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Novel sulfamethazine ligand used for one-step purification of immunoglobulin G from human plasma

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Abstract

To replace conventional affinity ligand like protein A or protein G, a pseudobioaffinity ligand seems to be an alternative for the purification of immunoglobulin G (IgG). In this study, sulfamethazine (SMZ) was chosen as novel affinity ligand for investigating its affinity to human IgG. Monodisperse, non-porous, cross-linked poly (glycidyl methacrylate) (PGMA) beads were employed as the support for high-performance affinity chromatography. SMZ was immobilized on PGMA beads using bisoxirane (ethanediol diglycigyl ether) as spacer. The resultant affinity media presented minimal non-specific interaction with other proteins. Results of high-performance frontal analysis indicated that the media showed specific affinity to human IgG with a dissociation constant on the order of 10^{-6} *M*. The SMZ affinity column proved useful for a very convenient one-step purification of IgG from human plasma. Antibody purity after a one-step purification was higher than 90%, as determined by densitometric scanninng of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified fraction under reducing condition. The results obtained indicate that SMZ is a valuable affinity ligand for purification of human IgG.

Keywords: Immunoglobulins; Sulfamethazine

1. Introduction

Human immunoglobulin G (IgG) is an important plasma protein produced worldwide on a large scale. The purification of human IgG is essential, especially for immunotherapeutic applications [1,2]. Of the numerous purification methods, high-performance affinity chromatography (HPAC) is becoming increasingly important owing to its specificity, rapidity and high resolution [3,4]. The basis of HPAC is the use of an appropriate immobilized ligand [5,6]. The conventional ligands used to purify IgG from different sources are protein A and protein G ligands. They are very specific as indicated by a good binding, however, degradation by microorganisms, sensitivity to organic solvents and pH change and difficulty in purification make the column packings not only short lived but also costly [7,8]. Protein A and protein G ligands are used mostly for analytical-scale in vitro/diagnostics use but not for large-scale production of therapeutic IgG from human plasma [1].

As most biospecific ligands are expensive or unstable in chromatographic systems, they are often replaced by pseudo-biospecific ligands, such as chelated metal ions [9,10], amino acids [11,12] and

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pharmaceuticals [13,14], which normally are simpler molecules. The pharmaceuticals have their corresponding receptors on human cell membranes, and the pharmaceutical receptor interactions are specific. Besides, before being released for clinical use, these compounds had already been studied in terms of both their physicochemical properties and their biological effects and toxicity. Thus, pharmaceuticals could well be rich sources of new ligands for purification of some important proteins. Affinity column packings made of such ligands should be of long life, low cost and free of toxicity. For example, sulfamethoxazolum (SMO) has been successfully used as a pseudo-biospecific ligand for the purification of trypsin [15].

In our previous paper, a QCM biosensor was used for a rapid screening analysis of the interaction between different proteins and the immobilized sulfamethazine (SMZ) ligand. The experimental results indicated that the SMZ-immobilized sensor showed specific interaction only with IgG [16]. In this paper, SMZ, a kind of antimicrobial pharmaceutical, was chosen as a novel pseudo-biospecific ligand for investigating its affinity to IgG. The affinity chromatography behavior of SMZ-linked poly(glycidyl methacrylate) (PGMA) beads for human IgG were evaluated. The application of the prepared media as high-performance affinity media to isolate IgG from human plasma is also described.

2. Experimental

2.1. Materials and equipment

The monodisperse non-porous PGMA beads were made in the laboratory [17]. Human IgG, goat IgG and mouse IgG in lyophilized form (electrophoresis purity) were obtained from Beijing Xin Jing Ke Biotechnology (Beijing, China). SMZ was from Beijing Medicine (Beijing, China). Other biochemical reagents were purchased from Sigma (St. Louis, MO, USA). Chemical reagents were of analytical grade and were purchased from Beijing Chemical Factory (Beijing, China) or Sigma. Water used to prepare aqueous buffers was triple distilled. All buffers and solutions were filtered through 0.22 μm filters before use. The loading buffer was 0.05 mol/l phosphate-buffered saline (PBS), pH 5.5. The elution buffer was 0.05 mol/l PBS, 0.25 mol/l NaCl, pH 5.5. The buffers were used in a step-wise mode. Buffers were stored at 4 °C.

The HPLC apparatus consists of a Model 345 ternary liquid chromatograph (Beckman Instruments, USA) with a Spectroflow 757 UV–Vis detector. A WDL-95 HPLC Workstation (Dalian Institute of Chemical Physics, Dalian, China) was used to process the data.

2.2. Preparation of SMZ immobilized affinity packing and blank packing

The preparation procedure for PGMA beads was described in a previous work [17]. The immobilization of SMZ on PGMA beads consists of activation of PGMA, ligand coupling and blocking residual active sites. The immobilization procedure is described as follows:

- PGMA (1.5 g) was suspended in a mixture solution consisting of 5 ml of 0.5 mol/l NaOH, 5 ml of ethanediol diglycigyl ether containing 10 mg of sodium borohydride. The reactions were performed at 30 °C for 8 h and stopped by washing the PGMA beads with large volume of water in a sintered glass funnel and vacuum-dried at room temperature [18].
- SMZ (0.5 g) was dissolved in 10 ml of PBS (50 m*M*, pH 10) and reacted with epoxy-activated PGMA at 50 °C for 36 h. Thus, SMZ was coupled to the PGMA through its amino group under alkaline conditions.
- Finally, 10 ml of Tris–HCl (pH 8.5, 1 mol/l) was used as a blocking agent for the prevention of nonspecific binding and applied to block the residual reacting sites for 3 h.

Blank packing was also prepared according to the immobilization procedure except the ligand-coupling step. The immobilization procedures are illustrated in Fig. 1.

The above two prepared packings were, respectively, packed into stainless steel columns (70×4.0 mm I.D.) under 16 MPa pressure.

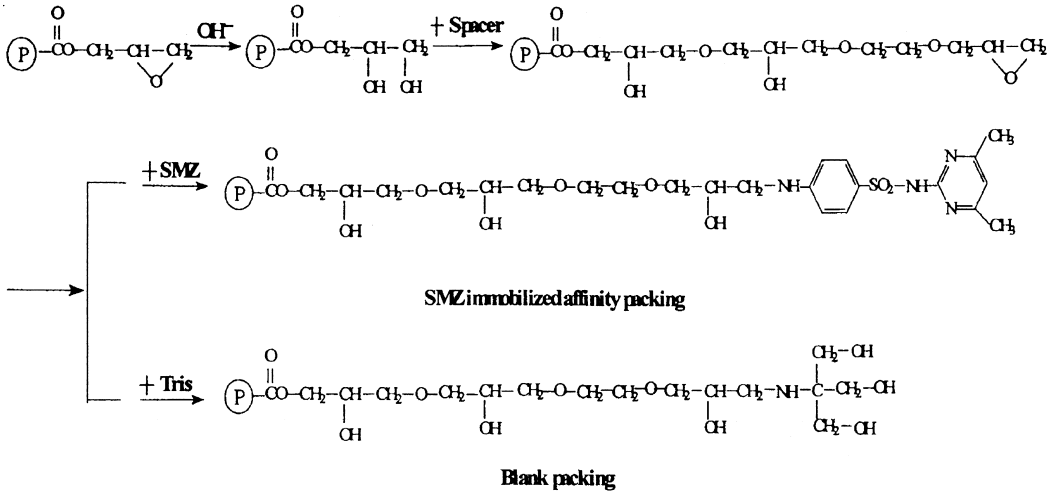

Fig. 1. The procedure for preparing SMZ immobilized affinity packing and blank packing.

2.3. Quantitative determination of SMZ immobilized on PGMA

The amount of SMZ immobilized on PGMA beads was determined as described by Wu and Liu [15]. A suitable amount of SMZ immobilized PGMA was well suspended in a mixture of water–ethanol–*n*-butanol (20:30:50) by ultrasonication. The absorbance of the solution at 254 nm was measured against hydrolyzed epoxy-activated PGMA as the reference. The standard curve can be made by quantitative additions of SMZ to the hydrolyzed PGMA and the absorbance measured under the same conditions.

2.4. Chromatographic conditions

All chromatographic experiments were performed at room temperature. Protein solutions were prepared in 1 mg/ml with the loading buffer (0.05 mol/l PBS, pH 5.5). All separations without the special description were carried out through the column by passing the loading solution for 10 min, the elution solution for 10 min, and then loading solution within 10 min at a flow-rate of 0.5 ml/min, after injection of the protein solution (50 μl). The chromatographic profiles were monitored at 280 nm. Fractions were collected if further measurements or experiments were necessary.

2.5. Separation of IgG from human plasma with SMZ-PGMA columns

Normal human plasma donated by healthy volunteers was a supply of Perking University Third Hospital, diluted 1:1 (v/v) with loading solution (0.05 PBS, pH 5.5) and filtered through a 0.45 μm filter. Then 50 μl of the treated plasma was injected into the test column. Unbound proteins were washed from the column with the loading buffer. The bound IgG was eluted out with 0.05 mol/l PBS buffer (pH 5.5) containing 1.0 mol/l NaCl. After elution was complete, the column was washed with the loading buffer to restore it to its initial baseline for the next experiment.

2.6. Characterization of purified IgG

IgG purity characterization was carried out on a Bio-Rad electrophoresis system (Richmond, CA, USA) with Mini-PROTEIN II electrophoresis cell (gel size: 7×8 cm) and PAC 300 power. The chromatographic fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under denaturing conditions on 12% gels using Blue Coomassie R-250 to stain the separated bands and protein markers from 97.4 to 14.4 kg/mol (Amresco, Solon, OH, USA). The following proteins were used as markers: phosphor-

ylase b, M_r 94 000; albumin, M_r 67 000; ovalbumin, M_r 43 000; carbonic anhydrase, M_r 30 000; trypsin inhibitor, M_r 20 100; and α-lactabumin, M_r 14 400.

The gels were scanned using a Shimadzu dual-wavelength flying spot scanning densitometer (Shimadzu, Tokyo, Japan) for quantification of protein bands.

3. Results and discussion

3.1. Immobilization of SMZ on PGMA beads

PGMA beads are cross-linked copolymers of glycidyl methacrylate and ethylene dimethacrylate. The beads are monodisperse hydrophilic material bearing free epoxy groups on their surface. The average size of the beads in diameter is 11 μm. Fig. 1 shows the immobilization of SMZ on PGMA beads via an arm of ethanediol diglycigyl ether. This bisoxirane could provide not only active epoxide groups for the coupling of ligand, but also an appropriate spacer. This long spacer or arm was of great importance in biomolecular interactions when the ligand was small and the affinity constant low [18].

3.2. Optimization of experimental conditions

The influence of pH on the interaction of human IgG with immobilized SMZ was investigated and presented in Fig. 2. Maximal adsorption was achieved at pH 5.5. The optimum pH was chosen as 5.5.

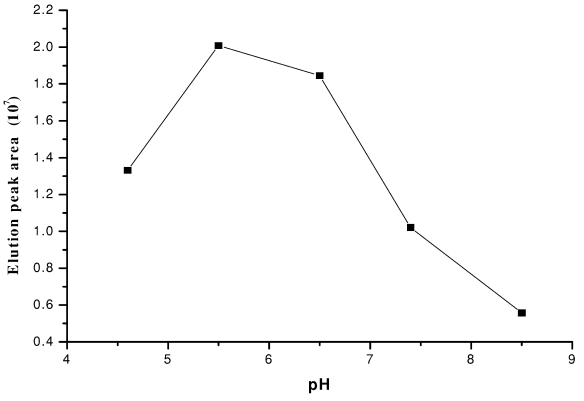

Fig. 2. Influence of pH on human IgG retention on SMZ immobilized affinity column.

The flow-rate also exhibited significant influence on the effective adsorption of human IgG. The optimum flow-rate was chosen as 0.5 ml/min, under which the maximal adsorption could be achieved.

Under the chosen conditions, the affinity column packed with SMZ immobilized PGMA beads showed specific adsorption for human IgG, while there was no retainment of IgG on the blank column at the same condition (shown in Fig. 3). Capacity determination was carried out by overloading the columns with samples containing 5 mg of human IgG and measuring the amount of adsorbed immunoglobulin after elution with 0.05 mol/l PBS buffer (pH 5.5) containing 0.25 mol/l NaCl. The quantity of IgG in the eluent was determined spectrophotometrically at 280 nm by the standard curve method. Binding capacity and protein recovery of the affinity column were determined and shown in Table 1.

3.3. Non-specific binding and selectivity of immobilized SMZ for IgG from different species

In order to study the specificity of the interaction of immobilized SMZ with human IgG, some proteins such as human and bovine serum albumin (HSA and BSA), protein A, concanavalin A (Con A), trypsin and antithrombin III (AT-III) were injected into SMZ immobilized affinity column and eluted under the elution conditions studied (shown in Fig. 4). The results have indicated that these proteins have not been retained on the column and the column has exhibited no any non-specific bindings for these proteins. The low non-specific binding of the SMZ column will be very useful in purification of IgG with high purity.

The binding of IgG from three different species to immobilized SMZ was also assessed (shown in Fig. 5). The marked species-dependent difference was observed and the affinity to immobilized SMZ lay in the sequence human IgG>goat IgG>mouse IgG.

3.4. Determination of the affinity constant between human IgG and immobilized SMZ

In order to understand quantitatively the interaction of SMZ matrix and human IgG, an ex-

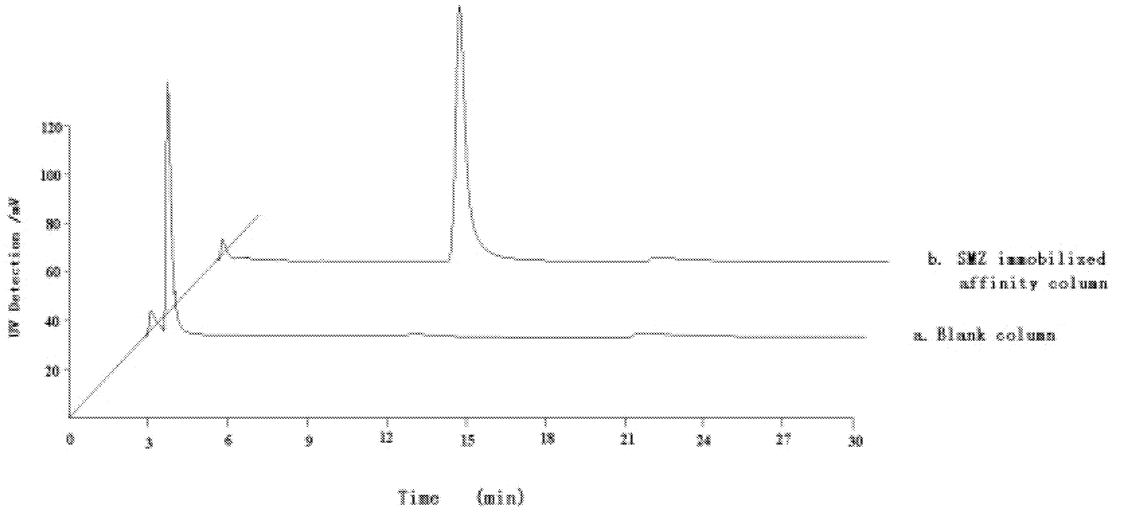

Fig. 3. Chromatographic behaviors of human IgG on SMZ immobilized affinity column and blank column. Chromatographic conditions: human IgG injected, 50 μg; flow-rate, 0.5 ml/min; detection, UV at 280 nm, 0.1 aufs; buffer A, 0.05 mol/l PBS, pH 5.5; buffer B, A + 0.25 mol/l NaCl, pH 5.5; eluted condition: 0–10 min buffer A, 10–20 min buffer B, 20–30 min buffer A.

Table 1
Binding capacity and protein recovery of SMZ-PGMA for IgG

Affinity adsorbent	SMZ bound on PGMA (mg/g beads)	Binding capacity (mg/g beads)	Protein recovery (%) IgG
SMZ-IgG	0.33	1.95	99.2

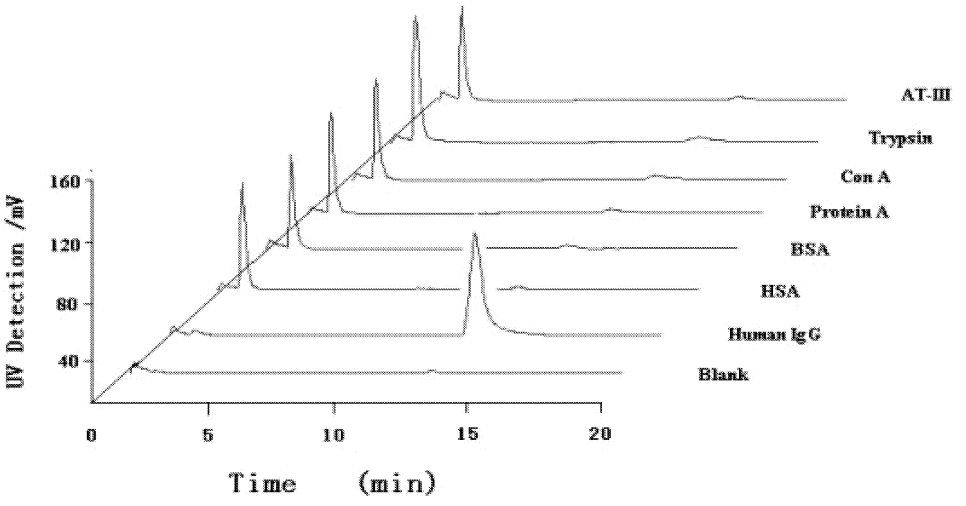

Fig. 4. Comparison of interaction of SMZ immobilized affinity column with various proteins (protein concentration: 1.0 mg/ml). Chromatographic conditions as in Fig. 3.

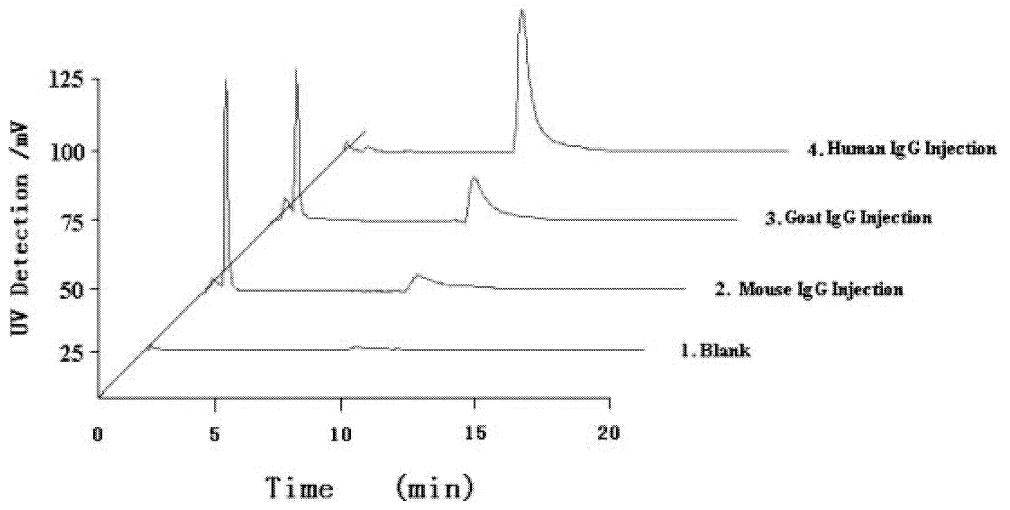

Fig. 5. Comparison of interaction of SMZ immobilized affinity column with IgG from different species (IgG concentration: 1.0 mg/ml). Chromatographic conditions as in Fig. 3.

perimental approach using analytical HPAC could be used [19–21]. Frontal analysis was performed by continuously applying various concentrations of human IgG to the SMZ immobilized affinity column at a flow-rate of 0.25 ml/min. Solutions of different concentrations, $[P]_0$, of mobile human IgG were passed through the column and monitored by UV absorbance (λ=280 nm) until a plateau of maximum absorbance was observed (shown in Fig. 6). At this time, saturation was achieved and the eluate had the same concentration of mobile IgG as the initial applied solution. The variation of elution volume $\overline{V}$ was plotted according to the equation [22]:

$$\frac{1}{\overline{V}-V_0}=\frac{K_d}{M_T}+\frac{1}{M_T}[P]_0$$

where $\overline{V}$ is elution volume at which the affinity matrix is half-saturated (ml), V_0 is void volume of the column (ml), K_d is the dissociation constant of complex of the immobilized SMZ and human IgG (mol/l), M_T is the total amount of immobilized SMZ (mol/g beads), and $[P]_0$ is the initial concentration of mobile human IgG (mol/l). From a plot of $1/(\overline{V}-V_0)$ vs. $[P]_0$ (shown in the inset of Fig. 6), K_d can be determined as a ratio: K_d = intercept/slope = $3.49\cdot 10^{-6}$ mol/l.

The correlation coefficient (r) was equal to 0.997. Apparently, human IgG showed higher affinity to immobilized SMZ in view of the lower K_d value. However K_d is within the range of 10^{-4} to 10^{-8} mol/l, which is suitable for affinity ligands [23].

3.5. Separation IgG from human plasma

A rapid and efficient isolation of IgG from human plasma was achieved with SMZ immobilized affinity column and the profile of human plasma proteins eluted from the column is shown in Fig. 7. The central fraction of the elution peak was collected and concentrated, and then analyzed by SDS–PAGE. The electropherogram in Fig. 8. has shown that IgG (M_r 150 000) has always appeared two major bands on M_r 55 000 and 24 000 due to dithiothreitol (DTT) cleaving IgG into two identical heavy chains (55 000) and light chains (24 000) [8]. It was showed that eluted fractions have high purity by SDS–PAGE analysis since no traces of other contaminant proteins were detected. Densitometric scanning of the gel lane containing the purified fraction indicated that these two bands accounted for 91.7% of the total protein. From the results it can be concluded that non-specific binding of proteins in human plasma on the SMZ immobilized affinity column can be negligible during the process of affinity separation in our experimental conditions.

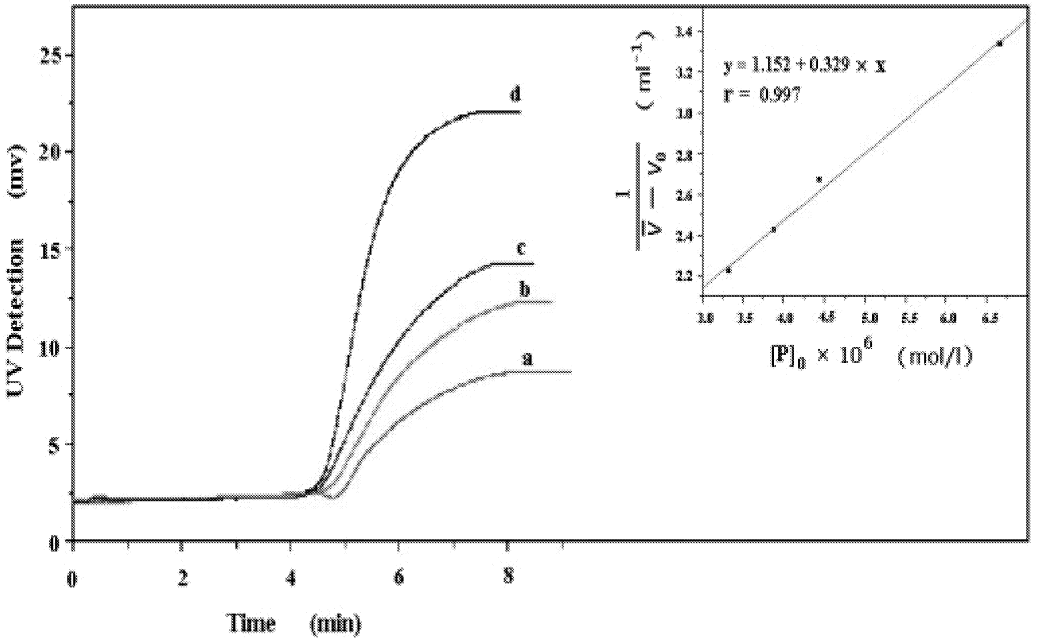

Fig. 6. High-performance frontal analysis experiment performed with IgG on the SMZ immobilized affinity column. The varying concentrations of human IgG were as follows: (a) $3.33 \cdot 10^{-6}$ mol/l; (b) $3.89 \cdot 10^{-6}$ mol/l; (c) $4.44 \cdot 10^{-6}$ mol/l; (d) $6.67 \cdot 10^{-6}$ mol/l. The flow-rate was 0.25 ml/min and the application buffer was 0.05 mol/l PBS (pH 5.5). The void time under same conditions was approximately 4 min. Inset: Plot of $1/(V - V_0)$ vs. $[P]_0$. The linear regression equation and correlation coefficient were shown in the inset.

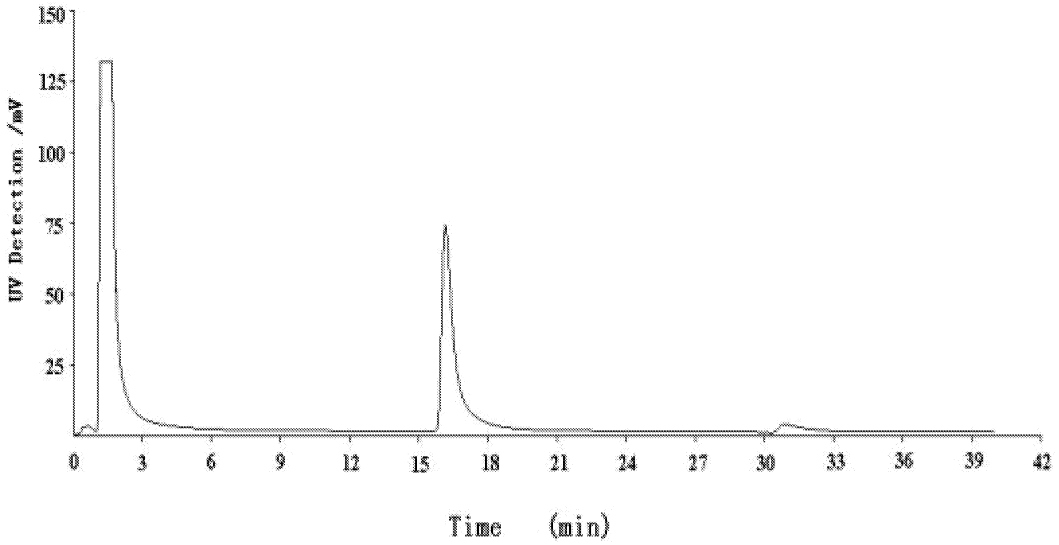

Fig. 7. Chromatogram of human plasma on SMZ immobilized affinity column. Chromatographic conditions: human plasma injected, 50 μl; flow-rate, 0.5 ml/min; buffer A, 0.05 mol/l PBS, pH 5.5; buffer B, A + 1.0 mol/l NaCl, pH 5.5; eluted conditions: 0–15 min buffer A, 15–30 min buffer B, 30–40 min buffer A. For other conditions, see Fig. 3.

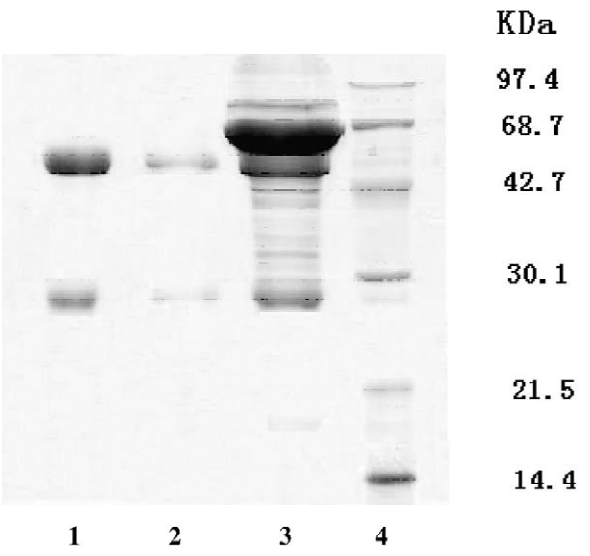

Fig. 8. SDS–PAGE analysis of fractions obtained from the purification of IgG from human plasma under reducing condition. Lanes: 1=standard human IgG; 2=IgG purified from the SMZ immobilized affinity column; 3=human plasma; 4=molecular mass markers.

3.6. Reproducibility of methodology

Reproducibility of the method was also investigated and the results are shown in Table 2. Relative standard deviations (RSDs) of elution peak areas were 1.28% ($n=6$) and 3.53% ($n=3$) for human IgG in standard solution and human plasma, respectively.

4. Conclusion

This study demonstrated that high-performance pseudo bioaffinity chromatography with immobilized SMZ as a ligand provided efficient ability for capture and purification of IgG directly from human plasma. It is an attractive alternative to conventional protein A or G affinity chromatography because of its very low cost as well the low molecular mass of SMZ made it free of any eventual denaturation. High-performance frontal analysis indicates that human IgG shows higher affinity to immobilized SMZ ligand ($K_d=3.49\cdot10^{-6}$ M). The use of SMZ as a novel affinity ligand will make IgG purification much more convenient and affordable, opening up new avenues in the study and application of this important class of immunoglobulins.

Table 2
Reproducibility of the methodology

Sample	Added	Elution peak area ($\cdot10^7$)	RSD (%)
Human IgG	50 μg	2.008, 2.022, 2.017, 1.998, 2.011, 1.987	1.28
Human plasma	50 μl	1.575, 1.524, 1.489	3.53

Acknowledgements

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