No.118

TOSOH

SEPARATION REPORT

TSKgel® FcR-IIIA-NPR Column for High-Performance Affinity Chromatography

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TOSOH CORPORATION

1. Introduction

Demand is growing for antibody drugs as therapeutics for cancer, autoimmune disorders, and the like. Because of this, production facilities for antibody drugs have been newly constructed or augmented all over the world. Antibody drugs are produced by animal cell expression system, and in recent years it has been determined that the structure of the glycans present in the Fc region influences an efficacy of drug. It has become clear that differences in glycan structure affect binding to the Fc receptor on the effector cells that mediate the immune response, thereby affecting the elicitation of the accompanying immune response. Because of this, strict control of glycan structure has been required when producing antibody drugs. At this time, we have commercialized the TSKgel FcR-IIIA-NPR, a high-performance affinity chromatography (AFC) column that can separate antibodies based on affinity for FcγRIIIA, an Fc receptor. In this report, we present the basic characteristics of the TSKgel FcR-IIIA-NPR and applications.

2. Basic characteristics

2-1. Packing material properties

The TSKgel FcR-IIIA-NPR is an AFC column wherein a PEEK column is filled with packing material in which recombinant FcγRIIIA protein has been introduced as a ligand onto a non-porous hydrophilic polymer material. The ligand of this column is a protein whose physicochemical stability was enhanced by multiple amino acid substitutions to the native protein sequence, the resulting FcγRIIIA protein then was overproduced in *E. coli* from the recombinant gene.

Because FcγRIIIA specifically binds to the hinge region of the Fc antibody, FcγRIIIA is able to bind and separate only antibodies, even when starting directly from a cell culture supernatant that contains contaminants or other materials. In addition, resolution has been greatly improved by using a non-porous resin.

Table 1 presents the specifications for this column.

Table1

Part No.		0023513
Packing materials	Base materials	non-porous hydrophilic polymers
	avg. particle size	5 μm
	Ligand	Recombinant human FcγRIIIA (produced by *E. coli*)
Column	Dimension	4.6 mm I.D. × 7.5 cm
	Materials	PEEK
	Shipping solvent	0.025 % ProClin® 300 + 0.65 mmol/L citric acid + 9.35 mmol/L trisodium citrate (pH 6.5)
Separation conditions	pH range	pH 4.0 ～ pH 8.0 (short term), pH 5.0 ～ pH 7.0 (long term)
	Temperature range	15 ℃～ 25 ℃
	Suitable flow rate	1.0 mL/min
	Max. pressure drops	9.0 MPa
Storage conditions		Replace the shipping solvent and keep cool at 2 ℃～ 8 ℃

"ProClin" is a registered trade name of Rohm and Haas Company.

2-2. Standard separation conditions

Because many antibodies bind to FcγRIIIA in the neutral pH range (pH 6.0 to 7.5) and elute under acidic conditions (pH 4.0 to 5.0), the pH gradient elution method is generally used for separation of antibodies by the TSKgel FcR-IIIA-NPR through the use of two buffers having different pHs.

While various buffers can be used for the eluent, a sodium citrate buffer solution that has buffering capacity in a broad pH range is most suitable. In particular, when analyzing human IgG_1 antibody prepared with generic CHO cells, typical eluents are a 50 mmol/L sodium citrate buffer solution (pH 6.5) as equilibration buffer (eluent A) and a 50 mmol/L sodium citrate buffer solution (pH 4.5) as elution buffer (eluent B). After equilibrating the column in advance with eluent A (allowing flow-through of five or more column volumes), the sample is injected and allowed to bind to antibody. Subsequently, the eluent is switched to eluent B by a linear gradient, and antibody is allowed to elute. Finally, by re-equilibrating with eluent A, the next measurement can be smoothly performed. While the FcγRIIIA ligand used in this column has a certain amount of acid tolerance, prolonged exposure to an acid buffer can reduce the column life.

In addition, nonspecific binding to the packing material may occur because of contaminants contained in some antibodies and samples, and retention times may gradually decrease. In many cases, this sort of adsorption stems from electrostatic interactions, and can be controlled by adding about 150 mmol/L NaCl to eluents A and B.

Table 2 presents examples of typical separation conditions. Because the optimal conditions may vary with the antibody, performing an investigation regarding conditions is recommended. **Figure 1** presents an antibody application wherein the change in pH is provided using a sodium citrate buffer solution (conditions presented in Example 1 in **Table 2**).

As for the column temperature during an analysis, it has been shown that the antibody affinity for the FcγRIIIA ligand is affected by temperature. Using a column oven to control the column temperature during analysis is strongly recommended to obtain reproducible results. **Figure 2** presents the change in retention when analyses are performed under column oven temperature conditions of 15, 20, and 25°C. It can be seen that by increasing the temperature in this manner, the affinity for antibodies decreases, and the antibody retention time is shortened.

Table 2 Examples of separation conditions

	Example 1	Example 2	Example 3
Eluent	A : 50 mmol/L sodium citrate buffer (pH 6.5) B : 50 mmol/L sodium citrate buffer (pH 4.5)	A : 50 mmol/L sodium citrate buffer + 150 mmol/L NaCl (pH 6.5) B : 50 mmol/L sodium citrate buffer + 150 mmol/L NaCl (pH 4.0)	A : 50 mmol/L sodium citrate buffer + 150 mmol/L NaCl (pH 6.5) B : 50 mmol/L sodium citrate buffer + 150 mmol/L NaCl (pH 4.0)
Gradient program	0 − 2 min B 0 % 2 − 20 min B 0 % → 100 %, linear gradient 20 − 25 min B 100 % 25 − 30 min B 0 %	0 − 2 min B 0 % 2 − 20 min B 0 % → 100 %, linear gradient 20 − 25 min B 100 % 25 − 30 min B 0 %	0 − 7 min B 0 % 7 − 25 min B 0 % → 100 %, linear gradient 25 − 30 min B 100 % 30 − 35 min B 0 %
Flow rate	1.0 mL/min	1.0 mL/min	1.0 mL/min
Detection	UV 280 nm	UV 280 nm	UV 280 nm
Injection volume	1 ～ 100 μL	1 ～ 100 μL	1 ～ 100 μL
Sample load	5 ～ 50 μg	5 ～ 50 μg	5 ～ 50 μg
Remarks	Standard conditions	Suited to samples with higher binding	suited to samples containing impurities like culture media

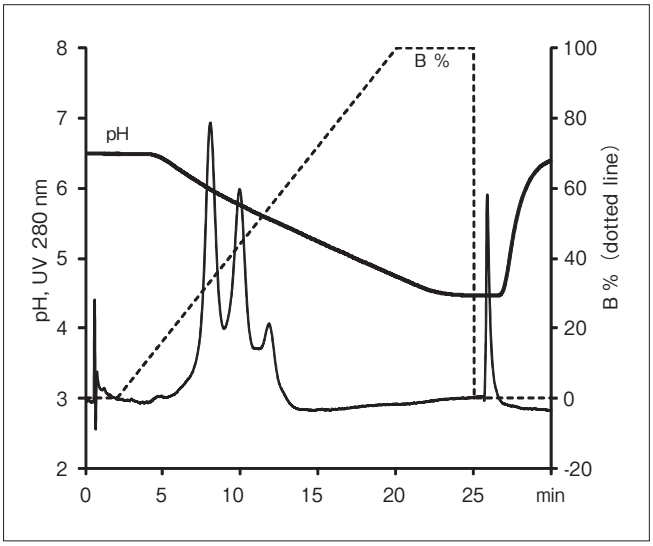


Figure 1. Separation of antibody under standard conditions

〈Conditions〉
Column: TSKgel FcR-IIIA-NPR (4.6 mm I.D. × 7.5 cm)
Eluent A: 50 mmol/L sodium citrate buffer (pH 6.5)
B: 50 mmol/L sodium citrate buffer (pH 4.5)
Gradient: 0 − 2 min B 0 %
2 − 20 min B 0 % → 100 %, linear gradient
20 − 25 min B 100 %
25 − 30 min B 0 %
Flow rate: 1.0 mL/min
Detection: UV 280 nm
Temperature: 25 ℃
Sample: human IgG_1 (Sigma-Aldrich), 10 μg

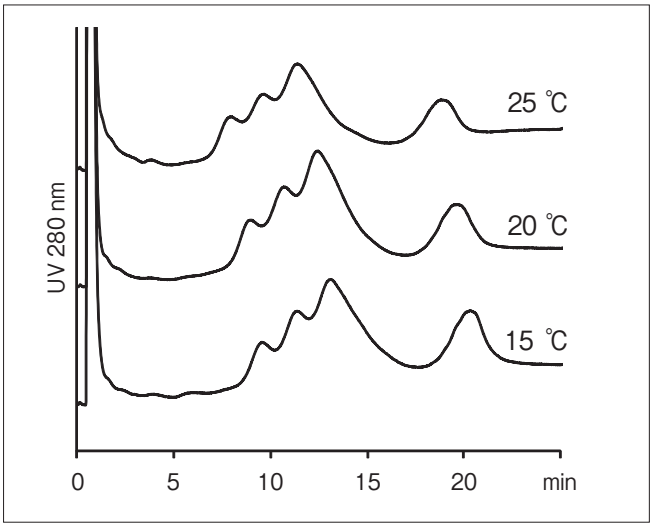


Figure 2. Effect of column temperature on measurement

〈Conditions〉
Column: TSKgel FcR-IIIA-NPR (4.6 mm I.D. × 7.5 cm)
Eluent A: 50 mmol/L sodium citrate buffer (pH 6.5)
B: 50 mmol/L sodium citrate buffer (pH 4.5)
Gradient: 0 − 2 min B 0 %
2 − 20 min B 0 % → 100 %, linear gradient
20 − 25 min B 100 %
25 − 30 min B 0 %
Flow rate: 1.0 mL/min
Detection: UV 280 nm
Temperature: depicted in the fig.
Sample: human γ- globulin, 20 μg

2-3. Effect of eluent composition

When analyzing antibodies that differ greatly in binding to Fc receptors, it is necessary to optimize the separation conditions. **Figure 3** presents a monoclonal antibody application in which the buffer concentration and NaCl concentration in eluent were changed. Antibody affinity for FcγRIIIA ligand is affected by ionic strength in addition to pH and temperature, and in many cases, retention times are shortened under high ionic strength conditions. On the other hand, care is needed when measurements are carried out with an eluent having an extremely weak elution strength, because antibodies may remain in the column and have an effect on subsequent measurements.

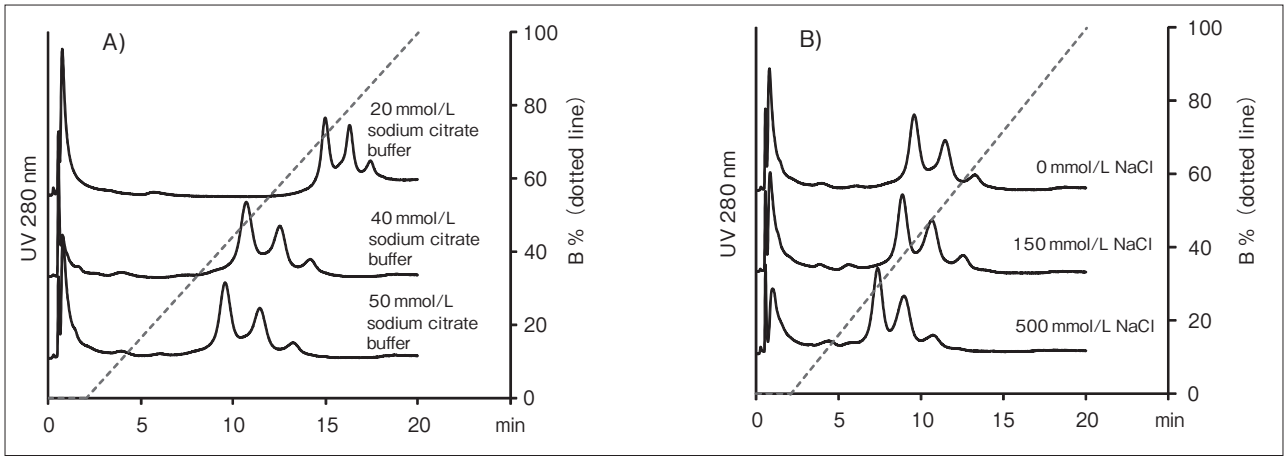


Figure 3. Effect of eluent composition on measurement

〈Conditions〉
Column: TSKgel FcR-IIIA-NPR (4.6 mm I.D. × 7.5 cm)
Eluent A: sodium citrate buffer (pH 6.5)
B: sodium citrate buffer (pH 4.5)
(buffer concentration is depicted in fig.)
Gradient: 0 − 2 min B 0 %
2 − 20 min B 0 % → 100 %, linear gradient
20 − 25 min B 100 %
25 − 30 min B 0 %
Flow rate: 1.0 mL/min
Detection: UV 280 nm
Temperature: 25 ℃
Sample: monoclonal antibody

〈Conditions〉
Column: TSKgel FcR-IIIA-NPR (4.6 mm I.D. x 7.5 cm)
Eluent A: 50 mmol/L sodium citrate buffer + NaCl (pH 6.5)
B: 50 mmol/L sodium citrate buffer + NaCl (pH 4.5)
(NaCl concentration is depicted in fig.)
Gradient: 0 − 2 min B 0 %
2 − 20 min B 0 % → 100 %, linear gradient
20 − 25 min B 100 %
25 − 30 min B 0 %
Flow rate: 1.0 mL/min
Detection: UV 280 nm
Temperature: 20 ℃
Sample: monoclonal antibody

2-4. Effect of sample load

Because the TSKgel FcR-IIIA-NPR uses a non-porous resin as base materials, the column exhibits good resolution. On the other hand, the maximum sample load is limited compared to common packing materials. Though capacity varies with detector sensitivity, when running at a flow rate of 1.0 mL/min, an antibody load of 5 μg to 50 μg is appropriate for a single measurement. **Figure 4** presents a chromatograms in which the antibody load was varied. In every case, the resolution of the peaks was the same; however, the antibody retention decreased with increasing antibody load.

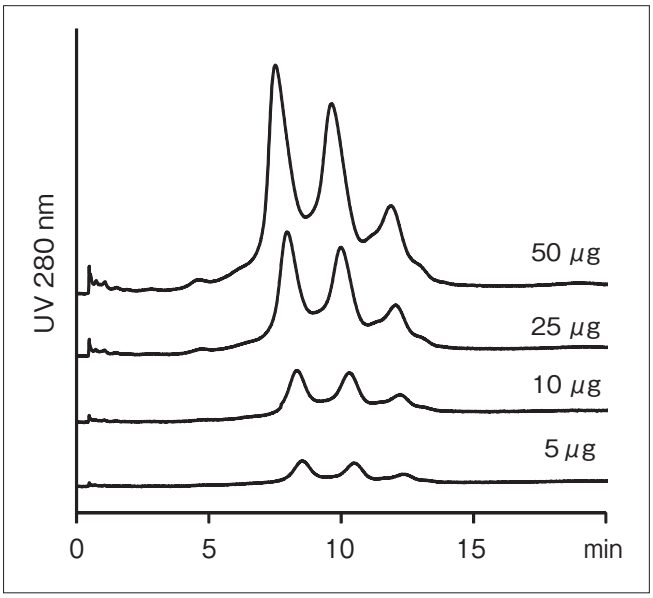


Figure 4. Effect of sample load on measurement

〈Conditions〉
Column: TSKgel FcR-IIIA-NPR (4.6 mm I.D. × 7.5 cm)
Eluent A: 50 mmol/L sodium citrate buffer (pH 6.5)
B: 50 mmol/L sodium citrate buffer (pH 4.5)
Gradient: 0 − 2 min B 0 %
2 − 20 min B 0 % → 100 %, linear gradient
20 − 25 min B 100 %
25 − 30 min B 0 %
Flow rate: 1.0 mL/min
Detection: UV 280 nm
Temperature: 25 ℃
Sample: human IgG_1 (Sigma-Aldrich), 10 μg

2-5. Durability

Two hundred consecutive measurements were carried out using samples of CHO cell culture supernatants containing monoclonal antibody. Condition 3 in **Table 2** was used to inhibit nonspecific binding of impurities and the like. **Figure 5** presents chromatograms for every 20 injections. The chromatograms indicate good durability with no noticeable changes in antibody retention time or resolution observed. However, mold and other microorganisms grow easily in sodium citrate buffer solutions, and the column pressure may suddenly increase due to the resulting clogging. To inhibit the growth of microorganisms, measures need to be considered, such as filtering the eluent with a 0.2 μm filter and periodically replacing the eluent with a new one. In addition, installing a line filter between the pump and injector is also effective.

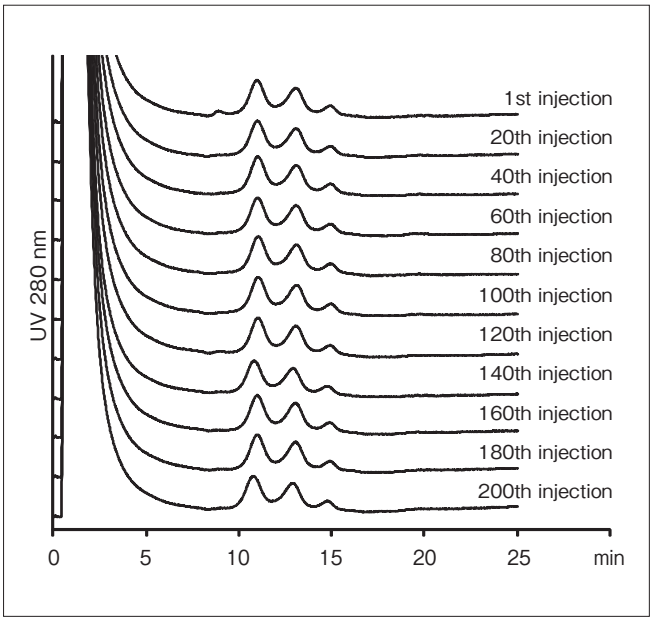


Figure 5. Chromatograms of durability test

〈Conditions〉
Column: TSKgel FcR-IIIA-NPR (4.6 mm I.D. × 7.5 cm)
Eluent A: 50 mmol/L sodium citrate buffer + 150 mmol/L NaCl (pH 6.5)
B: 50 mmol/L sodium citrate buffer + 150 mmol/L NaCl (pH 4.0)
Gradient: 0 − 7 min B 0 %
7 − 25 min B 0 % → 100 %, linear gradient
25 − 30 min B 100 %
30 − 35 min B 0 %
Flow rate: 1.0 mL/min
Detection: UV 280 nm
Temperature: 20 ℃
Sample: cell culture supernatant containing antibody

2-6. Cleaning method

When the eluent consists of a buffer solution that does not have sufficient eluting ability, it is possible that the surface of the packing material may become contaminated, which may affect subsequent measurements. For example, when 40 mmol/L sodium citrate buffers (eluent A, pH 6.5; eluent B, pH 4.5) are used for a sample of CHO cell culture supernatant containing monoclonal antibodies, certain part of the sample remains within the column, and retention ability decreases with repeated measurements. When such a phenomenon occurs, retention ability may be recovered by injecting a buffer solution containing 500 mmol/L NaCl or a buffer solution containing 20 vol% ethanol. **Figure 6** presents chromatograms that show a decrease in retention times due to column contamination, as well as a recovery in retention time as a result of cleaning.

Please note that because the FcγRIIIA ligand used in this product does not have sufficient alkali resistance, alkaline solutions such as sodium hydroxide solution cannot be used as cleaning solutions.

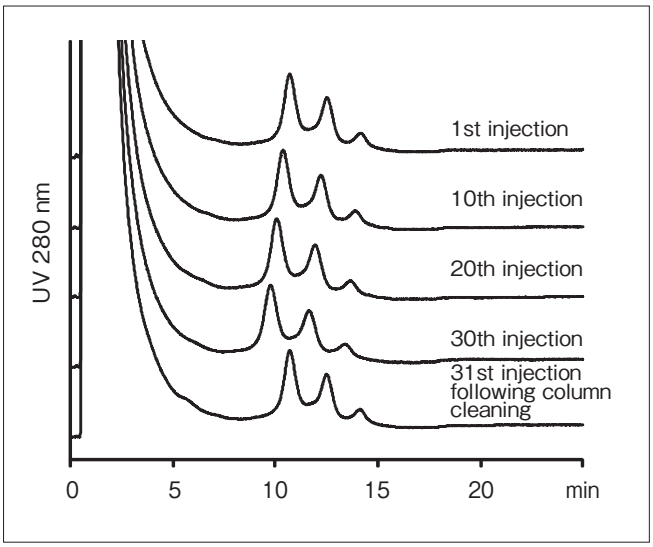


Figure 6. Reduction in retention ability due to poor elution and the effect of column cleaning

〈Conditions〉
Column: TSKgel FcR-IIIA-NPR (4.6 mm I.D. × 7.5 cm)
Eluent A: 40 mmol/L sodium citrate buffer (pH 6.5)
B: 40 mmol/L sodium citrate buffer (pH 4.5)
Gradient: 0 − 7 min B 0 %
7 − 25 min B 0 % → 100 %, linear gradient
25 − 30 min B 100 %
30 − 35 min B 0 %
Flow rate: 1.0 mL/min
Detection: UV 280 nm
Temperature: 20 ℃
Sample: cell culture supernatant containing antibody
Cleaning solvent: 40 mmol/L sodium citrate buffer + 500 mmol/L NaCl (pH 4.5)
Cleaning procedure: running eluent A, inject 50 μL of cleaning solvent for 3 times.

2-7. Lot-to-lot variance of packing materials

Figure 7 presents chromatograms of measurements of IgG_1 using three lots of packing material. No major differences between lots were observed in peak shape and elution position, and good reproducibility was obtained.

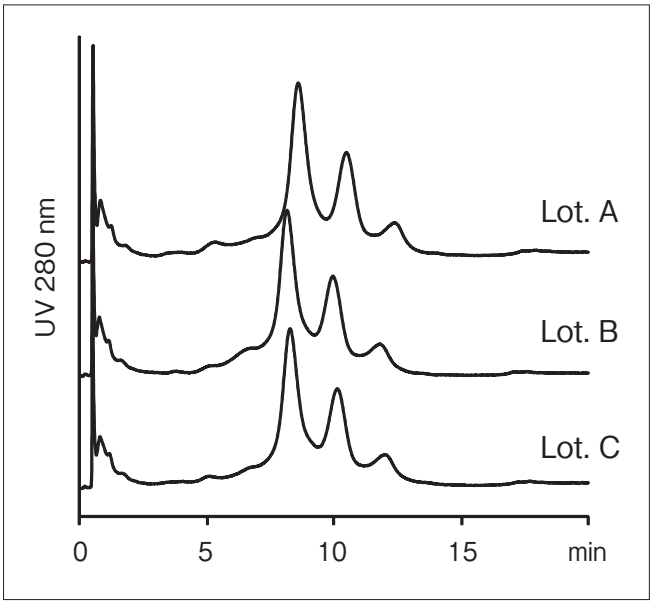


Figure 7. Comparison of differences between packing material lots

〈Conditions〉
Column: TSKgel FcR-IIIA-NPR (4.6 mm I.D. × 7.5 cm)
Eluent A: 50 mmol/L sodium citrate buffer (pH 6.5)
B: 50 mmol/L sodium citrate buffer (pH 4.5)
Gradient: 0 − 2 min B 0 %
2 − 20 min B 0 % → 100 %, linear gradient
20 − 25 min B 100 %
25 − 30 min B 0 %
Flow rate: 1.0 mL/min
Detection: UV 280 nm
Temperature: 25 ℃
Sample: human IgG_1 (Sigma-Aldrich), 10 μg

2-8. Stability in storage

Figure 8 shows the change of retention time and theoretical plates of colum as a function of % rate when the TSKgel FcR-IIIA-NPR is stored in cool condition with shipping solvent. It can be seen that in all cases, no major change was observed over a 12-month period, and the immobilized FcγRIIIA ligand on the packing material was stable. However, because the ligand of this product is a protein, a degradation in performance is expected from interactions with various proteases and microorganisms, and hence, appropriate controls during storage are required.

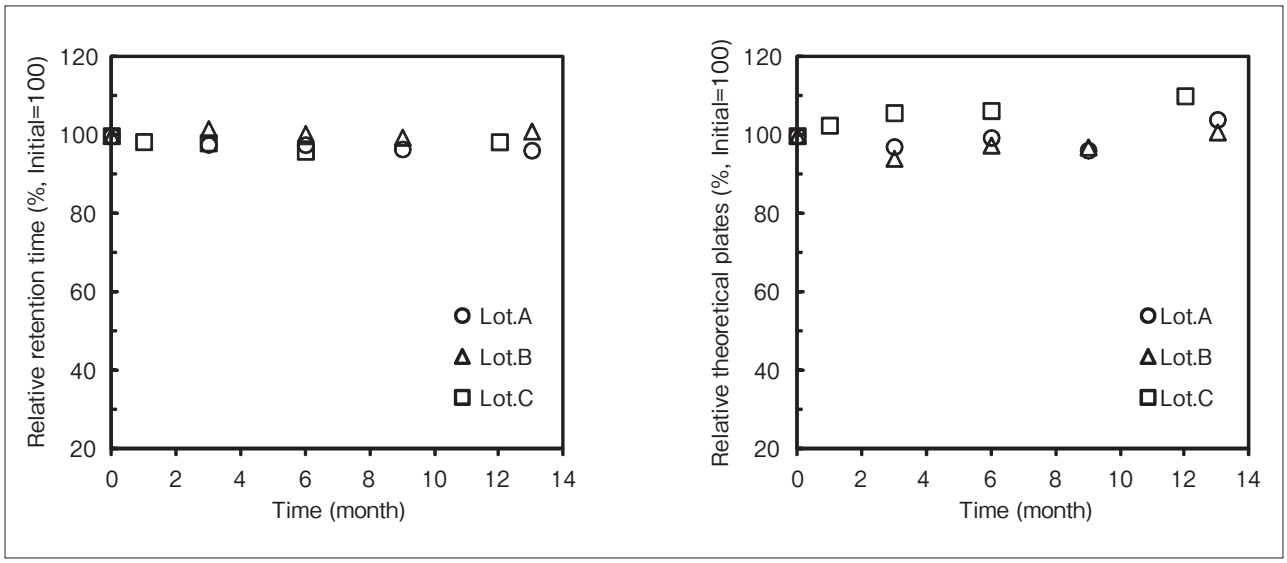


Figure 8. Column stability in storage

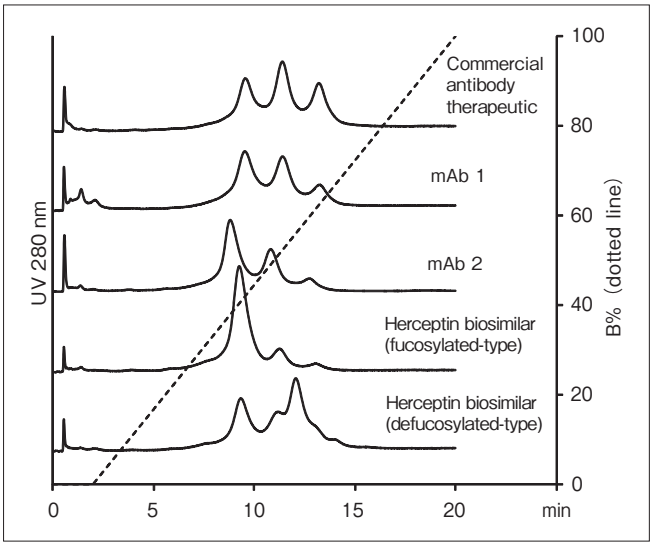
〈Conditions for retention time measurement〉
Column: TSKgel FcR-IIIA-NPR (4.6 mm I.D. × 7.5 cm)
Eluent: 50 mmol/L sodium citrate buffer (pH 5.1)
Flow rate: 0.3 mL/min
Detection: UV 280 nm
Temperature: 25 ℃
Sample: human IgG_1 (Sigma-Aldrich), 20 μg
※ Calculated retention time of the 3rd peaks

〈Conditions for packing stability measurement〉
Column: TSKgel FcR-IIIA-NPR (4.6 mm I.D. × 7.5 cm)
Eluent: 100 mmol/L sodium phosphate buffer +
100 mmol/L sodium sulfate (pH 6.7)
Flow rate: 1.0 mL/min
Detection: UV 280 nm
Temperature: 25 ℃
Sample: 1 % acetone aqueous solution, 10 μL

3. Applications

3-1. Antibody applications

Results of various antibody measurements are presented in **Figure 9**. With standard monoclonal antibodies prepared with CHO cells, three main peaks eluted in most cases. In addition, the peak area ratios differed greatly by antibody sample. In comparing chromatograms of fucosylated-type and defucosylated-type Herceptin biosimilars, it was clear that the defucosylated-type tended to have a larger quantity ratio of components having a longer retention time. Generally, it has been reported that antibodies having defucosylated glycans have higher ADCC activity, and the results obtained were in agreement with this information.



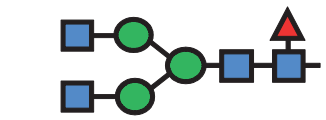
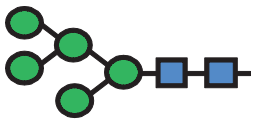
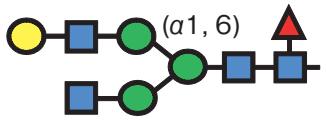
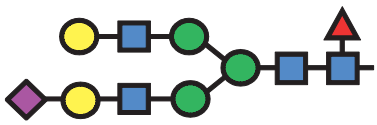
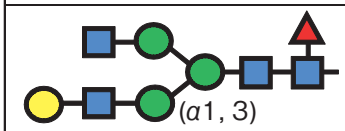
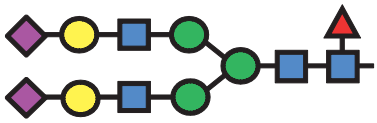
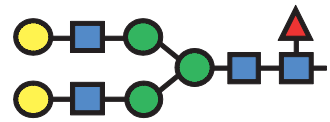
〈Conditions〉
Column: TSKgel FcR-IIIA-NPR (4.6 mm I.D. × 7.5 cm)
Eluent A: 50 mmol/L sodium citrate buffer (pH 6.5)
B: 50 mmol/L sodium citrate buffer (pH 4.5)
Gradient: 0 − 2 min B 0 %
2 − 20 min B 0 % → 100 %, linear gradient
20 − 25 min B 100 %
25 − 30 min B 0 %
Flow rate: 1.0 mL/min
Detection: UV 280 nm
Temperature: 25 ℃
Sample: monoclonal antibodies, 10 μg each

Figure 9. Separation profiles of five monoclonal antibodies

3-2. Relationship of antibody glycan structure and retention ability

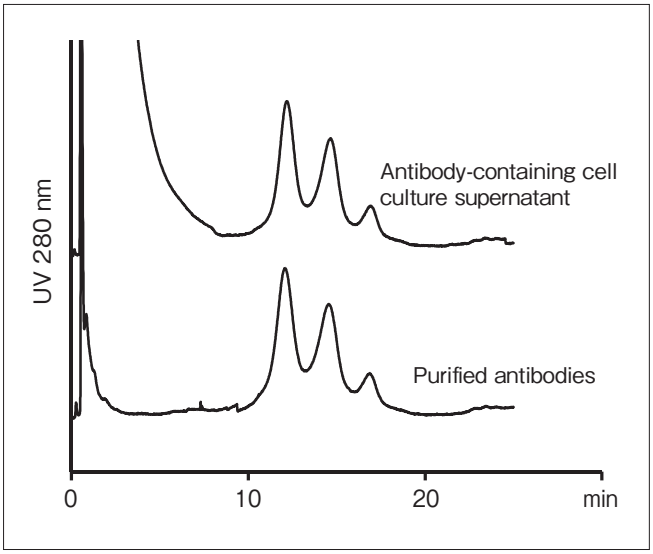
A large FcγRIIIA-immobilized column was prepared for the preparative separation of three peaks that eluted during analysis of monoclonal antibodies prepared with CHO cells, and each component was isolated with 80% or greater purity. The glycan structure of the fractionated components obtained was analyzed, and the trends in retention ability were inspected for each glycan structure. Results are presented in **Table 3**. According to these results, the TSKgel FcR-IIIA-NPR recognized the antibody glycans and separated the components based on their glycan structures. In addition, the presence or absence of galactose at the end of the glycan gave a particularly large effect on affinity.

Table 3. Relationship of antibody glycan structure and retention ability

Glycan structure	Abbreviation	Retention	Glycan structure	Abbreviation	Retention
	G0F	Weak		Man5	Weak
	G1F	Strong		SG2F	Strong
	G1'F	Weak		S2G2F	Strong
	G2F	Strong			

3-3. Analysis of antibodies within cell culture supernatant

Because components other than antibodies do not bind to TSKgel FcR-IIIA-NPR, it is possible, by establishing the appropriate separation conditions, to directly measure antibodies even in samples such as a cell culture supernatant containing a large quantity of various impurities. **Figure 10** presents a chromatogram of antibodies in a cell culture supernatant. In comparing the chromatogram with purified antibody, similar results are obtained even when a sample contains a large quantity of impurities. Such a technique for directly separating antibodies within a culture supernatant is expected to be useful as a monitoring technique in the culturing process for antibody drug manufacturing.



〈Conditions〉
Column: TSKgel FcR-IIIA-NPR (4.6 mm I.D. × 7.5 cm)
Eluent A: 50 mmol/L sodium citrate buffer +
150 mmol/L NaCl (pH 6.5)
B:50 mmol/L sodium citrate buffer +
150 mmol/L NaCl (pH 4.5)
Gradient: 0 - 7 min B 0 %
7 - 25 min B 0 % to 100 %, linear gradient
25 - 30 min B 100 %
30 - 35 min B 0 %
Flow rate: 1.0 mL/min
Detection: UV 280 nm
Temperature: 20°C
Sample: Antibody-containing cell culture supernatant, purified antibody

Figure 10. Comparison of separations of antibody-containing cell culture supernatant and purified antibody

4. Precautions for use

Table 4 lists precautions for use.

Table 4. Precautions for use

HPLC system	1. Internal system cleaning before use (perform prior to connecting the column) 1) Systems where the internal system cleaning is sufficient Send 0.1 mol/L citric acid solution through at 1.0 mL/min for approximately 30 minutes. 2) Old systems and systems where internal system cleaning is insufficient (1) Send 0.1 mol/L sodium hydroxide solution through at 1.0 mL/min for approximately 30 minutes. (2) Send purified water through at 1.0 mL/min for approximately 30 minutes. (3) Send 0.1 mol/L citric acid solution through at 1.0 mL/min for approximately 30 minutes. After performing either of the aforementioned cleanings in 1) and 2), replace liquid phase with eluent A and send the liquid through at 1.0 mL/min for approximately 15 minutes, and then run a blank gradient. Subsequently, connect and equilibrate the column. After running a blank gradient, start performing analyses. 2. Line filter Installing a line filter between the pump and injector is recommended. ・Line filter kit PEEK (Product No.: 0018014) 3. Column oven equipped with cooling function The column operating temperature is 15 to 25°C. Because the elution time for samples is affected by temperature, it is recommended to perform analyses while maintaining a constant temperature with a column oven equipped with a cooling function.
Eluent	When using a citrate buffer solution as the eluent, preparing the buffer solution from citric acid and sodium citrate is recommended. After preparing the eluent, filtering of the eluent with a 0.2-μm-pore-size filter is recommended. In addition, because microorganisms easily grow in the eluent, it is recommended to prepare eluent at the time of use.
Sample solution	It is recommended to inject sample solution filtered with a 0.2-μm-pore-size filter.
Column cleaning	Inject eluent containing 0.5 mol/L NaCl or eluent containing 20% ethanol multiple times from the injector.
Column storage	After replacing with preloaded solvent (0.025% ProClin® 300 + 0.65 mmol/L citric acid + 9.35 mmol/L trisodium citrate (pH 6.5)), place in refrigerated storage (2 to 8°C).
Column expiration date	The column has an expiration date. The expiration date is noted on the column box and in the Analysis Report accompanying the column.

5. Conclusion

We have presented an overview of the new TSKgel FcR-IIIA-NPR column intended for antibody drugs. Conventionally, expensive equipment and complicated work operations were required to analyze the glycan structure or to evaluate the activity of antibody drugs. However, the new analysis methods using this column are extremely easy and can provide results with good reproducibility in a short amount of time. In addition, this column can be used not only for antibody drug quality control, but also for screening of cell strains for production use, optimization of the composition of cell culturing medium, process analysis of culturing processes, and the like.

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