ELSEVIER

Journal of Chromatography A, 743 (1996) 171–180

JOURNAL OF CHROMATOGRAPHY A

# Influence of column type and chromatographic conditions on the ion-exchange chromatography of immunoglobulins

Yan-Bo Yang*, Kervin Harrison

*The Separations Group, Inc., 17434 Mojave Street, Hesperia, CA 92345, USA*

**Abstract**

Immunoglobulins are often purified by affinity chromatography. However, this technique is costly, can result in poor resolution for subclasses (or is only group specific), and leads to possible leaching of contaminants into the purified products. Ion-exchange chromatography has shown great potential and has found an increased usage in the purification of immunoglobulins. The aim of this study is to further understand the separation mechanism with emphasis on the influence of column type and chromatographic conditions on the peak shape, selectivity and changes in the elution patterns. Included are strong cation-exchange, strong anion-exchange and weak anion-exchange columns. Five immunoglobulin G antibodies were used as test probes. Some sera and ascites were also used in the study. Among the chromatographic conditions examined were mobile phase pH, buffer type, buffer concentration, gradient rate, and column temperature. Significant differences in the chromatographic behavior (elution pattern, peak shape and selectivity) of the test samples are discussed in regard to the column type and the chromatographic conditions.

*Keywords:* Mobile phase composition; Stationary phases, LC; Immunoglobulins; Antibodies; Proteins; Albumin

## 1. Introduction

Immunoglobulins (antibodies) are a group of biologically active proteins produced by plasma cells in response to the presence of foreign substances. They continuously attract the attention of scientists because of their important biological functions and their growing interest for diagnostic and therapeutic applications [1,2]. Studies of their properties and usage require highly purified antibodies. A good separation method is indispensable for homogeneous preparations of monoclonal as well as polyclonal antibodies. The existence of subclasses, the differences in glycosylations [3,4] and possible post-translational changes [5] impose a great challenge for their separations. In addition, it is vitally important to separate any antibodies that have changed (modified and denatured) during storage.

HPLC has provided a very useful means to purify antibodies. Affinity chromatography, size-exclusion chromatography (SEC), ion-exchange chromatography (IEX), hydrophobic interaction chromatography (HIC), hydroxyapatite chromatography and thiophilic interaction chromatography have been used [2,6–11] for the separation. Traditionally, immunoglobulins are most often purified by affinity chromatography. It has been found [6] that this technique is costly, can result in poor resolution for subclasses (or is only group specific), and leads to possible leaching of contaminants into the purified products. Ion exchange chromatography has shown [10,12] great potential and an increased usage for the

*Corresponding author.

0021-9673/96/$15.00 
*PII* S0021-9673(96)00255-5

purification of immunoglobulins. Almost all antibodies have isoelectric points near neutral pH [13]. This enables one to purify them using both cation- and anion-exchange columns. Due to their similarity and diversity, many influential factors need to be better understood in order to optimize the separation of antibodies.

The newly developed IEX columns [14,15] used in this study not only provide both chemical stability and high efficiency, but also require a lower salt concentration for elution. This enables the use of these columns for the study of chromatographic behavior of antibodies.

The aim of this study is to better understand the influence of column type and chromatographic conditions on peak shape, selectivity and changes in elution patterns. Included in the study are a strong cation-exchange (SCX), a strong anion-exchange (SAX) and a weak anion-exchange (WAX) column. Five immunoglobulin G (IgG) antibodies were used as test probes. Some antibody-containing sera and ascites were also used in the study. Significant differences in the chromatographic behavior (elution pattern, peak shape and selectivity) of the test samples were observed in regard to the column type and the chromatographic conditions.

## 2. Experimental

### 2.1. Apparatus

Chromatographic experiments were carried out with an assembled chromatographic system. The system consists of two ConstaMetric-III metering pumps (LDC/Milton Roy, Riviera Beach, FL, USA), a dynamic on-line solvent mixer of 2.4 ml volume (Beckman Instruments, Fullerton, CA, USA), a 7125 syringe-loading sample injector (Rheodyne, Cotati, CA USA), a SpectoMonitor D variable-wavelength UV detector (LDC/Milton Roy), a 486 IBM-compatible PC computer (Equus/Systems, Hesperia, CA, USA) with an OkiData printer (Oki America, Mount Laurel, NJ, USA). An EZChrom chromatography data system (Scientific Software, San Ramon, CA, USA) was used to control the chromatographic system and for data acquisition. A TSK-6097 column thermostat (NOVEX, San Diego, CA, USA) was used to control the column temperature.

### 2.2. Columns and chromatographic procedure

All chromatographic separations were performed using Vydac 400VHP575, 300VHP575 and 301VHP575 ion exchange columns (Separations Group, Hesperia, CA, USA), as described in Table 1. The column dimension of each of the three columns was 5 cm×0.75 cm I.D. The packing materials were highly crosslinked polystyrene–divinylbenzene (PS–DVB) beads with a modified hydrophilic surface and appropriate functional groups attached [14,15]. The mean particle size was 5 $\mu$m and mean pore diameter 900 Å. All chromatography was performed at ambient temperature, except when the specific temperatures are indicated. A flow rate of 1.0 ml/min was used throughout the study. The elution profiles were monitored with the UV detector at 280 nm.

### 2.3. Chemicals and reagents

Five purified IgGs from normal serum, purified monoclonal mouse IgG1, kappa (MOPC 21) and its clarified ascites, as well as sheep serum were purchased from Sigma (St. Louis, MO, USA). More detailed information of these antibody samples is listed in Table 2. Protein standards of carbonic anhydrase, conalbumin, ovalbumin and soybean trypsin inhibitor were also from Sigma. N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES, p$K_a$ 7.55), 2-(N-morpholino)ethanesulfonic acid (MES, p$K_a$ 6.15), 2-(cyclohexylamino)ethanesulfonic acid (CHES, p$K_a$ 9.5) were obtained from CalBiochem. The reagent grade tris-(hydroxymethyl)aminomethane (Tris, p$K_a$ 8.3) was from Sigma under the trade name of Trizma Base.

Table 1
Vydac columns used in the study

| Column name | Column type | Functional group |
|---|---|---|
| 400VHP575 | Strong cation exchange | Sulfonic |
| 300VHP575 | Strong anion exchange | Quarternized amine |
| 301VHP575 | Weak anion exchange | Tertiary amine |

Table 2
Antibody samples used in this study from Sigma

| Product No. | Description |
|---|---|
| I5131 | Sheep IgG, reagent grade |
| I4131 | Rat IgG, reagent grade |
| I5006 | Rabbit IgG, reagent grade |
| I4381 | Pig IgG, reagent grade |
| I5506 | Bovine IgG, reagent grade |
| M7894 | Clarified ascites of mouse IgG, Kappa (MOPC 21) |
| M9269 | Purified mouse IgG, Kappa (MOPC 21) |
| S3772 | Normal sheep serum |

Sodium chloride (NaCl) (99+%) was from Aldrich (Milwaukee, WI, USA). Hydrochloric acid (HCl) and "Baker Analyzed Reagent" grade triethylamine (TEA) were from J.T. Baker (Phillipsburg, NJ, USA). Deionized water was obtained in-house by treating the tap water with a carbon filter, reversed-osmosis, a mixed bed of ion exchangers, and a 0.45 $\mu$m filter. All proteins and chemicals were used as received.

## 3. Results and discussion

### *3.1. Influence of column type and the changes in mobile phase pH*

Because most IgGs have p*I* points in the range of 7 to 8 [13], ion-exchange chromatography of IgGs can be carried out on both anion-exchange (AX) and cation-exchange (CX) columns. It is believed that there are some differences between IgGs from different sources and that there exist heterogeneous components in the commercially available purified IgGs [3], particularly in the polyclonal antibodies. The chromatography of the five purified IgGs from normal serum was scouted first on the three different ion-exchange columns using the same buffered mobile phase. Fig. 1 shows the chromatograms of the five IgGs on a WAX, a SAX and a SCX column at pH 7.34 using Tris buffer as the mobile phase. A gradient of increasing salt (NaCl) concentration was used to elute the IgGs. At this near neutral pH, all IgG samples used had some portion retained on the

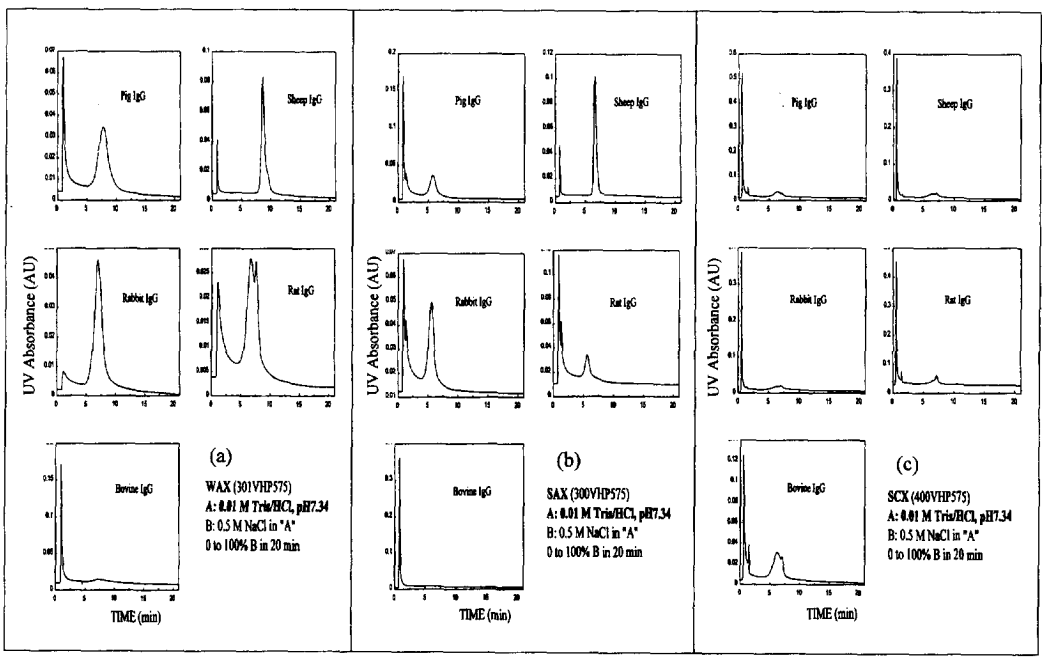


Fig. 1. Chromatograms of five IgGs on different IEX columns at pH 7.34. (a) WAX, (b) SAX and (c) SCX columns. For additional chromatographic conditions, see Section 2.

WAX and SAX columns except bovine IgG. The relative quantities retained depended on the specific IgG. Bovine IgG was eluted at the column void volume with a small amount retained. Sheep IgG had the longest retention time on both WAX and SAX columns. However, on the SCX column (Fig. 1c), only a very small portion of the IgG sample was retained for pig, sheep, rabbit and rat, and most was eluted at the column void volume. More bovine IgG was retained. The elution profiles were different for IgGs from different sources, even though all IgGs have similar structure [16] and properties. This implies that the purification of each different IgG needs a different separation scheme. Fig. 1 shows some similarities in the elution profile between rat, rabbit and pig IgGs. Differences were also observed between the WAX (Fig. 1a) and the SAX (Fig. 1b) columns. For instance, a small shoulder behind the major peak is visible for sheep IgG on the WAX column, but not on the SAX column. Rabbit and rat IgGs were retained more on the WAX column than on the SAX column. Rat IgG showed two retained peaks on the WAX column, but only one on the SAX column (but two peaks were eluted near the column void volume). It is unclear whether the difference in the retentivity and the peak height ratio are caused by the difference in the resolving power or in the adsorption kinetics of the columns.

A question raised from the data in Fig. 1 was whether the separation can be improved if the IgGs are retained longer in the column. The retentivity of IgGs was increased by adjusting the mobile phase pH away from neutrality. On the WAX and SAX columns the mobile phase was changed from pH 7.34 to 8. The mobile phase pH was reduced from 7.34 to 6 for the SCX column. The chromatographic results are shown in Fig. 2. On both WAX (Fig. 2a) and SAX (Fig. 2b) columns, all IgGs were more retained, but less separation was obtained compared to the result shown in Fig. 1. On the WAX column, almost nothing eluted at the column void volume for rabbit, rat, sheep and pig IgGs. On the SAX column, almost nothing eluted at the column void volume for rabbit and rat IgGs. The significant changes occurred on the SCX column (Fig. 3c). All five IgGs were fully retained with visible shoulders on the main peak. Noteworthy is the sheep IgG which exhibited the narrowest peak on both WAX and SAX columns

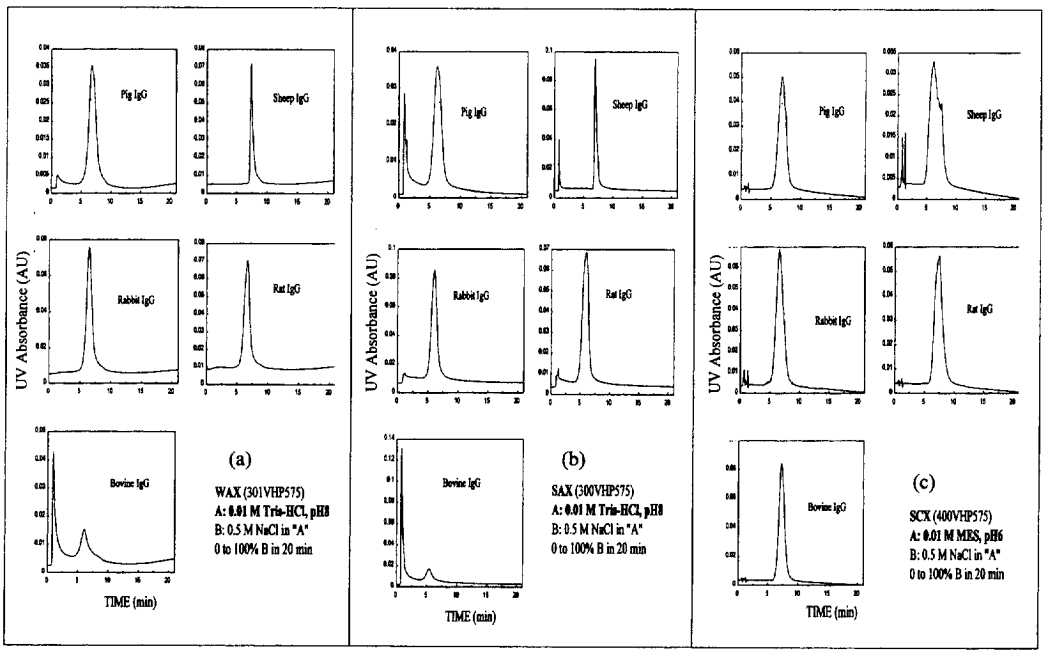


Fig. 2. Chromatograms of five IgGs on different type of IEX columns at different pH values. (a) 301VHP575 (WAX), pH 8.0; (b) 300VHP575 (SAX), pH 8.0 and (c) 400VHP575 (SCX), pH 6.0. For additional chromatographic conditions, see Section 2.

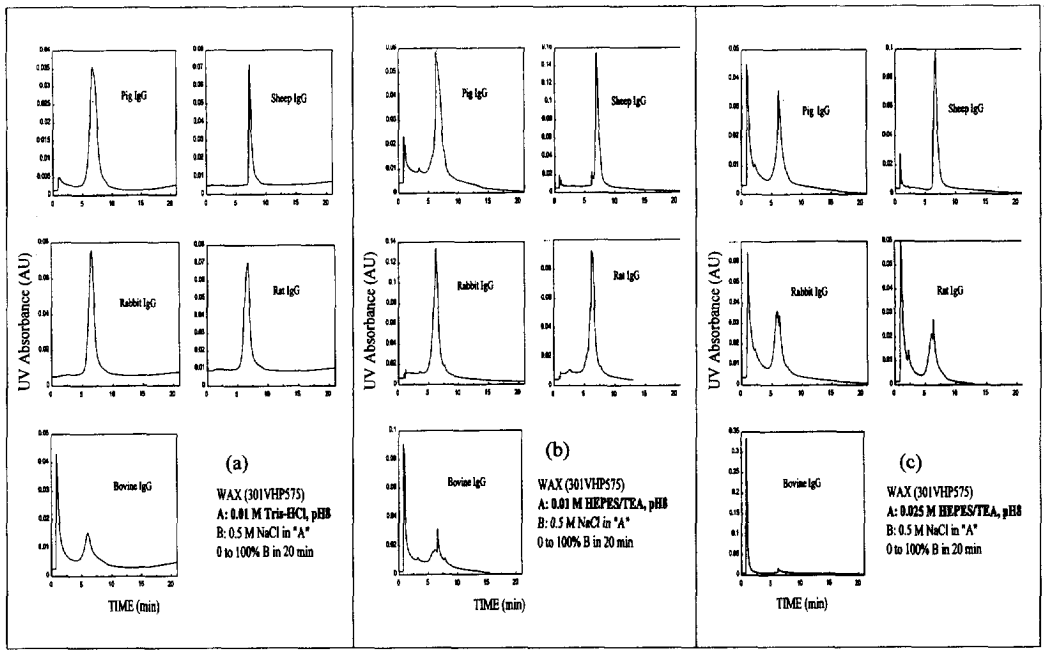


Fig. 3. Influence of buffer type and buffer concentration of the chromatography of IgGs on WAX column. (a) Tris buffer, (b) HEPES buffer and (c) HEPES buffer of higher concentration. For additional chromatographic conditions, see Section 2.

but gave the broadest elution profile on the SCX column with multiple peaks. One interesting point is that a narrower peak may not be better than a broader peak when trying to resolve multiple components which have closely related properties, such as IgGs (which contains both heterogeneity and subclasses). A broader peak may not always be an indication of a bad column, but simply implies that the resolution is improved, but not enough to fully resolve all the components. The results confirm that IgGs can be chromatographed on both anion and cation exchange columns. The selection of the column depends on the specific antibody and need. SCX and AX columns may provide complementary selectivity (e.g., sheep and bovine IgGs).

### 3.2. Effects of buffer type and buffer concentration

HEPES and Tris buffer were used to compare the influence of the buffer type on the chromatography of IgGs. The comparison was done at the same pH (pH 8) using the same buffer concentration and other chromatographic conditions. Fig. 3a and Fig. 3b show the chromatograms of the five IgGs obtained using the two different buffers. More peaks and shoulders were clearly visible for most of the IgGs using the HEPES buffer (Fig. 3b). The most significant change from Fig. 3a to Fig. 3b occurred on the bovine IgG. Peaks of bovine IgG were very sharp and more peaks were visible when the HEPES buffer was used (Fig. 3b).

To explore the buffer concentration effect on the separation, the HEPES buffer concentration was increased from 0.01 to 0.025 *M*. The column was fully equilibrated with this mobile phase before the chromatography runs. Comparing the chromatograms in Fig. 3b and Fig. 3c, we can see that the separation of rabbit and rat IgGs was improved when the initial buffer concentration was increased 2.5 times. However, it appeared that fewer peaks and shoulders of pig, sheep and bovine IgGs were resolved with the increased buffer concentration.

### 3.3. Separation of IgGs at high pH on both strong and weak anion-exchange columns

Attempts were made to improve the separation of possible multiple components within the commer-

cially available purified IgGs. The mobile phase pH was adjusted above 8 to enhance the total net charges on the IgG surface with the intention of obtaining better separation on the AX columns. Fig. 4 shows the chromatograms of the 5 IgGs on both WAX (Fig. 4a) and SAX (Fig. 4b) columns. The 5 IgGs were well retained on both columns with a single eluted peak. However, the separation did not improve compared to the results at pH 7.34 and 8. In addition, shorter retention time was obtained for all five IgGs on the WAX column at this higher pH. This is reasonable considering that the degree of ionization of tertiary amine functional groups on the surface of the stationary phase can be partially suppressed at pH 9.53, even though the IgGs become more charged. For the SAX column, the charge on the quarternized amine functional groups at the stationary phase surface should not be affected at pH 9.53. Fig. 4b shows a slight increase in the retention of the IgGs on the SAX column. It was noticed that no improvements in the separation were made at this elevated pH even on the SAX column. This result is in an agreement with the other results the authors obtained on the separation of hemoglobins [17]. In order to better understand this chromatographic result, four well resolved protein standards (carbonic anhydrase, conalbumin, ovalbumin and soybean trypsin inhibitor) were also chosen to check this pH influence on the SAX column. As illustrated in Fig. 5, an increase in retention time with the increase in mobile phase pH occurred on all the protein standards. However, the degree of the retention time increase differed significantly among the proteins used. The result indicates that the retention of proteins with higher p*I* values increased more than that of proteins with lower p*I* values. Consequently, the proteins in this mixture moved close together at pH 9.53 with decreased resolution. Further examination was done by plotting the retention time of the five IgGs at different pHs, as shown in Fig. 6. The slope of the curve represents the resolution between the IgGs, the greater the slope the better the resolution. On the WAX column, as shown in Fig. 6a, retention time and resolution decreased with increase in mobile phase pH from 8 to 9.53. On the SAX, as shown in Fig. 6b, resolution also decreases, although retention time increases, with increase in mobile phase pH. These results indicate that the optimum

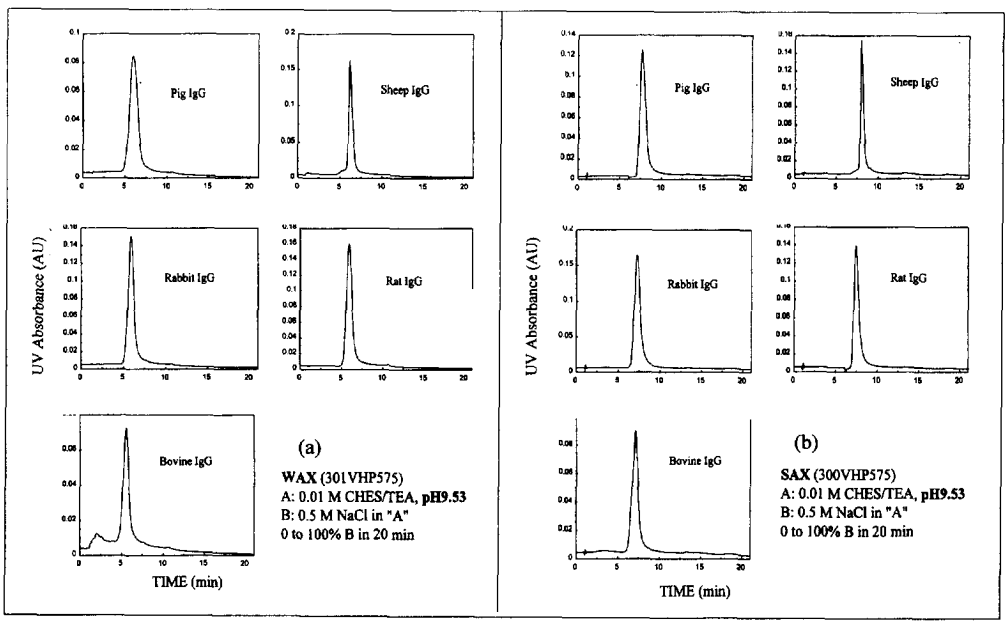


Fig. 4. Chromatograms of five IgGs on both (a) WAX and (b) SAX columns at pH 9.53. For additional chromatographic conditions, see Section 2.

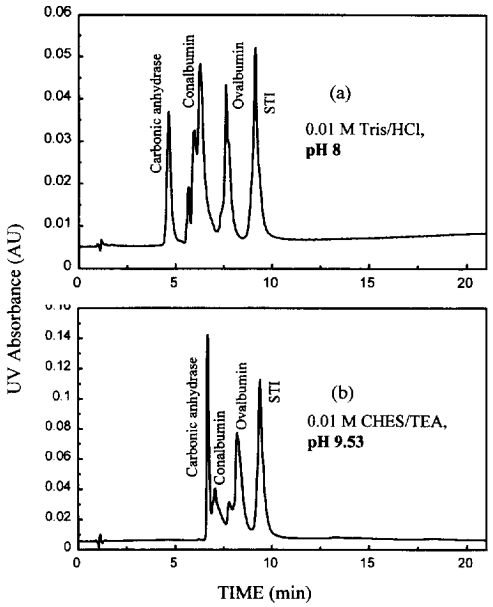


Fig. 5. Separation of four protein standards on the SAX column at (a) pH 8 and (b) pH 9.53. Column: 300VHP575; mobile phase: A, 0.01 *M* Tris–HCl pH 8; B, 0.5 *M* NaCl in "A". Flow-rate: 1.0 ml/min; gradient: 0 to 100% B in 20 min. Samples: bovine carbonic anhydrase (p*I* 7.3), conalbumin (p*I* 6, 6.3, 6.6), ovalbumin (p*I* 4.7) and soybean trypsin inhibitor (STI) (p*I* 4.5).

mobile phase pH for best resolution appears to be around pH 8 and pH 7 for WAX and SAX column, respectively. However, we probably can not generalize this phenomenon for every case, because the specific properties of each protein determines its chromatographic behavior. Even with similar p*I* values, proteins can differ in their chargeable functional groups in respect to the total number of charges, the type of charges, the relative number of positive and negative charges and their distribution within or on the surface. These differences can all affect the retention properties of each individual protein.

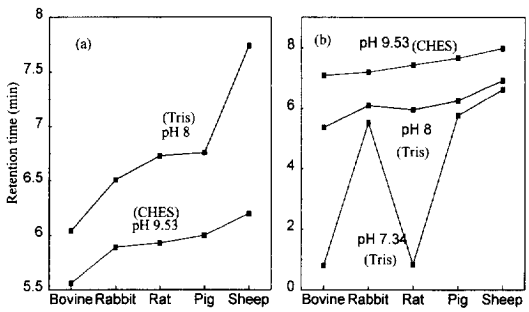


Fig. 6. Retention time versus the types of IgGs at different pH values on (a) WAX and (b) SAX columns. Columns: (a) 301VHP575 (WAX) and (b) 300VHP575 (SAX); Mobile phase: A, 0.01 *M* Tris–HCl, Tris–HCl and CHES–TEA for pH 7.34, 8 and 9.53 respectively; B, 0.5 *M* NaCl in "A". Gradient: 0 to 100% B in 20 min.

### *3.4. Column temperature influence on the separation*

Column temperature control is often used not only for keeping a constant column temperature for a better run-to-run reproducibility, but also for optimizing a separation. A temperature range of 7.5 to 60°C was examined for the separation of rat IgG, as shown in Fig. 7. Several observations can be made from this experiment. First, the resolution of three major peaks was improved with more distinguishable shoulders when the column temperature increased from 7.5 to 59.3°C. Secondly, the retention time increased with the increase in column temperature. This indicates some possible conformation changes of the IgG molecules in the temperature range tested, otherwise the retention should decrease with the increase in temperature [18]. Finally, the peak heights decreased with the increase in the column temperature. The second peak decreased faster than the third peak, resulting in a change of the peak height ratio between the second and the third peaks. This peak height decrease accelerated at higher temperatures. One explanation is that the broader peak elution profile, due to the better resolution at higher temperatures, contributed to the decrease in peak height. Two other possibilities are (1) that the conformation change may cause spectroscopic property change at 280 nm, and (2) that the possible denaturation affects the mass recovery of the IgG. It is noteworthy that these conformational changes started at a temperature as low as 7.5°C in a short elution time of around 10 min. This information and technique may provide a valuable means to monitor the stability of IgGs at different conditions for short term and long term storage.

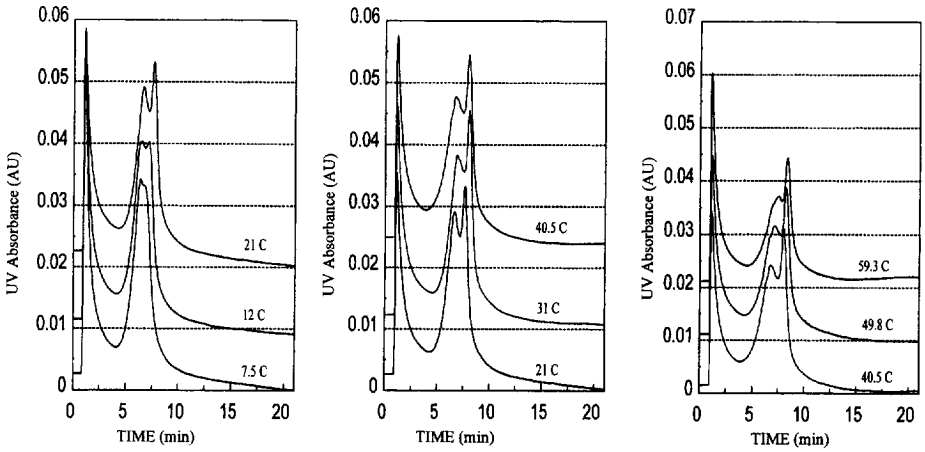


Fig. 7. Influence of column temperature on the separation of IgGs. Column: 301VHP575 (WAX); mobile phase: A, 0.01 *M* Tris–HCl, pH 7.34; B, 0.5 *M* NaCl in "A"; gradient: 0 to 100% B in 20 min.

### *3.5. Influence of gradient slope*

It has been known [19] that the gradient steepness affects the resolution of the separation. The less steep the gradient, the better the resolution. The influence of the gradient rate on the separation of IgG was examined using rat IgG as the probe, as illustrated in Fig. 8. The four gradients tested were (a) 0 to 100% B, (b) 0 to 40% B, (c) 0 to 20% B and (d) 0 to 10% B in 20, 40, 40 and 80 min, respectively. They were equivalent to the gradient changes of 5% B/min (or 25 m*M*/ml), 1% B/min (or 5 m*M*/ml), 0.5% B/min (or 2.5 m*M*/ml) and 0.125% B/min (or 0.625 m*M*/ml). By decreasing the gradient slope 10 times from 25 m*M*/ml (Fig. 8a) to 2.5 m*M*/ml (Fig. 8c), the resolution improved only slightly, as indicated by the appearance of two small shoulders before and after the major peak. The separation improved more significantly when the gradient slope decreased to 0.625 m*M*/ml (Fig. 8d). A barely observable shoulder at the top of the major peak in Fig. 8c was separated out as a visible peak (Fig. 8d). The small shoulder behind the major peak in Fig. a, b and c was separated (Fig. 8d) from the major peak (only half of the peak was recorded in the chromatogram due to the limit of date acquisition time set up). These results indicate that shallowing the gradient can affect the IgG separation, and that

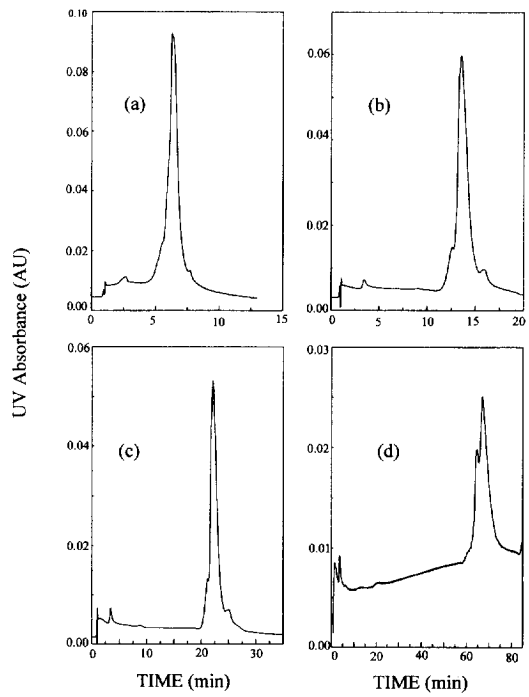


Fig. 8. Influence of gradient slope on the separation. Column: 301VHP575; mobile phase: A, 0.01 *M* HEPES–TEA pH 8; B, 0.5 *M* NaCl in "A". Flow-rate: 1.0 ml/min; gradient: (a) 0 to 100% B in 20 min, (b) 0 to 40% B in 40 min, (c) 0 to 20% B in 40 min, and (d) 0 to 10% B in 80 min; sample: rat IgG.

significant improvement occurs when the gradient slope is very shallow.

### 3.6. Example separations

#### 3.6.1. Purified mouse IgG1, κ and its ascites

The purified mouse IgG1, κ and mouse IgG1, κ ascites were chromatographed on the WAX column to examine the applicability of purifying the IgG from its ascites. It was first noticed that the IgG peak from the purified mouse IgG1, κ sample was broader than that from the ascites (data not shown). Further optimization of the separation resolved an early eluting peak (14.83 min) from the major peak (15.76 min) with purified mouse IgG1, κ sample, as shown in Fig. 9a. Under the same chromatographic conditions, a sharp narrow IgG peak (15.40 min) was obtained from ascites (Fig. 9b). The possible explanations are (a) the ascites of the purified IgG1, κ contained heterogeneous IgGs and the purification method the supplier used was not sufficient to resolve them, and (b) the purified IgG1, κ changed after it was purified (e.g.: due to instability). In either case, these separation conditions may provide a possible approach to monitoring heterogeneity and/or changes.

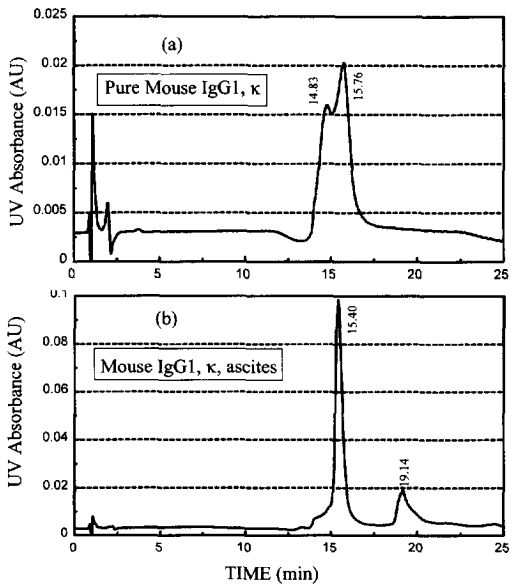


Fig. 9. Chromatograms of (a) purified mouse IgG1, κ and (b) mouse IgG1, κ ascites. Column: 301VHP575; mobile phase: A, 0.025 *M* HEPES–TEA, pH 8.0; B, 0.5 *M* NaCl in "A"; gradient: 0 to 20% B in 20 min.

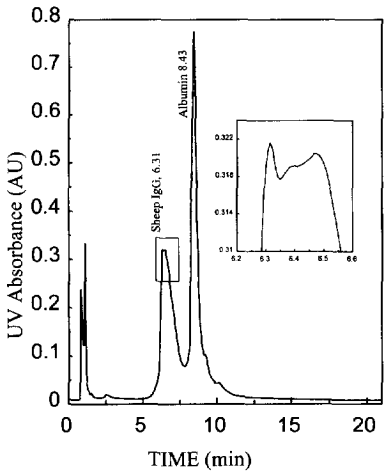


Fig. 10. Separation of sheep serum. Column: 301VHP575; mobile phase: A, 0.01 *M* HEPES–TEA, pH 8.0; B, 0.5 *M* NaCl in "A"; gradient: 0 to 100% B in 20 min.

#### 3.6.2. Sheep serum and bovine IgG

Fig. 10 shows a chromatogram of sheep serum on the WAX column. The insert shows multiple components in the IgG peak (6.31 min). Fig. 11 shows the separation of bovine IgG and bovine serum albumin. These chromatograms demonstrate that the albumin can be easily separated from the IgG. The separation can be used not only to remove the albumin from IgGs, but also to monitor the albumin content. For instance, when the albumin is used as an additive in the final formulation of IgG containing drugs, quantitation of albumin becomes necessary.

## 4. Conclusion

Chromatography of IgGs is strongly affected by the chromatographic conditions, e.g.: column type, mobile phase buffer type, mobile phase buffer concentration, temperature and gradient slope. Each of these factors influence the separation in a different manner. Mobile phase pH was a prominent factor influencing the separation. However, mobile phase

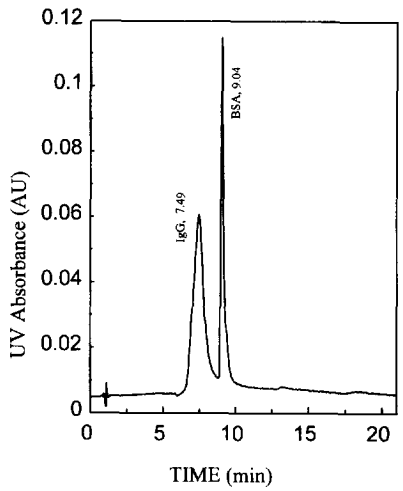


Fig. 11. Separation of bovine IgG and bovine serum albumin. Column: 300VHP575; mobile phase: A, 0.01 *M* CHES–TEA, pH 9.53; B, 0.5 *M* NaCl in "A"; gradient: 0 to 100% B in 20 min.

with pH higher than 8 did not improve the separation on either WAX or SAX columns. HEPES buffer gave better resolution than the Tris buffer. A slight increase in the initial buffer concentration (HEPES) enhanced the resolution for some IgGs, which seems more effective than shallowing the gradient. The increase in the column temperature increased the resolution and the retention, and affected the peak height ratio in a temperature range of 7.5 to 60°C. This may suggest that one can monitor the stability of IgGs at different temperatures. All three types of ion-exchange columns (WAX, 301VHP575; SAX, 300VHP575 and SCX, 400VHP575) can be used for the separation of IgGs depending on the individual IgG and specific needs. Further experiments are required to identify the different components (or peaks) in the chromatograms and gain more insights into the IgG samples.

## Acknowledgments

We wish to thank Carol MacHarg, Angel Burnell, William Campbell, John Kindsvater and Holly Keith for reviewing the manuscript with many helpful suggestions.

## References

[1] P.R. Hamann, L.M. Hinman and J. Upeslacls, presented at the Fifth International Conference on Monoclonal Antibody Immunoconjugates for Cancer, San Diego, CA, USA, March 15–17, 1990, Abstract number 99.
[2] W. Jiskoot, J.J.C.C. Van Hertrooij, A.-M.V. Hoven, J.W.T.M. Klein Gebbinck, T. Van der Velden-de Groot, A.J.A. Crommelin and E.C. Beuvery, J. Immunol. Methods, 138 (1991) 273.
[3] R.C. Grebenau, D.M. Golddenberg, C.-H. Chang, G.A. Koch, D.V. Gold, A. Kunz and H.J. Hansen, Mol. Immunol., 29 (1992) 751.
[4] R. Parekh, Am. Biotechnol. Lab., Nov. (1992) 8.
[5] C. Schoneich, A.F.R. Huhmer, S.R. Rabel, J.F. Stobaugh, S.D.S. Jois, C.K. Larive, T.J. Siahaan, T.C. Squier, D.J. Bigelow and T.D. Williams, Anal. Chem. 67 (1995) 155R.
[6] S.I. Sivakoff, in K.M. Gooding and F.E. Regnier (Editors), HPLC of Biological Macromolecules: Methods and Applications, Marcel Dekker, New York, 1990, Ch. 19, p. 487.
[7] G.A. Perry, J.D. Jackson, T.L. McDonald, D.A. Crouse and J.G. Sharp, Prep. Biochem., 14 (1984) 431.
[8] B. Pavlu, U. Johansson, C. Nyhlen and A. Wichman, J. Chromatogr., 359 (1986) 449.
[9] S.W. Burchiel, J.R. Billman and T.R. Alber, J. Immunol. Methods, 69 (1984) 33.
[10] P. Clezardin, J.L. McGregor, M. Manach, H. Boukerche and M. Dechavanne, J. Chromatogr., 319 (1985) 67.
[11] J.E. Hale and D.E. Beidler, Anal. Biochem., 222 (1994) 29.
[12] O. Kaltenbrunner, C. Tauer, J. Brunner and A. Jungbauer, J. Chromatogr., 639 (1993) 41.
[13] P.G. Righetti, T. Caravaggio and K. Ek, J. Chromatogr., 220 (1981) 115.
[14] Y.-B. Yang, K. Harrison and J. Kindsvater, J. Chromatogr., 723 (1996) 1.
[15] Y.-B. Yang, U.S. Pat. Appl., 919 518, Filed on July 24, 1992.
[16] L. Stryer, Biochemistry, W.H. Freeman and Company, New York, 3rd ed., 1988, Ch. 35, p. 898.
[17] Y.-B. Yang and K. Harrison, presented at the Eighth Symposium of The Protein Society, San Diego, CA, USA, July 9-13, 1994, Abstract number 273T. Protein Science, Vol. 3, Suppl. 1 (1994) 99.
[18] L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley, New York, 2nd ed., 1979, Ch. 8, p. 426.
[19] L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley, New York, 2nd ed., 1979, Ch. 16, p. 680.