drop. The mixture was stored for one hour at –20 ˚C and centrifuged for 5 minutes in 2,700 g. Red colored upper phase was discarded and white precipate was dissolved in buffer PBS 0.01 M.

Coupling of native human hemoglobin, heme-less globin and denatured globin to Sepharose

If the activated matrix is incubated in the presence of amino groups, under certain conditions, it reacts with them and finally covalent binding takes places. For this purpose, 20 mg of freeze-dried hemoglobin, globin and denatured globin were independently dissolved in 20 mL of PBS buffer (with no amino group) and incubated in the presence of 15 ml of activated gel (2:1.5). To protect the possible sugar recognition sites on the proteins, coupling step was accomplished in the presence of 0.1 M glucose, galactose, mannose, fructose and maltose. The extent of protein covalent binding to the solid phase was assessed through sampling from the upper phase and determination of amount of remaining protein. The resulted Hb-gel/globin-gel were washed several times with distilled water and then incubated in the presence of 2 M glycine or 2 M urea for 12 hours till remained active groups on the gel to be neutralized by the binding of glycine or urea. After washing with distilled water, the gels were used to fill the glass columns. To separate unbound proteins, the gels were washed with three volumes (compared to the bed volume of the gels) of 1M NaCl and absorption of outlet at 280/415 nm, were checked.

hCG interaction with Hb-Sepharose

Given the unique properties of hCG and with knowledge of the characteristics of Hb, quality and quantity of hormone retention/absorption by Hb-Sepharose (as well as globin-sepharose) columns were investigated. For this purpose, the serum of pregnant women containing at least 200 mIu/ml of the hormone was examined. Initial hCG and eluted-hCG levels were measured/compared using IRMA and RIA methods (Diasorin kit).

Evaluation of radioactive hCG (I^{125}-hCG) absorption of Hb-Sepharose

In this survey, the amount of I^{125}-hCG uptake was calculated with measurement of radioactivity (CPM) of outlet solution compared to the initial loaded solution.

Serum hCG absorption capacity of Hb-Sepharose

0.5 ml of hCG (seum) was loaded into Hb-Sepharose column and also washed with equilibrating buffer, then 25 ml of column output was collected in one balloon. Then the column was washed with 10 ml of 1M NaCl and the same volume was collected again. Hormone-binding capacity of the column was examined through the measurement of hormone levels in each of the above samples in comparison with the prototype (serum).

Comparison of hCG absorption capacities of DEAE-Sepharose/Hb-sepharose

For this comparison, a column of DEAE-cellulose (1×8 cm) was prepared and then equilibrated with 50 mM Tris-HCl (pH 7.5) buffer. 0.5 ml serum containing hCG was loaded and extent of hCG (and serum) absorption was measured as whatever done for Hb-sepharose column.

Evaluation of hCG uptake (absorption) strength by Globin-Sepharose and Denatured Globin-Sepharose and Sepharose-Denatured Globin compared with normal Hb

In order to determine mechanism of hCG binding to Hb, ability to absorb this hormone by column containing heme-less globin (Globin-Sepharose) and denatured-globin by 8 M urea (Denaturant Globin-Sepharose) was examined, as to what was mentioned for Hb-Sepharose.

Evaluation of hCG affinity to Hb-sepharose under the effect of different NaCl concentrations

0.5 ml serum containing hCG was loaded to the Hb-sepharose (1×8 cm) column after equilibrating with Tris-HCl buffer, then was washed with 25 ml of the same buffer. The adsorbed proteins were eluted using a step by step gradient of 0.1–1.0 M NaCl in the same buffer (volume of each fraction was 10 ml). Fractions containing hCG were pooled and

hormone concentration in each fraction for estimating critical concentration of NaCl, required for detaching of bound hCG, was determined.

Evaluation of absorption capacity of prepared Hb-sepharose and DEAE-cellulose columns toward hCG and growth hormone (GH)

Unlike hCG, somatotropin (GH) is a polypeptide of 191 amino acids (molecular mass, 21,500 Daltons), possesses two disulfide bonds and with no sugar moity (15). Regarding acidic pI values of hCG (3.5) and GH (5.2), a growth hormone uptake by mentioned columns were studied. For this purpose, 0.5 ml of serum (containing GH) was entered into both columns (with the same dimensions (1×8 cm)) and washed with 25 ml of equilibrating buffer. Hormone concentration in each fraction was measured compared to the serum sample using ELISA technique.

Evaluation of the I^{125}-hCG affinity to free Hb

In order to ensure the mutual affinity of hCG and Hb, a column of sephacryl S-200 (1×50 cm) was equilibrated with 50 mM Tris-HCl buffer (pH 7.5), then 0.5 ml of human hemoglobin (20 mg/ml) was loaded into the column and was washed with the same buffer. Outlet fractions from t=0 with an interval of 1 minute were collected with a fraction collector and their spectroscopic absorption at 280 nm were registered. In an independent experiment, retention time for I^{125}-hCG was recorded by counting of radioactivity of collected fractions using a Gamma Counter. In another attempt, a mixture of Hb (0.4 ml) and I^{125}-hCG (0.1 ml) was prepared and incubated for 2 hours at 4°C. The sample was then loaded on sephacryl column and retention times of both Hb and I^{125}-hCG were registered.

Evaluation of I^{125}-hCG affinity to free (heme-less) globin

0.5 ml of qualified globin solution (20 mg/ml) in 50 mM Tris-HCl (pH 7.5), was loaded into sephacryl S-200 column which previously equilibrated with the same buffer, followed by its washing with mentioned buffer. The retention time was calculated by using measuring absorbance of fractions at 280 nm. A mixture of globin (0.4 ml) and I^{125}-hCG (0.1 ml) was prepared and incubated for 2 hours at 4°C at the same conditions. The sample was then loaded on sephacryl column and retention times of both globin and I^{125}-hCG were registered, separately.

RESULTS

Electrophoresis on cellulose acetate is shown in Figure 1. As also indicated in electrophoretogram and based on scanning of the protein bands at complementary wavelength (525 nm), crude Hb solution contained 96.6% HbA and 3.4% HbA_2. After successful chromatography on DEAE-Cellulose column, HbA with ~100% purity was obtained.

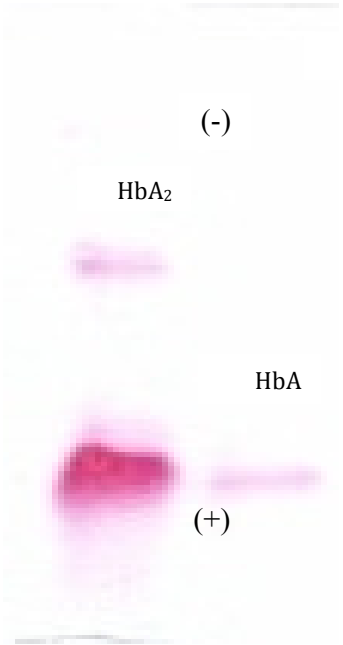

Figure 2. Hb electrophoresis on cellulose acetate gel before/after purification by anion-exchange chromatography.

Sepharose activation and coupling of HbA

When Hb was incubated in the presence of activated sepharose in PBS (as coupling buffer), Hb transition from solution phase to the solid phase occurred. Comparison of solution absorbances at 280/415 nm, before and after coupling reaction, indicates that more than 93% of the Hb is covalently bound to sepharose. Hb-sepharose was then entered into a glass column and washed with 1M NaCl solution. Outlet solution showed no significant absorption at 280 and/or 415 nm, indicating that Hb (as well as globin) molecules are being covalently attached to the sepharose matrix.

May serum proteins interact with hemoglobin or globin?

In two separate experiments, a solution containing Hb alone and a mixture of Hb plus human serum was loaded onto the sephacryl S-

200 column. As indicated in Figure 3, the Hb retention time decreased from t=31 to t=24 (minutes), indicating that at least some Hb molecules bind to specific proteins of serum. Different observations were made for heme-less globin. There was no difference between retention times of globin alone and globin in "mixed globin-serum" system (data not shown).

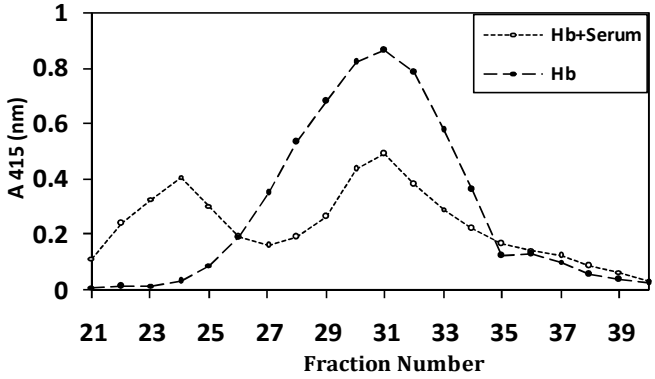

Figure 3. Effect of serum proteins on the retention time (RT) of human Hb when it was loaded (with or without serum) on sephacryl S-200 (1×50 cm) column. 0.5 mL of each sample entered into the column and washing was accomplished with phosphate buffer. About 35% of the hemoglobin population exit quickly from the column possibly due to interaction with special proteins of serum. Size of each fraction and flow rate were set at 15 drops and 30 drops per minute, respectively.

I^{125}-hCG interaction with free Hb

To ensure that there is a mutual affinity between hCG and Hb, purified hemoglobin was mixed with radioactive hCG (RIA kit) and the retention times (RTs) of samples (I^{125}-hCG alone and I^{125}-hCG +Hb) were analyzed/compared using sephacryl S-200 column (1×50 cm). As indicated in Figure 4, the retention times for free Hb (MW 65kD), I^{125}-hCG (MW 22kD), and I^{125}-hCG+Hb were t=33, t=41, and t=28 min, respectively. Decreased RT for "I^{125}-hCG+Hb", based on CPM and A_{415} nm, may reminiscent of non-covalent interaction between Hb and the hormone.

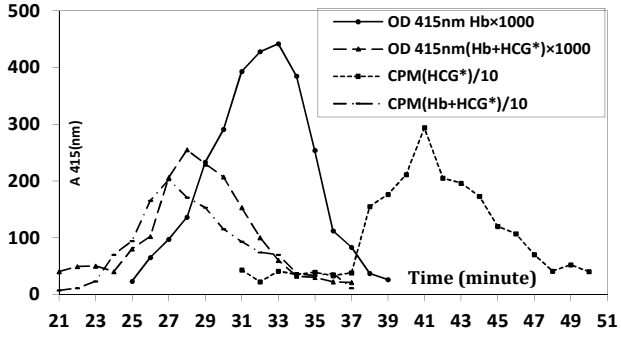

Figure 4. Comparison of Retention times on sephacryl S-200 (1×50 cm) column, free hemoglobin (min 33), radioactive-hCG* (min 41) and a mixture of Hb and radioactive-hCG* (min 28).

I^{125}-hCG absorption by immobilized Hb (Hb-Sepharose column)

Radioactive hCG (I^{125}-hCG, RIA kit) was loaded on Hb-sepharose column and CPM of inlet/outlet samples were compared. After washing, bound I^{125}-hCG was eluted by 1 M NaCl solution. As indicated in tables 1-3 (with focus on table 3), the amount of I^{125}-hCG absorption by the column, was estimated over 80%.

Evaluation of hCG absorption by Hb-Sepharose column

After sample loading followed by washing with salt solution, the eluted sample was collected. As indicated in tables 1 and 2, over 90% of serum proteins showed no affinity to column, but in a limited part of proteins bound to immobilized-Hb. As Table 2 shows, hCG can be purified 31 to 34 times with a yield of over 80%, only in one step hCG.

Table 1. Comparison of hCG/ serum proteins adsorption by Hb-Sepharose (Numbers in parentheses indicate percentage).

	hCG (CMP)	**Other serum proteins (A_{280})**
Initial sample	7724 (100%)	1.71 (100%)
Washing step	780 (10.9%)	1.56 (91.2%)
Eluted by NaCl	6944 (89.9%)	0.121 (7.076%)

Table 2. Study of Hb-Sepharose and hCG interaction.

Step (sample)	**Total Activity (CPM)**	**Total protein (A_{280})**	**Specific activity**	**Fold purification**
Initial sample	193100	42.75	4516	1
Washing step	19500	39	500	0.11
Eluted by NaCl	173600	1.21	143471	31.76

Is hCG-Hb interaction electrostatic or non-specific?

The question remains whether hCG binding to column can be interpreted in the frame of an affinity chromatography? For this purpose, the hCG-rich serum was loaded on Hb-sepharose column followed by successful washing. Then the column-bound samples were eluted using NaCl-containing buffer with increasing concentration of salt and in a step by step manner. As evident from Figure 5, significant elution (detaching) of hCG take places at 0.2 M NaCl and continues at higher concentrations confirming electrostatic nature of hCG-Hb interaction. Also NaCl at 0.4 M is able to detach the homone completely indicating relatively tight binding of hCG to the immobilized Hb.

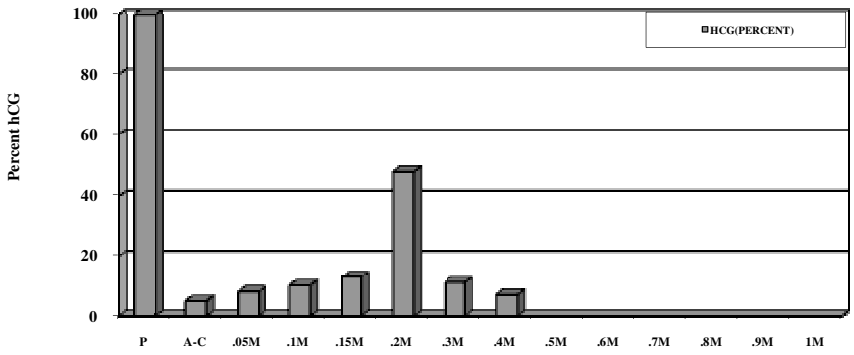

Figure 5. Effect of increasing NaCl concentration on the elution profile of hCG. The value of hCG in prototype (p) is 100%. Washing with equilibrating buffer with different concentrations of NaCl (0.05 to 1 M) showed that 48% of bound hCG is detached while eluting accomplished with 0.2 M NaCl.

Extent of hCG and GH absorption by Hb-sepharose and DEAE-cellulose columns. Gorner et al. showed that hemoglobin affinity column may be designed regarding strong interaction between Hb and polyanions [7]. There is the possibility that hCG and Hb interaction be also the affinity type. To assess this possibility, and since hCG is glycoprotein (with 35% carbohydrate and 10% sialic acid), and GH has no carbohydrate moiety in its structure; their binding extent to DEAE-Cellulose (anion exchanger) column and Hb-Sepharose (affinity column) were compared. As it is evidenced from Figure 6, both hCG and GH significantly bind to DEAE-Cellulose. On the other hand, only hCG binding by Hb-affinity column was observed (see also Table 3).

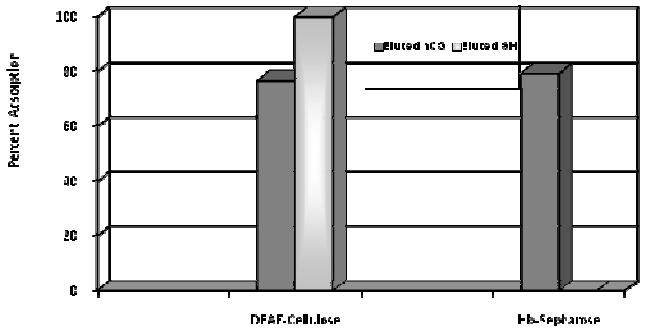

Figure 6. Comparison of the extent of GH and hCG binding to DEAE-cellulose and Hb-sepharose columns. Although both hormones bind to DEAE-cellulose column, but Hb-sepharose catches only hCG (not GH).

Table 3. Comparison of the GH and hCG (and other serum proteins) binding extents to DEAE-Cellulose and Hb-Sepharose. (Numbers in parentheses indicate percentage)

Other serum proteins		**hCG (mIU/ml)**		**GH (mIU/ml)**		
Column/sample	Hb-Sepharose	DEAE-Cellulose	Hb-Sepharose	DEAE-Cellulose	Hb-Sepharose	DEAE -Cellulose
Initial value	1.131 (100%)	1.131 (100%)	1714 (100%)	1714 (100%)	10.09 (100%)	10.09 (100%)
washed sample	1.023 (90.45%)	0.194 (17.15%)	406 (23.68%)	426 (24.85%)	9.90 (98.11%)	0 (0%)
Eluted sample	0.115 (10%)	0.945 (83.55%)	1358 (79.22%)	1316 (76.77%)	0 (0%)	10.15 (100%)

Comparison of Hb-Sepharose column with Globin-Sepharose, Denatured Globin-Sepharose and Sepharose-Denatured Globin columns

To ensure that natural structure of Hb plays a major role in hCG binding to the Hb-column; different globin-based columns were prepared and their ability for hCG catching were compared to Hb-sepharose. As indicated in table 4, correct quaternary structure of Hb is essential for proper hCG binding.

Table 4. Comparison hCG binding to Hb-Sepharose, Globin-Sepharose, Denatured Globin-Sepharose and Sepharose-Denatured Globin.

Type of column	**Hb-Sepharose**	**Globin-Sepharose**	**Denatured Globin-Sepharose**	**Sepharose-Denatured Globin**
Initial value (mIu/ml)	1714 (100%)	1714 (100%)	(100%) 1714	(100%) 1714
Washed sample (mIu/ml)	406 (23.68%)	(56.59%) 970	(71.82%) 1231	1260 (73.51%)
Eluted sample (mIu/ml)	1358 (79.22%)	(39.43%) 676	(26.72%) 458	406 (23.68%)

Evaluation of I^{125}-hCG binding to globin when the heme group is separated from the globin, Hb is break down to αβ dimmers so that BPG binding site is disturbed. To prove whether natural structure of Hb has central role in the ligand (Hb)-hCG interaction, extent of I^{125}-hCG binding to hemoglobin/globin was also assessed. The retention times for free globin, I^{125}-hCG and globin in the presence of I^{125}-hCG was 62, 39, and 62 minutes, respectively (Figure 7). It is documented that retention time of globin samples in the absence and presence of radioactive hCG, is the same so it can be concluded that there is no interaction between hCG and globin.

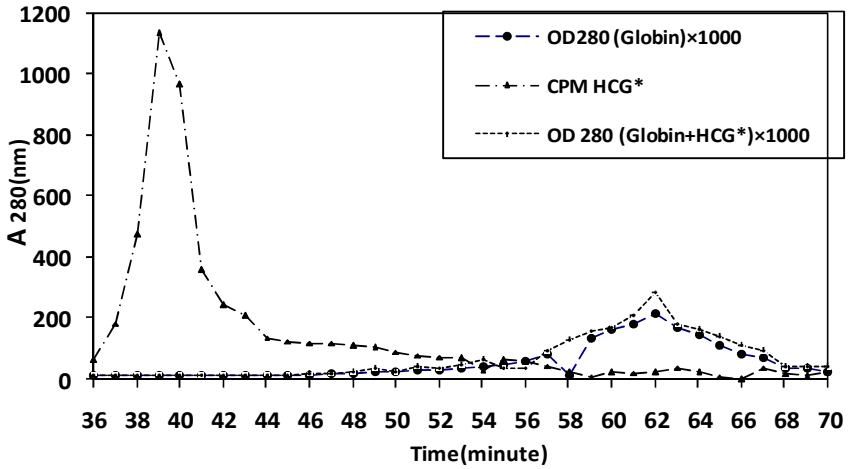

Figure 7. Comparison of retention time of various samples from Sephacryl S-200 (1×50 cm) column. Globin (min 62), HCG* (min 39) and mixture of them (min 62).

DISCUSSION

Affinity chromatography is a unique method for purification of biological molecules. In this regard, inexpensive, more specific and efficient methods are of particular interest [2]. Hb is the most intracellular protein in red blood cells (RBC), thus its purification is very fast and takes the least time. Another advantage of this protein is its (usually unknown) different unusual properties [6].

Cyanogen Bromide method was used for Hb coupling to sepharose due to efficiency, speed, high efficiency and low cost. The generated active groups easily react with ligand amino groups, are caused the covalent bond between the solid phase and hemoglobin with large number of ligand binding [15]. Assuming that all reactive positions on the activated sepharose are not neutralized by binding amino groups of Hb/globin, glycine or urea were used to fill the residual reactive sites. Lack of protein in the outlet sample, after treatment of the Hb column with NaCl solution, indicated that Hb is being covalently bound to the sepharose. Additionally, major part of serum proteins (90%) passed throghth the Hb column with no significant binding.

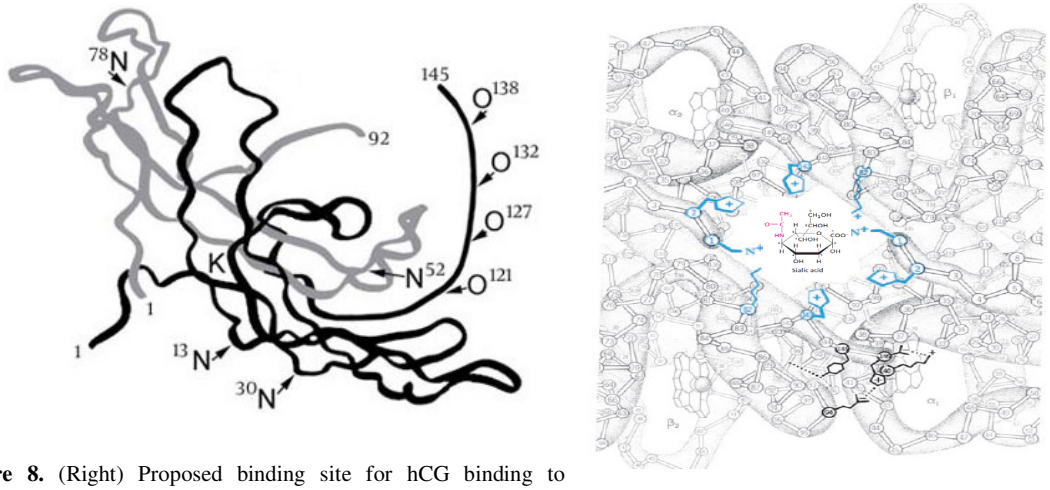

Figure 8. (Right) Proposed binding site for hCG binding to hemoglobin. Central cavity of hemoglobin contains positively charged side chains that may complementary interact with negatively charged sialic acid carboxyl groups of hCG. (Left) Crystal Structure of Deglycosylated hCG. The symbol N indicate the site of attachment of N-linked oligosaccharides, and the symbol O the attachment site of O-linked oligosaccharides. The α-subunit is shown in grey, and ß-subunit in black.

Comparison of Hb and globin data may inspire us that Hb structure and its intact natural binding sites have central role to catch hCG. According to the unique properties of hCG as well as awareness of human Hb structure, absorption extents of hCG by globin- and Hb-sepharose columns were evaluated. Using the Hb-based column and only in one step, hCg was purified (fold purification was ~34) with 80% efficacy. Also hCG-Hb interaction did not detach at NaCl concentrations below 0.2 indicating hCG tight binding to Hb. Also GH failed to connect Hb column confirming that carbohydrate moieties of hCG have critical/key role in hCG-Hb interaction. Disability of all types of heme-less globins in binding to hCG convinced us to propose BPG binding site (a central positively charged cavity) in Hb as possible binding site of hCG (sialic acids) to Hb (Figure 8).

The role of BPG site in purification of charged heteropolysaccharides [11, 12], electrophoretic behavior of some types of Hb in the presence of amylopectin charged groups (impure agarose) [17] have been repeatedly reported [3, 13]. Assuming that hCG binds to Hb through its sialic acid moities, sialic acid may be considered as eluant. Since we observed I^{125}-hCG-Hb (not globin-I^{125}-hCG) interaction, this property of native Hb led us to propose that the constructed column is an affinity (not simple ion exchanger) column. In future works, we try to set up more for convenient purification of target molecules.

REFERENCES

1. Freifelder D. Physical biochemistry, applications to biochemistry and molecular biology, W.H. Freeman. 1976; 257-265.
2. Wilchek M, Jakoby WB. The literature on affinity chromatography, Methods Enzymol. 1973; 34:3-10.
3. Lis H and Sharon N. The biochemistry of plant lectins (PHA), Annu. Rev. Biochem. 1973; 42:541-547.
4. Sharon N. Lectins, Scientific American, 1979; 120:180-191.
5. Barodes SH. Lectins: Their multiple endogenous cellular function, Annu. Rev. Biochem. 1981; 50:207-231.
6. Bunn HF and Forget BG. Hemoglobin: Molecular, Genetic and Clinical Aspects, W.B. Saunders Company, Philadelphia. 1986; 13.
7. Gorner T, Gissinger P, Leonard M, Dellacherie E. New support for the affinity chromatography of hemoglobin, j. of Chromatography B. 1997; 694 39-48.
8. Stryer L. Biochemistry – fourth edition. 1995.
9. Devlin T M. Text book of biochemistry with clinical correlation. 1982.
10. Bodensky O and Lanter AL, Advanced in clinical chemistry 1972; (15).
11.Wu VY and Cohen MP. Purification of glycated Hb, Methods Enzymol. 1994; 231:65-75.

Dedicated to the Memory of My Deceased Professor, Dr **Hamid Rahi** Who Passed away in 2000. **(R. K.)**