International Journal of Biomedical Science and Research
ISSN: 3068-9244

PRIME OPEN ACCESS

Volume 2, Issue 3

Research Article

Date of Submission: 06 August, 2026
Date of Acceptance: 31 August, 2026
Date of Publication: 07 September, 2026

Fast and Cheap Purification Several Significant Blood Plasma Proteins by Casmac: Conclusions from the Two Practical Developments

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Citation: Volkov, H. L., Havryliuk, S. P., Gavryliuk, O. S. (2026). Fast and Cheap Purification Several Significant Blood Plasma Proteins by Casmac: Conclusions from the Two Practical Developments. *Int J Biomed Sci Res, 2*(3), 01-13.

Abstract

Recently we have demonstrated the possibility of using a number of high-affinity peptides to isolate both the total pool of IgG1-4 and specific IgG against several human viruses. Our published list of seven peptides that high specificity interacted with IgG1-4 at seven different points, which was specially made to tightly bind a large immunoglobulin molecule and deprive it mobility in order to avoid denaturation during high-temperature (35-45 °C) virus-inactivation directly in the chromatographic column. The affinity-7-peptide-chromatographic gel was named 7-PEPs-WorkBeads. The affinity peptide for aRUBIgG was developed on the base of interaction of amino acid sequence of RV capsid proteins which are possible the sites of RV interaction with target cell membranes or mitochondrial proteins or RV cell receptor MOG. In a similar way, affinity peptides to IgG against viruses rabies, measles, poliomyelitis and herpes were developed.

Methods: Affinity peptides, designed for chromatographic purification other plasma proteins, also revealed favorable properties for specific capture of Fg, Cpm, ProThr and A1AT family as for high types of buffer flow, as well as high virus inactivation temperature. The study aimed to present highly selective peptides as ligands for separating groups and individual proteins and possibility the "ledges" for the cascade chromatographic separation of human plasma proteins on the basis of the peptide-affinity gels. Human total IgG and subsequent specific anti-virus IgGs (anti-Rubella, anti-Mumps, anti-Measles, anti-Polio and anti-Varicella zoster viruses IgGs) as well as human blood plasma proteins (fibrinogen, ceruloplasmin, prothrombin and alpha-1-antithrombin family) were purified by two specific affinity cascade chromatography methods.

Results: The content of varicella-zoster, mumps, rubella, polio and rabies viruses IgGs were shown corresponded to dates of other experiments with minor deviations. The enough satisfactory purity (>94%) of four plasma proteins and their high yield (>84%) were achieved not only due to the specific affinity of the synthesized gels, but also due to a significant reduction in the buffer volumes, which ensured a significant reduction in the time of oxidation, denaturation and hydrolytic degradation of proteins, that is, the cascade chromatography method used.

Conclusion: As a result, the examples presented in the paper strongly support the transformation of biologics purification towards multi-column chromatography instrumentation due to its beneficial effects on product quality, time savings, and reduction of production costs. Moreover, these methods can be easily scaled to industrial processes.

Keywords: Calculated Affinity Peptide, Peptide-Affinity Cascade Chromatography, In Column Virus Inactivation

List of Abbreviations

RT - residence time
DBC – dynamic binding capacity
TDC – temperature-dependent binding capacity
aRUBIgG - anti-Rubella virus human IgG
aMUMIgG - anti-Mumps virus IgG
aMEAIgG - anti-Measles virus IgG
aPOLIgG - anti-Poliovirus IgG
aVARIgG - anti-Varicella zoster virus IgG
ProThr - prothrombin
Thr - thrombin
Cpm – ceruloplasmin
Fg - fibrinogen
A1AT family – (A1AT+A1ACT+PPC1I)
A1AT – alpha-1-antitrypsin
A1ACT - alpha-1-antichymotrypsin
PPC1I - plasma protease C1 inhibitor

Introduction

The treatment of casualties by the plasma derived biological medicines was beginning from the Second World War. And despite the unstoppable development of the biotechnology of recombinant preparations, proteins from blood plasma continue to be the main ones for medicine due to a number of continuing advantages. And despite the unstoppable development of the biotechnology of recombinant preparations, proteins from the blood plasma continue to be the main ones for medicine due to a number of continuing advantages.

First, the blood plasma of donors has a huge therapeutic potential, which modern medicine uses hardly 15-20%. Even today, we are not entirely confident in identifying around 700 unique proteins in plasma, and with 99% certainty we can name a little more than half [1,2]. As for use, most of it concerns diagnostic studies. Among all these proteins, four of them have gained commercial importance for production since the 1940s thanks to the historical Kohn method [3]. These four products are: albumin, IgG, factor VIII, and Factor IX.

Secondly, it is precisely due to the significant diversity in one source that plasma proteins retain and, we are sure, will continue to hold leadership in value in the commercial market for a long time to come. There is no need to prove this position. We calculated over 15 years ago the cost of the sequential purification of 10 plasma proteins when were scale-lab methods developing for. The only thing that should be noted is that each new protein technologically added to the noted ones leads to a decrease in the cost of each [4].

Third, the starting material for the production of biological drugs from plasma is natural which is different from synthetic starting material. So, the quality of plasma as starting material plays an important role in the quality of final products [5-7].

Since long ago, protein biochemists have strived to develop improved methodologies to separate and polish proteins extracted from human plasma. The most usual systems of protein separation were initially based upon chemical or physical precipitation and partitioning tools, using different chemicals or pH/temperature variations. The liquid chromatography was introduced as a more selective and refined fractionation and purification procedure, initially mostly relying upon the use of ion-exchange, hydrophobic interaction, and size-exclusion chromatography as an auxiliary post-treatment of produced proteins, later as an independent technology [8-10].

The most significant advance was made through the use of affinity chromatography, which was characterized by a much higher specificity for families and individual proteins. Perhaps along this path, the theoretical possibility of separating proteins of complex biological mixtures, such as insects hemolymph or mammalian blood plasma, on serially connected chromatographic columns (cascade) packed with different affinity gels appeared [11,12].

Here we have to deviate from the core of the publication and define the terminology. What does the technical term «cascade» mean? A cascade is a natural or artificial waterfall that falls down ledges. Let stress here "ledges". The technical term "cascade" means "a group of similar devices connected in series". So, we understand under term "plasma proteins cascade separation" a group of chromatographic columns packed with gels have the specific affinity to different plasma proteins connected in series, those a waterfall (blood plasma) successively falls onto several ledges (specific affinity gels), on which one of the plasma proteins is captured.

The idea of such a chromatographic cascade was offered by Berkowitz publication in 1989 and was realized after two years by Porath and Hansen [11,13]. The effectiveness of group-resolving power of cascade-mode multi-affinity column chromatography (CASMAC) was demonstrated with human serum. More than 99% of the serum proteins were adsorbed in the same high salt-containing buffer on a tandem column consisting of (1) immobilized Zn^{2+} on triscarboxymethyl

diamine gel followed by (2) thiophilic (T) gel, (3) Zn^{2+} bound to the dipicolylamine agarose, (4) hexyl-thioether C6-S agarose and (5) Ni2+-dipicolylamine agarose. After the adsorption step the immobilized metal ion affinity gels were attached to the top of tandem columns of other adsorbents (T gel, Sephadex G-25 for desalting and Mono-Q) and the elution conditions were selected such that further group separation was achieved. High resolution, high recovery, easy manipulation and high capacity are characteristic features of the cascade process with these adsorbents. The advantage of CASMAC is particularly striking when, with a given number of adsorbents, the overall number of operations involving adsorption, desorption, washing, buffer change and substance concentration can be effectively minimized. Perhaps the only one but significant drawback was the insufficiently high affinity of metal-affinity and hexyl-thioether C6-S gels. In fact, on average, each gel adsorbed more than 30-50 proteins. Most likely, several additional steps were required for the final purification of individual proteins.

Over the last few years, impressive developments in ligand chemistry and peptide synthesis as well as in bioinformatics have led to the availability of a wide range of affinity gels or membranes for extracting and purifying plasma-derived protein therapeutics[14-18]. This certainly revolutionary step raised hopes for the possibility of creating a system for the cascade separation of plasma proteins.

Separation proteins of human plasma is a challenge due to the presence of albumin (35-50 $g \times L^{-1}$) and immunoglobulins (all classes together >20 $g \times L^{-1}$) which can mask and capture or adsorb the low abundant proteins – 1^{st} group. The low abundant proteins interested for purification and exploitation in medicine is possible to divide on other 3 group according to their content in human plasma: 2^{nd} group 0.25-5 $g \times L^{-1}$ (fibrinogen, alpha-2-macroglobulin, transferrin, haptoglobin, alpha-1-antitrypsin+alpha-1-antichymotrypsin+plasma protease C1 inhibitor, alpha-1-acid glycoprotein and ceruloplasmin); 3^{rd} group, 40-200 $mg \times L^{-1}$ (antithrombin III, histidine-rich alpha-2-glycoprotein, prothrombin, ferritin and C-reactive protein); 4^{th} group, 0.5-25 $mg \times L^{-1}$ (mostly coagulation factors and proteins C, S and Z). The proteins content in human plasma shown here according to Schenk et al. and Kliuchnikova et al. [19,20].

The problem of the high abundant proteins in the analytical area is solved by a simple discharge of plasma on columns selective for albumin and immunoglobulins [21]. However, efficient manufacturing requires maximum products recovery with minimum impurities. Therefore, at the first steps of the cascade plasma proteins isolation development the following question arises: isolate high abundant proteins at the very beginning of the process or, if it does not create difficulties for the interaction of low abundant proteins with a chromatographic matrix, postpone work with albumin and immunoglobulins at a later stage. Moreover, the methods for preventing of proteins interaction have already been tested today (Ca^{-2} ions removing, relatively high ionic strength due to monovalent cations, "softening" of hydrophobic interactions by adding 5-10% glycerol) and a correctly selected affinity peptide(s) will provide highly selective binding of the desired protein to the chromatographic gel.

The second question for cascade separation is how many peptide-affinity chromatographic columns can be included in the cascade. Every chromatographer knows that even one column, as the target protein binds on the gel (ceteris paribus), shows an increase in back pressure, which, firstly, begins to reduce the buffer flow rate, and, secondly, threatens the life of the column itself, since they are often designed for a maximum working pressure of 5 bar.

The third question for cascade is which column have to be first with more target protein loading or less to avoid significant back pressure buildup.

Considering recent developments in chromatographic gels with high dynamic capacity, which means a high buffer flow rate with a fairly short residence time, we hope for the successful solution of the three problems described above [22]. This article presents highly selective peptides as ligands for separating groups and individual proteins. On the basis of these peptide-affinity gels, possible "ledges" for the cascade chromatographic separation of human plasma proteins are considered.

Material and Methods

Reagents, Equipment, Materials

All reagents were purchased from Sigma-Aldrich (Green Chemistry LLC, distributor in Mongolia) unless otherwise indicated. Reagents and equipment from other manufacturers are indicated below. Human (donors) plasma, obtained by plasmapheresis, was bought from Ulaanbaatar (Mongolia, Ulaanbaatar) and Kyiv (Ukraine, Kyiv) blood collection centers. Activated WorkBeads 40/1000 ACT matrix was received from Bio-Works Technologies AB, Uppsala, Sweden. All buffers for peptide-affinity chromatography were identical in all experiments: equilibration and application - 0.020 M Na-citrate buffer, pH 6.8, included 150 mM NaCl and 2.6 mM CaCl2; first washing - 0.020 M Na-citrate buffer, pH 6.8; second washing (if process not includes virus inactivation) - 0.020 M Na-citrate buffer, pH 4.5, included 150 mM NaCl; elution - 0.020 M Na-citrate buffer, pH 2.5. If chromatographic process includes virus inactivation directly in column between first and second washing the RSD-temperature washing by 0.020 M Na-citrate buffer, pH 6.8, includes 1% TnBP and 2,5% Triton X-100, was added. RSD-temperature washing means washing at the rising system T°, stable high system T° and decreasing system T° to the initial.

Chromatographic experiments were performed with glass different size ECO/ECOPLUS (YMC Europe GmbH, Germany, Dinslaken) or BPG Ø200×500 columns by ÄKTA*explorer* 100/ÄKTA*pilot* systems (GE Healthcare AB, Uppsala, Sweden). Protein sample diafiltration and/or concentration were done by KrosFlo RS-20 TFF system with SIUS TFF cassette for sterilization and MW cutoffs 50 or 100 kDa (Repligen corp., distribution office in China, Shanghai). All tests carried on with analytical equipment Typhoon Trio Imager, Biotrak II visible plate reader and UV/VIS-spectrophotometer Ultrospec 3300*pro* (GE Healthcare AB, Sweden, Uppsala).

Peptide Array Libraries, Array and Fmoc Solid Phase Peptide Synthesis, Spot Peptide Array Experiments
Peptide array library was calculated according to 7 developed principles described before using nucleotide sequence of the human genes and amino acid sequences of the human IgG (all including chains classes 1-4), fibrinogen (alpha-chain – P02671, beta-chain – P02675, gamma-chain – P02679), prothrombin (P00734), ceruloplasmin (P00450), alpha-1-antitrypsin (P01009), alpha-1-antichymotrypsin (P01011), plasma protease C1 inhibitor (P05155) from Protein Knowledgebase - UPKB (UniProtKB/Swiss-Prot). Proteins and peptides sequences numeration was done according to the whole molecule represented in UniProtKB [15].

The synthesis and SPOT peptide array experiments were carried on as described before [15,17,18].

Synthesis of the Peptide-Affinity Chromatographic Gels
All developed by early publish method 7- and 8-mer peptides or thrombin-like protein (Ancistron B) were coupled with WorkBeads 40/1000 ACT matrix according to manufacturer's recommendations [15,17-18]. In case of synthesis peptide affinity gels for immunoglobulin and fibrinogen purification the WorkBeads 40/10000 ACT matrix was used, for other proteins with smaller MW - WorkBeads 40/1000 ACT matrix. A peptide/protein 1% solution in Na-carbonate-bicarbonate buffer, pH 9.4, was mixed with 10 volumes of WorkBeads 40/1000 or 40/10000 ACT matrix during 48 hours at 4 °C. All other manipulations were carried on according to recommendation of activated gel manufacturer. The peptide density on the WorkBeads gel 40/1000 (or 40/10000) was determined by quantifying the difference between total peptide quantity in start solution and unbound peptide by fluorescence of tryptophan in the spacer measured by Typhoon Trio.

Target Proteins Determination
The protein's quantity was measured by the standard methods with Human Fibrinogen ELISA Kit (ab108841), Human Prothrombin ELISA Kit (ab108889), Human Thrombin ELISA Kit (Factor II) (ab108909), Ceruloplasmin Human ELISA kit (Abcam, ab108817), Human alpha-1-Antitrypsin ELISA Kit (ab108799), Human alpha-1-Antichymotrypsin ELISA Kit (ab157706), Human C1 inhibitor ELISA Kit (ab224883) – all from China Abcam, Shanghai, China; Human Immunoglobulin G (IgG) ELISA Kit (EI7200-1), Human Immunoglobulin M (IgM) ELISA Kit (EI7301-1), Human Immunoglobulin A (IgA) ELISA Kit (EI7001-1) – all from AssayPro, St. Charles, USA; Human Rubella virus IgG/IgM ELISA Kit (ESR129G), Mumps Virus IgG ELISA Kit (ESR103G), Varicella-Zoster Virus IgG ELISA Kit (ESR104G) – all from QED Bioscience, San Diego, USA; Human Poliomyelitis Virus IgG (PV-IgG) ELISA Kit (abx052879) – from Abbexa, Milton, UK; Human Rabies Virus antibody IgG ELISA Kit (DEIA1027) – distributor of Creative Diagnostic, Shirley, USA, in China Abace Biology, Beijing.

The protein's activity was measured by the standard methods with Human Thrombin Chromogenic AssaySense Activity Assay Kit (CT4010) – AssayPro, St. Charles, USA; Ceruloplasmin Colorimetric Activity Kit (K035-H1) – distributor of Arbor Assays LLC in China ZhongHao.

The total protein in the collected samples was determined by Bradford method with Stoscheck modification [23,24].

Determination of the Residence Time (Rt), Dynamic (Dbc) and Temperature-Dependent Binding (TDC) Capacities, and Apparent Kd for Peptide-Affinity Chromatographic Gel
The RT, DBC and TDC of the peptide-affinity gels were determined on Ø15×120 mm columns packed with 3 mL gel, bed Ø:height 15×15 mm (packing density factor 1,15-1,20). The RT determination and optimization carried on according to the method described by Björkman for "Protein A"-affinity chromatographic gel. DBCs were determined according to standard procedure described by Challener [22,25]. TDCs were determined by the following way. The quantity of protein samples was calculated 10% lower than maximum level of adsorbent DBC at 20°C. After sampling and washing the column/buffer temperature were slowly raised on 5 degrees each step and determined the target protein quantity in eluates. 5 or more independent experiments were performed according to chromatographic conditions stipulated above.

The apparent Kd of each protein-peptide interaction was determined by standard procedure described by Baja et al. for intact proteins interactions with development affinity peptides [26].

Human Total Igg and Subsequent Specific Anti-Virus Iggs Purification Directly from Plasma with In-Column Virus Inactivation/Elimination
20 L defrosted plasma was diluted with 5 L 0.020 M Na-citrate buffer, pH 6.8, included 150 mM NaCl and 2.6 mM $CaCl_2$ and applied on equilibrated pilot glass BPG column Ø200×500 mm packed with 13,8 L of 7-PEPs-WorkBeads 40/10000 gel to the bed height 44 cm (packing density factor 1,15-1,20) [18].

The plasma application was carried on with flow rate 1 Vc×h^{-1} that provided sufficient RT for the IgG and peptide ligand interaction. After application the column was washed with 3 Vc 0.020 M Na-citrate buffer, pH 6.8, included 150 mM NaCl, and with 5 Vc 0.020 M Na-citrate buffer, pH 4.5, included 150 mM NaCl, flow rate 2-2.5 Vc×h^{-1}. IgG elution was carried on 2 Vc 0.020 M Na-citrate buffer, pH 2.5, flow rate 1.5 Vc×h^{-1}. Unbounded and eluted IgG solution was automated neutralizing to pH 6.8 by 0.010 M NaOH and diafiltrated against 0.020 M Na-citrate buffer, pH 6.6-6.8, to I=5-7 mS×cm^{-1}/concentrated to 10% protein content (~10 and 3.5 L, correspondently).

Obtained total IgG solution was applied on the equilibrated with 0.020 M Na-citrate buffer, pH 4.5, five Ø25×750 columns connected in series "one by one" and packed with: 1) RDHHGTHE-WorkBeads 40/10000 gel for isolation anti-Rubella virus human IgG (^{aRUB}IgG) [18], 2) FDLLKARLLY-WorkBeads 40/10000 gel, for isolation anti-Mumps virus IgG (^{aMUM}IgG), 3) YRQTTQDSFT-WorkBeads 40/1000 gel for isolation anti-Measles virus IgG (^{aMEA}IgG), 4) CVLRKWLPPM-WorkBeads 40/10000 gel for isolation anti-Poliovirus IgG (^{aPOL}IgG) and 5) QRCGAHSANF-WorkBeads 40/10000 gel for isolation anti-Varicella zoster virus IgG (^{aVAR}IgG). The last four gels were developed according described before procedure [15,18]. All five column were packed with 230 mL gels to the bed height 47 cm (packing density factor 1,15-1,20). Total IgG sample was applied with flow rate 2 Vc×h^{-1} that provided sufficient RT for the IgG and peptide ligand interaction. After application columns were subjected to the first washing (10 Vc), flow rate 5 Vc×h^{-1}. Then columns were disconnected and proteins coupled by gels were subjected to the direct in-column virus inactivation/elimination. All conditions were early described in detail [18]. The chromatographic process was finished by second washing (15 Vc), flow rate 5 Vc×h^{-1} and specific IgG elution (2 Vc), flow rate 2-3 Vc×h^{-1}.

Human Fibrinogen, Prothrombin, Ceruloplasmin and Alpha-1-Antitrypsin Family Proteins Subsequent Purification with In-Column Virus Inactivation/Elimination Target Proteins Application and In-Column Accumulation

Unbounded on 7-PEPs-WorkBeads gel plasma fraction after concentration was applied on four equilibrated (10 Vc, flow rate Vc×h^{-1}) connected in series "one by one" Ø50×750 mm columns with flow rate 2 Vc×h-1 that provided sufficient RT for the IgG and peptide ligand interaction. All columns were packed with 1150 mL of peptide-affinity gels to the bed height 60 cm (packing density factor 1,15-1,20) as following: 1) VVIVPADR-WorkBeads 40/1000 (ProThr purification), 2) HKLLLKRT-WorkBeads 40/1000 (Cpm purification), 3) FRHDWYLL-WorkBeads 40/1000 (A1AT family proteins purification), 4) FFFFRIF-WorkBeads 40/10000 (Fg purification) [18]. Affinity-peptides as ligands for Cpm and A1AT family were developed according to early described method [15]. After equilibration and application columns were subjected to first washing (2 Vc), flow rate 5-10 Vc×h-1 and disconnected. First column with ProThr, second column with Cpm and third column with A1AT proteins family were moved to the refrigerator at 4 °C. The fours column was directly moved to virus inactivation process.

Human Prothrombin Activation by Immobilized A-Specific Thrombin-Like Enzyme

ProThr was eluted from first column after 15 times application, 2 Vc with flow rate 3 Vc×h^{-1} and automated adjusted pH=7.6-7.8 and transfer to Thr activation, on gel coupling and virus inactivation.

Collected pure ProThr was pumped in a closed circle through tandem Ø50/450 and Ø50/750 columns with flow rate 25-50 mL×min^{-1}: a) first packed with 575 mL Ancistron-B-Work Beads 40/1000 gel to the bed height 25 cm (packing density factor 1,15-1,20) for hydrolyze ProThr by thrombin-like protein to Thr and b) second packed with1150 mL PFLRAWAI-WorkBeads 40/10000 gel to the bed height 60 cm (packing density factor 1,15-1,20) for specific coupling Thr by peptide-affinity gel developed early [18,27]. Process activation was completed 24 h later. This method of thrombin activation, however, with immobilized thrombin, was proposed by Strukova [28]. Then column was transfer to Thr virus inactivation process.

Target Proteins Virus Inactivation Directly in Column

Target proteins, Fg, Thr, Cpm, and A1AT family coupled by gels were subjected to the direct in-column virus inactivation/elimination. All conditions were early described in detail [18]. The chromatographic process was finished by second washing (15 Vc), flow rate 5 Vc×h^{-1} and proteins were eluted (2 Vc) with flow rate 2-3 Vc×h^{-1} and with automated pH adjusted to pH=7.2-7.4 or 6.8-7.0 in Thr case in order to maximally reduce activity and autohydrolysis.

Target Protein Purification on Reverse Column Configuration

The selection of Fg, Cpm, A1AT and ProThr in the case described above "one by one columns" were located in that chain according to the accumulation of a specific protein from the max amount to the min in approximate percentage ratio 100:50:25:6. But due to binding protein accumulation, this ratio gradually changed to 100:100:50:25, 100:100:100:50 and 100:100:100:100. Considering the sufficiently significant gel bed height in each column (60 cm), the change in the physical state of the columns as the affinity gels are loading with proteins (increase in back pressure), it was important to assess whether there is a dependence of the back pressure on the initial upper or lower load of the series-connected columns.

To clarify this question, it was enough to simply reverse the application, wash and elution flows and monitor the back pressure of the four-column design.

Results

Mechanical Properties of Chromatographic Gel in 4× Or 5× "One-By-One" Design

Columns combining into a sequential "one-by-one" design actually means an increase in the gel bed height, which in our case specific IgG purification 5×47 cm is 235 cm, and in the case of plasma proteins purification 4×60 cm is 240 cm. 5-10 years ago, we would not even theoretically test the possibility of setting up such an experiment, given that even the best gels for low-pressure liquid chromatography (soft gels) had a strict column pressure limit of up to 3 bar due to completely banal physical destruction. In addition, the column design itself allowed the operating pressure to be no higher than the same 3 bar. However, the last seven-year period was marked by the appearance of gels with a high dynamic capacity (rigid gels), i.e. it became possible to carry out the chromatographic process with much higher buffer flow rates, which economically justified the creation of columns operating at a pressure of 5-10 bar and even higher. To relate the ability of a chromatographic gel to operate at high buffer flows with column back pressure and gel bed height, it is enough to look again at the relationships described by well-known formula below.

$$\Delta P = \frac{\eta F L}{K^0 \pi r^2 d_p^2},$$ where:

ΔP – column back pressure difference, K^0 – specific permeability,
η – buffer/sample viscosity, π – 3.14…,
F – buffer/sample flow rate, r – column radius,
L – gel bed height, d_p – particle diameter.

In fact, if other things being equal, there are three factors - viscosity, buffer flow rate and gel bed height - that can directly proportionally affect the pressure in the chromatographic column.

Practically, the back pressure in the column Ø25 mm with bed height 47 cm was 0.31 bar during the equilibration with 0.020 M Na-citrate buffer at a flow rate 300 cm×h^{-1}, and 0.36 bar during the 10% IgG solution application. According to the WorkBeads manufacturer at the recommended max flow rate 900 cm×h^{-1}, the back pressure in a Ø25 mm column with a bed height 20 cm was 2 bar, which practically coincides with our data [29]. The design "one-by-one" of 5 columns Ø25 mm with a total bed height of 235 came resulted in backpressure up to 1.7 bar during a buffer pumping and up to almost 2.0 bar during the 10% IgG solution application. In the case of 4 columns Ø50 mm combined into the "one-by-one" system with the total bed height 240 cm during the 10% solution of the plasma protein mixture application the back pressure was significantly lower – 1.4 bar. In the case of flows redirection from a column with a higher protein load to a column with a lower load, significant changes in the back pressure were not recorded.

The obtained results made it possible to intensify the process of protein purification by increasing the flow of chromatographic buffers to achieve a back pressure about 2.5-2.8 bar, since WorkBeads 40/1000 (or 40/10000) gel has an allowable pressure of 5 bar, and the used ECO columns - 10-15 bar [30].

Total And 5 Specific Iggs Purification from the Human Plasma by Peptide-Affinity Chromatography

Total IgG and IgG subclasses concentrations followed either a relative to the normal distribution from one plasma pool to another provided by the blood transfusion center. IgG4 demonstrated by far the widest range of concentrations between pools (130-fold: 0.014–1.83 g×L^{-1}). The content of IgG2 was the most stable and fluctuated up to 20% (7.02-8.65 g×L^{-1}), as well as the content of total IgG (12.66-15.71 g×L^{-1}). Variations in the content of IgG1 and IgG3 did not exceed 40% (3.97-6.44 and 1.09-1.82 g×L^{-1}, respectively).

The results of total IgG isolation on the 7-PEPs-WorkBeads gel column from the most characteristic content of both total and subclasses of IgG plasma pool are presented in Table 1.

IgGs in:	IgG total and subclasses content in the plasma and purified total IgG by peptide-affinity chromatography (g×L^{-1})				
	IgG total	IgG_1	IgG_2	IgG_3	IgG_4
Plasma (one pool)	14.59	5.11	7.53	0.68	1.27
Eluted IgG fraction	11.98	4.21	6.05	0.57	1.03
IgG yield (%)	81.70	82.39	80.35	83.82	81.10

Table 1: Total and Subclasses Iggs Content in the Separate Pool of the Human Plasma Bought from Regional Blood Center (Ulaanbaatar, Mongolia) and Yield After Purification on 7-Peps-Workbeads Gel Peptide-Affinity Chromatography

The dates shown in the Table 1 suggested that with 7-PEPs-WorkBeads gel it was possible to isolate more than 80% of the total IgG contained in the plasma pool. It should also be noted that in the total IgG eluate were detected IgM 1.03 g×L^{-1} (in terms of volume of used plasma) and traces of IgA, which were contained in plasma 1.59 and 2.29 g×L^{-1}, respectively. After diafiltration and concentration IgG eluate to protein content 8-10% the quantity and ratio IgG/IgM and IgG subclasses are still the same, but IgA was not detected. The total IgG purity was not less 95,61% and given that the main impurity was IgM (1.51 g×L^{-1}) and IgA traces, the purity of immunoglobulins was more than 97%.

During the chromatographic process the virus inactivation/elimination directly in column was carried on as reported early [17,18,31]. It was shown on the example of the total IgG that such virus inactivation is practically safe by removing/destroying possibly present viruses, decreasing virus concentration factor of (FVD) as a log10 was >9.6 [17,18].

The obtained virus-inactivated/eliminated IgG fraction (~25 L) was concentrated to the protein content 8-10%, which reduced the total volume to 3.5 L, and simultaneous diafiltration to set pH 6.6-6.8, electrical conductivity 5-7 mS×cm^{-1}. Specific IgGs were obtained with 5 columns connected in series "one by one" and packed with peptide-affinity gels. The results of specific IgGs isolation are presented in Table 2.

It is important to note that the IgG subclasses were isolated practically in almost the same ratio as in plasma.

Specific IgGs content and purified from donors plasma						
title	in plasma	applied on columns	eluted from columns, yield			
	IU×mL^{-1}	IU	IU	%	mg	IU×mg^{-1}
^{aVAR}IgG	249	4070154	2625656	64.51	517	5079
^{aMUM}IgG	147	2402862	1642837	68.37	357	4602
^{aRUB}IgG	94	1536524	955564	62.19	158	6048
^{aPOL}IgG	8	130768	79690	60.94	27	4843
^{aRAB}IgG	0.62	10135	6965	68.73	6	1669

Table 2: Five Specific Iggs Isolation on 5 Columns Connected in Series "One by One" and Packed with Peptide-Affinity Chromatographic Gels

The yield of specific immunoglobulins ranged from approximately 71 to 79%, which were in mass from 9 to 655 mg per immunoglobulin and correlated with their specific antiviral activity in plasma. On the other hand, regularity between the concentration of specific IgGs in plasma was not found. Anti-rubella virus IgG had the highest specific antiviral activity, anti-rabies virus IgG had the lowest activity. Other IgGs against varicella zoster, mumps, and polioviruses had fairly close specific activities of approximately 4500 to 5200 IU×mg^{-1}.

No specific IgG cross-contaminations were found in terms of the sensitivity of the used ELISA kits.

Fibrinogen, Prothrombin, Ceruloplasmin and Alpha-1-Antitrypsin Family Proteins Purification by Peptide-Affinity Chromatography

The choice of plasma proteins for the isolation was determined both by the desire to see the adsorption properties proteins with masses (our conventional groups II and III, see "Introduction"), and by the practical importance of proteins used in medicine.

Previously, the content of ProThr, Fg, Cpm and A1AT family (A1AT+A1AHT+PPC1I) in 10 plasma pools obtained from Ukraine (5 pools) and Mongolia (5 pools) was compared. There was no statistically significant difference in the content of target proteins in plasma pools from both countries. The only one thing that noted: the spread in the data of Mongolian donors was significantly higher than that of Ukrainian ones, which naturally affected the statistics.

The content of the target proteins in donor plasma (10 pools) detected by test systems shown in the Table 3 same as their quantity purified by cascade-affinity chromatography. On each column from cascade was received more than 80% yield. It was a fairly high result, taking into account the intensive washing and equally intensive virus inactivation/elimination process under at a sufficiently high temperature. The purity of all four proteins was more than 94% without cross-contamination according to the accuracy of the used test systems.

Purified ProThr was subjected to closed cycling activation by Ancistron B (nature prothrombin activator, group A: no cofactors, purified authors from the Agkistrodon blomhoffii ussuriensis venom) coupled to gel beads column[1]) [27] . Activated Thr very fast at the closed cycling pumping was coupled by FLRAWAI-WorkBeads colum[2]). This coupling preserved Thr from self-hydrolysis and represented them as new point of ProThr activation. Thus activation process was accelerated more and more until reached 100% results. For this it was necessary 4 °C and no more than 24 hours.

Target plasma proteins	Proteins ($mg \times L^{-1}$) in/from donors plasma			
	content	purified		
	M±m	M±m	Yield (%)	Purity (%)
Fg	3632±174	3076±129	84,7	/ 94,4
A1AT[f]	2027±215	1704±141	84,1	97,3
Cpm	327±13	282±18	86,2	95,0
ProThr	89±7	78±8	87,6	94,9
ProThr closed cycling activation on tandem columns packed with Ancistron-B-WorkBeads[1)] (ProThr activation into Thr) and FLRAWAI-WorkBeads[2)] (Thr coupling)				
ProThr ⟶ Thr		72±6	97,1	98,9

A1AT[f]: A1AT family (A1AT+A1ACT+PPC1I).

Table 3: The Content and Cascade Affinity Chromatography Yield of Target Proteins in/from Donors Blood Plasma Obtained in Ukraine And Mongolia

The high yield and purity (Table 3) further confirmed the complete ProThr activation and the activated Thr protection against self-hydrolysis by affinity gel binding.

Discussion

Recently we have demonstrated the possibility of using a number of high-affinity peptides to isolate both the total pool of IgG_{1-4} and specific IgG against rubella virus (^{aRUB}IgG) [18]. The calculation of high specific affinity peptides was made on the basis of literature dates from an experimental study of the IgG_{1-4} sites interaction with affinity proteins A, G, L, IgG Fc receptors, C1q, etc. The testing of 360 theoretically calculated peptides revealed several highly specific peptides for all four classes of IgG. The published investigations lists seven such peptides that high specificity interact with IgG_{1-4} at seven different points, which was specially made to tightly bind a large immunoglobulin molecule and deprive it of mobility in order to avoid denaturation during high-temperature (35-45 °C) virus-inactivation directly in the chromatographic column [18,31]. The affinity-7-peptide-chromatographic gel was called 7-PEPs-WorkBeads gel [18].

The presence of IgM (< 9%) in the eluate of total IgGs was due to the identical structure and the corresponding interaction of the monomolecular immunoglobulin, which forms 5- or 6-dimensional IgM structure, with specific peptide ligands of the 7-PEPs-WorkBeads gel. Although it should be noted that, most likely, the availability of IgM binding sites in the proposed chromatographic system is spatially limited, as evidenced by its significantly lower yield - 64.78%. However, the presence of IgM, according to its concentration in the plasma, does not in any way interfere with the use of IgG_{1-4} activity, on the contrary, it can enhance this activity, because it is known that in response to antigen, more active IgM appears first than the second IgG [32-34]. As for the trace impurities of IgA, it is also possible that it interacts with the affinity peptides of the chromatographic gel due to the similarity of the possible sites on the CH-1 and CH-2 fragments of the molecule. We are sure that in the following experiments we will be able to get rid of even trace impurities of IgA in IgG_{1-4} by applying the elution of immunoglobulins with a falling pH gradient.

The quantitative ratio of the obtained $Ig_{1,2,3,4}$ subclasses, which was identical to their ratio in blood plasma, indicates that the 7-PEPs-WorkBeads gel developed earlier, has almost the same affinity properties in relation to all IgG subclasses [18].

The affinity peptide for ^{aRUB}IgG was developed on the base of interaction of amino acid sequence of RV capsid proteins which are possible the sites of RV interaction with target cell membranes or mitochondrial proteins or RV cell receptor MOG. After the testing the 8-mer peptide RDHHGTHE was selected as a ligand for peptide-affinity gel synthesis [18,31]. The ^{aRUB}IgG purified from the IgG_{1-4} pull included about 81% of total plasma ^{aRUB}IgG with specific activity ~800 $IU \times mg^{-1}$ [18].

In a similar way, affinity peptides to IgG against virus's rabies, measles, poliomyelitis and herpes were developed and tested as well as the synthesis of peptide-affinity gels FDLLKARLLY-WorkBeads (^{aRAB}IgG), YRQTTQDSFT-Work Beads 40/1000 gel (^{aMUM}IgG), CVLRKWLPPM-WorkBeads 40/1000 gel (^{aPOL}IgG) and QRCGAHSANF-WorkBeads 40/1000 gel (^{aVAR}IgG) [unpublished dates].

The content of varicella-zoster, mumps, rubella, polio and rabies viruses IgGs shown in the Table 2 were corresponded to dates of other experiments with minor deviations, which can be explained by the different level population immunity of the different countries to the corresponding viruses, the different level of immunization, etc [35-38].

It is also known that antibodies to Polio, Rabies, and Varicella zoster viruses circulate in plasma for a long time and at a noticeable level, which was confirmed by our studies [39-41]. For example, the content of anti-RAB IgM and IgG over the years can be 0.125 IU/ml and 0.313 IU/ml, respectively. But the authors stressed that the presence of anti-RAB IgM, determined in some individuals, was most likely provided by passive immunization. In our study, the content of anti-RAB IgG in donor blood plasma was almost 2 times higher, 0.620 versus 0.313 IU/ml, than found by Zajac et al. [40]. According to the indirect dates of Zajac et al. study took place in USA, in the country with highly developed health care system and scheduled vaccination of the population. In Ukraine, since the 2010s, the epizootic situation with rabies has significantly worsened. Active centers of the natural type exist in all regions, which is a prerequisite for the formation of urban-type rabies centers and increases the threat of the emergence and spread of this infection among the population. Thus, in Ukraine, about 110,000 people seek medical help for animal bites every year, and almost 20,000 of them receive anti-rabies vaccinations [42]. Thus, the high level of anti-RAB IgG determined by us in this study is caused not only by passive, but also by high active immunization of the population.

In fact, the affinity anti-viral peptides used in this and other our works were developed as an improved sense sequences of cellular protein sites or anti-sequence of viral sites that interact with each other in the way of invasion or intracellular activity of the virus. Considering the above mentioned, it can be forced that such peptides can later be used as antiviral medicines in their native form or as part of a small polypeptide, which will allow faster and massive introduction of the drug into the animal/human body compared to vaccination. After all, it is known that the goal of vaccination is throw for the cell "a model" and push it to synthesize an amino acid sequence packed in the variable part of the antibody against throwed "model" [43]. But only 1) confirmation of the antiviral activity of the peptide in special experiments in vitro (cell culture) 2) the same experiments in vivo on animals will make it possible to expect a positive result for the treatment of viral infections in humans.

Affinity peptides, designed for chromatographic purification other plasma proteins, also revealed favorable properties for specific capture of Fg, Cpm, ProThr and A1AT family as for high types of buffer flow, as well as high virus inactivation temperature.

The very satisfactory purity (>94%) of proteins and their high yield (>84%) were achieved not only due to the specific affinity of the synthesized gels, but also due to a significant reduction in the buffer volumes, which ensured a significant reduction in the time of oxidation, denaturation and hydrolytic degradation of proteins, that is, the cascade chromatography method used.

Based on the above, we believe that we managed to answer the four main questions of cascade chromatography in this work:

- In general, is it possible to achieve such high separation specificity to isolate proteins with sufficiently similar chemical and physical properties [44];
- Isolate high abundant proteins at the very beginning of the process or, if it does not create difficulties for the interaction of low abundant proteins with a chromatographic matrix [11,12];
- How many peptide-affinity chromatographic columns can be included in the cascade [11,12];
- Which column have to be first: with more target protein loading or less to avoid significant back pressure buildup [11,12].

Q1. The answer to the first question had to wait a long time. The pioneering work of Edelman and Gall was published more than 53 years ago [44]. During the next 10 years Porath and Hansen, Porath were determined that this is indeed possible, but only thanks to affinity chromatography [11,45]. Another 12 years later, Peng, Yan, and Pappas showed that not only proteins, but also entire related cells can be separated due to their interaction with specific proteins or sites of such proteins, that is, amino acid sequences [46]. The last 15 years were marked by stormy works using short peptides (6-15 amino acids) as affinity ligands for chromatographic adsorbents. And today we can responsibly say "YES", the peptide-affinity chromatography creates the most favorable conditions for the separation of any proteins. You only have to determine the most specific sites in the protein and develop specific peptides for them, which generally represent their antisense amino acid sequences. We give an example of such affinity peptides for the separation of five anti-viral IgGs from the blood plasma of immunized donors. In the 150 kDa IgG molecule, a unique site has been identified and against which an antisense sequence has been developed, which makes it possible to separate molecules that are very similar in properties.

Q2. Again the answer is "YES". It is always necessary to carry out preparatory, "rough" work in order to get the expected result. At one time, the use of affinity adsorbents for the removal of albumin and immunoglobulins from plasma samples made it possible to obtain impressive 2D electrophoresis with the ability to identify more than a thousand proteins. In the presented work, two plasma fractions were previously separated, one of which contained massive immunoglobulins, and the other contained albumin. In the future, no more "rough" manipulations were needed.

Q3. The notorious back pressure! It did force the development of low-pressure liquid chromatography columns that can withstand a pressure of 5-10-15 bar. For a cascade from 4 or 5 columns with a total bed height 235-240 cm, the back pressure was max 1,5 bar due to new porous gels (in our case WorkBeads 40/1000 or 40/10000). Thus, the cascade could be supplemented with several more columns.

Q4. Our answer is "It doesn't matter". We specifically started the application of both IgGs and proteins fractions in such way that the most massive specific IgG or protein was adsorbed on the first column. It is clear that the question was a derivative of back pressure. But with peptide-affinity chromatography, non-specific adsorption is not observed, or almost not observed. Therefore, there is no "massive load", that is, clogging of the chromatographic gel and an increase the back pressure.

Conclusion

Thus, by applying the latest advances in the development of chromatographic gels with high dynamic capacity, which means a high flow rate of buffer with a fairly short residence time, we successfully solved all four questions that questioned the application of cascade chromatography [22]. This article as a several before presents highly selective peptides as ligands for the separation of groups and individual proteins. Peptide-affinity gels, along with new gel matrices, are the basis for the use of cascade chromatographic separation of human plasma proteins. This application is only an initial example for the penetration of cascade-mode multi-affinity column chromatography (CASMAC) into the industrial production of a variety of proteins from any biological source.

May be someone has doubts about why to use CASMAC together with virus-inactivation in a chromatographic column, which requires new rigid gels and new columns that can withstand back pressure up to 5-15 bar, when there are already standard operating methods and less expensive gels and columns. Without entering into a broad discussion, we can note in this work that the proposed cascade method of isolation, including in-column virus inactivation, provides the possibility of full automation of the chromatographic process and, thus, the exclusion of any influence of the human operator, that is, the factor of a person beyond the process of isolation of a pharmaceutical product. Isn't this what the administrative bodies that monitor compliance with production standards demand from the manufacturer?

Scientists and developers of pharmaceutical products can be directed to a thoughtful reading of the very "fresh" work of Heino and Breton [47]. Recently authors showed that converting the process from batch chromatography to multi-column chromatography has a great potential to reduce costs and shorten production times for the capture and purification of high-quality mAbs. Moreover, in the case of using a two-step mAb purification process, it was reproduced very closely to our chromatographic process of mAb purification - purification with in-column virus inactivation. With the difference that we captured immunoglobulins of classes 1-4 and specific immunoglobulins in several stages on successive columns (we assume that instead of purification by the cited authors). According to the results of the authors, a two-step process using the Octave BIO system can provide satisfactory overall mAb recovery (94.1% yield), effective viral clearance (≥ 3.23 log reduction of adventitious virus), and increased process productivity [47]. As a result, the examples presented in the paper strongly support the transformation of biologics purification towards multi-column chromatography instrumentation due to its beneficial effects on product quality, time savings, and reduction of production costs. Moreover, these methods can be easily scaled to industrial processes.

Acknowledgement

Authors express profound gratitude to the staff of the pilot plant of scientific and manufacturing firm Shijir International Co. Ltd, Sukhbaatar sq., Bodi Tower building, Ulaanbaatar, Mongolia, for the opportunity to intervene in the real manufacturing processes and to test the represented method on the pilot plant in the Raining (Boroo) Valley of Mongolia.

Statements and Declaration

Ethical Approval

Not applicable.
This article does not contain any studies with human participation or animal performed by any of the authors.

Consent to Participate

Not applicable.
This article does not contain any studies with human participation.

Credit Authorship Contribution Statement

Serhiy P. Havryliuk – written the first draft of the manuscript, methodology.
Heorhii L. Volkov – written first draft and original of the manuscript.

Olena S. Havryliuk – written revue and editing.

All authors contributed to the study conception and design, material preparation, dates collection and analysis, commented on previous versions of the manuscript
All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest. The study was performed at a time when authors were employees of Neutromics Ukraine TOV and working together at the biotechnology Pilot plant at Raining (Boroo) Valley, Mongolia. The authors have no relevant financial or non-financial interests to disclose.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

It was fully paid by Neutromics Ukraine TOV (Kyiv, Ukraine) at the own expense. The scientific dates and practical results obtained by authors belong to Neutromics Ukraine TOV and fixed by the agreement and the priority certificate for the granted patent of Ukraine.

The Neutromics Ukraine TOV has allowed publication in the open press with the transfer of rights on the article to the appropriate publisher.

The using of research and production facilities of the Pilot plant at the Raining (Boroo) Valley belongings to Shijir International Co. Ltd, Sukhbaatar sq., Bodi Tower building, Ulaanbaatar, Mongolia, by Neutromics Ukraine TOV is grounding on the non-finance cooperation between both parties. This agreement was signed due to the fact that as the project of Pilot plant same as all started technologies and plant staff education were designed and implemented by Neutromics Ukraine TOV according to the order by Shijir Int. Co. Ltd. The active cooperation of two companies will continue on the base that new methods and/or technologies developed by Neutromics Ukraine TOV firstly are offering for Shijir Int. Co. for buying and start-upping on the own decision.

The authors declare that nobody of them had not receive any financial support (funds, grants or other support) from third parties or private/public/foreign investors, and that they had no financial obligations to the above-mentioned organizations or investors during the preparation of this manuscript.

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process
During the preparation of this work the authors did not use ani AI and AI-assisted technologies in order tin the writing process.

Availability of Data and Materials

Not applicable.

Consent for Publication

Funding Information: This study was funded by Neutromics Ukraine TOV (Kyiv, Ukraine) at the own expense. The scientific dates and practical results obtained by authors are belonging to Neutromics Ukraine TOV and fixed by the agreement and the priority certificate for the granted patent of Ukraine. The company has allowed publication in the open press with the transfer of rights on the article to the appropriate publisher.

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Conflict of Interest Statement Authors declare no conflict of interest. The study was performed at a time when authors were employers of Neutromics Ukraine TOV and working together at the biotechnology pilot plant in Raining (Boroo) Valley, Mongolia.

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