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RESEARCH PAPER

Use of Cyclodextrin as a Novel Agent in the SEC-HPLC Mobile Phase to Mitigate the Interactions of Proteins or Peptide or their Impurities with the Residual Silanols of Commercial SEC-HPLC Columns with Improved Separation and Resolution

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ABSTRACT

Purpose Accurate quantification of the intact proteins, antibodies or peptides and their impurities without interaction to silanols of HPLC column.

Methods Hydroxypropyl ß Cyclodextrin (HPCD) is added in the mobile phase at different concentrations. Different commercial SEC-HPLC columns and biologics with a molecular weight ranging from 5.8 kDa to 150kDa were assessed with and without cyclodextrin.

Results Addition of non-ionic sugars such as Hydroxypropyl ß Cyclodextrin in the mobile phase, resulted improved peak performance such as theoretical plates, peak resolution, peak width, peak height, and improved quantification of aggregates in biologics such as antibodies Humira and Actemra, and peptides such as insulin. There is an increase in peak height, reduced retention time, increased plate and reduced peak width with increasing concentration of cyclodextrin studied.

Discussion High ionic strength, basic amino acids such as arginine, organic solvents (with a concentration low enough not to precipitate protein), sodium perchlorate and ion pairing agents in the mobile phase used for separation of peptides, proteins and antibodies to prevent silanol interaction. These commonly used solutions are not always successful, as they not only interact with the biologic, but are sometimes, not compatible. The non-ionic cyclodextrin itself does not cause protein aggregation but prevents the nonspecific binding or interaction of protein itself and thereby allowing for improved resolution, and accurate quantification of aggregates in antibodies, and peptides. The data on the separation in presence of cyclodextrin in the mobile phase showed higher peak resolution, improved peak shape, accurate apparent molecular weight, improved efficiency, and less peak tailing for biological products.

Conclusion Hydroxypropyl ß Cyclodextrin in the mobile phase, resulted improved SEC-HPLC resolution, and quantitation of aggregates in biologics by preventing the interaction of biologics to silanol of the commercial SEC-HPLC columns.

KEY WORDS proteins and peptides · SEC-HPLC column interaction · silanol · hydroxypropyl ß Cyclodextrin

ABBREVIATION

°C	Degree Centigrade
AUC	Area under the curve
BEH	Ethylene-bridged hybrid
CHO	Chinese Hamster Ovary
DAD	Diode-Array Detector
EP	European Pharmacopeia
FDA	Food and Drug Administration
HCl	Hydrochloric acid
HMW	High Molecular Weight
HPCD	Hydroxypropyl ß Cyclodextrin
IgG	Immunoglobulin G
kDa	Kilo Dalton
mL	Milli liter
mM	Milli molar
PBS	Phosphate buffered saline
PI	Isoelectric point
pH	Potential Hydrogen
SEC-HPLC	Size-Exclusion High-Performance Liquid Chromatography

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INTRODUCTION

For the separation of biologics, pharmaceutical industry uses commercial analytical SEC-HPLC columns to assess, the major peaks, aggregates and degradants, either during the processing of biologics, during purification or QC release of the drug product. The commercial SEC-HPLC columns routinely used porous silica columns. These columns were slightly acidic showing affinity to basic amino acids of proteins or peptides (1). As commercial SEC-HPLC columns are Silica based, the residual silanol of the column interacts with the biologic analytes and prevents the analyte from column elution. One strategy often used is the addition of a chaotropic agent arginine to the mobile phase to reduce secondary interactions (2,3). Arginine acts as a binder to the analyte in solution, thus preventing it from interacting with the stationary phase. It was found that an increased aggregate recovery was noticed, when arginine was added to the mobile phase (3). Other studies have also shown improvement in peak shape with the use of arginine as a mobile phase additive (2). Arginine in the SEC-HPLC mobile phase has been investigated for both large and small proteins, such as insulin (4–6). However, Arginine may not be compatible with some protein resulting the artifacts. Similarly, another chaotropic agent sodium hypochlorate in the mobile phase has been reported for seven commercial antibodies and shown that these antibodies exhibited higher HMW protein compared to sample tested with European pharmacopeia (EP) method (7). TSKgel G3000SWxl column eluted with 40 mM phosphate-0.2 M NaCl-0.005% NaN3, pH 7.0 is considered as EP method. Though FDA (7) stated, that oxidation by perchlorate is negligible at neutral pH, it has been reported to dissociate protein multimers (9–11), and perchlorate is known to be an oxidizing agent (8) hence the usage on the suitability of using perchlorate as a chaotropic salt in the eluent for analyses of proteins by SEC-HPLC (12) is questionable. In most cases, the separation of proteins in neutral pH are not suitable, and it favors more column interaction and more streaking. These interactions can be predicted based on the relationship between mobile phase pH and the isoelectric point of the protein. Varying pH of the mobile phase can perturb the three-dimensional conformation of the protein, resulting in changes in interactions with the stationary phase. Golovchenko *et al.* (13) showed that at low ionic strengths, ion exchange effects were observed at pH values below the pI of the protein, while ion-exclusion effects were observed at pH values above the isoelectric point of the protein (13).

Preconditioning of the column by proteins to prevent the column interactions have been explored by several scientists to adsorb and saturate the binding sites on the stationary phase. Such preconditioning affects the pore size of the stationary phase, altering the performance characteristics of the column by reducing peak resolution and separation range (7). Moreover, the adsorbed protein may bleed into the mobile phase showing baseline drift and affect the results of the analysis of the protein product and resulting data will not accurately reflect the actual aggregate content of the protein in the drug product (12,14–16).

As aggregation or high molecular weight species in a biological product is known to cause adverse events, including immunogenic reactions, hypotension, and anaphylaxis (7,17–21), an under estimation of HMW protein due to adsorption to column may compromise the safety and efficacy of the protein. To address this problem, we have investigated the use of a novel cyclodextrin in the mobile phase for SEC-HPLC analysis of therapeutic proteins by preventing silanol interaction to the biologics through complexation of silanol with cyclodetextrin. It is known, that when cyclodextrin is used as an excipient for proteins and peptide, it keeps the protein in native form even after stress such as shear, preventing the protein-protein interaction due to hydrophobic interface of protein or peptide (23,24). The experiments were performed with columns from different vendors and using different therapeutic proteins with a molecular weight ranging from 5.8 kDa to 150kDa. The addition of appropriate amount of cyclodextrin in the eluent showed higher peak resolution, improved peak shape, right apparent molecular weight, improved efficiency, less peak tailing etc.

MATERIALS AND METHODS

Materials

Humulin N (Eli Lilly, Indianapolis, IN USA) formulated in 0.35 mg of protamine sulfate, 16 mg of glycerin, 3.78 mg of dibasic sodium phosphate, 1.6 mg of metacresol, 0.65 mg of phenol, and 0.025 mg zinc ion to provide prolonged duration of action was sourced from Express scripts St Louis, MD USA. Human Insulin powder was purchased from Sigma Cat # 91077C-10G. Purified rat polyclonal IgG formulated in PBS at pH 7.4 was purchased from Innovative Research Novi, MI 48377. Recombinant monoclonal antibody Humira (Adalimumab) formulation containing 40 mg adalimumab, 4.93 mg sodium chloride, 0.69 mg monobasic sodium phosphate dihydrate, 1.22 mg dibasic sodium phosphate dihydrate, 0.24 mg sodium citrate, 1.04 mg citric acid monohydrate, 9.6 mg mannitol, 0.8 mg polysorbate 80, and Water for Injection, USP and 20 mg/mL. Actemra (Tocilizumab) is formulated in an aqueous solution containing disodium phosphate dodecahydrate and sodium dihydrogen phosphate dehydrate (as a 15 mmol/L phosphate buffer),

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Table 1 Chromatographic Peak Performance of Human Insulin (Sigma)

Conditions	Area mAU	% Agg	Peak height mAU	RT min	Plates	Peak Width min
0% HPCD human Insulin	4324	0.28	97	9.33	975	0.70
2% HPCD human Insulin	4495	0.62	107	9.26	1174	0.63
10% HPCD human Insulin	4419	1.50	160	9.11	2674	0.41
0% HPCD human Insulin 1 day at 50° C	3991	0.90	90	9.34	1024	0.68
2% HPCD human Insulin 1 day at 50° C	4210	1.75	100	9.29	1216	0.62
10% HPCD human Insulin 1 day at 50° C	4136	3.50	149	9.12	2005	0.41

polysorbate 80 (0.5 mg/ml), and sucrose (50mg/mL) were obtained from Abbvie North Chicago, IL.60064, USA and Genentech South San Francisco, CA 94080) respectively. The source and formulation for a 55 kDa protein and two antibodies studied are not disclosed due to proprietary reason and these proteins are recombinant proteins synthesized from CHO cells, Di sodium hydrogen phosphate mono hydrate, mono sodium di hydrogen phosphate anhydrous, sodium sulfate, Arginine hydrochloride, sodium chloride, 1 N sodium hydroxide, and 1 N hydrochloric acid were obtained from JT Baker supplied by Fisher Scientific (Waltham, MA). Hydroxypropyl ß cyclodextrin was obtained from Cargill, Inc., Wayzata, Minn. USA; The 55 kDa protein and two other antibodies formulations and source were not disclosed due to proprietary reason. The molecular weight marker reference was obtained from Biorad,

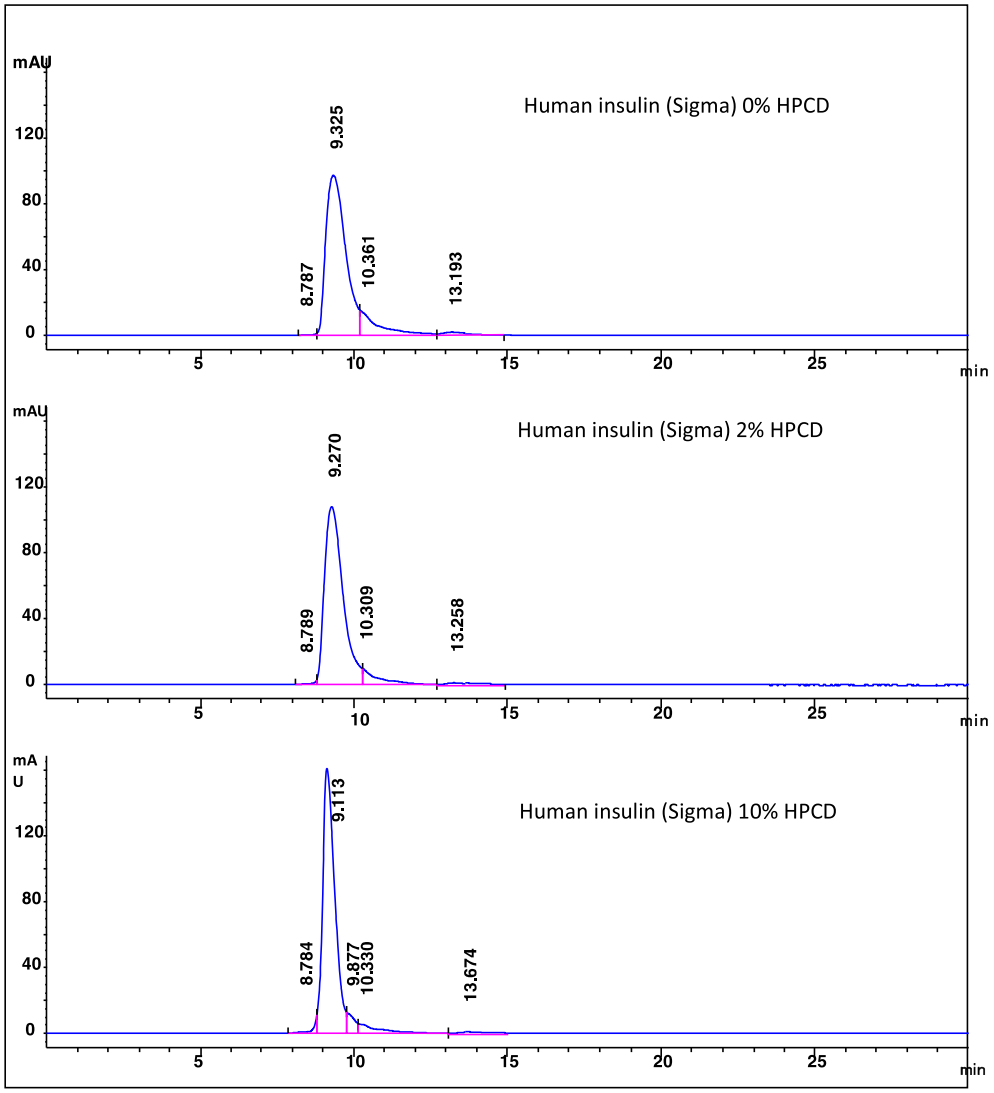

Fig. 1 Overlay chromatogram of 0.5 units equivalent of Human insulin (Sigma) with the elution buffer containing 100 mM sodium acetate and 100 mM sodium sulfate at pH 6.0 with 0, 2 and 10% HPCD.

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Hercules, CA 94548. HPLC grade water was obtained from Pharmco-Aaper CT, USA. Agilent 1100 or 1260 HPLC systems were used for this study.

Sample Preparation

To analyze Humulin N placebo, which contains Protamine sulfate and phenol, the insulin crystals from Humulin N was centrifuged at 10,000 rpm for 10 min and clear supernatant free of insulin crystal was used for SEC- HPLC analysis. The HPLC profile of placebo was confirmed with the freshly prepared inactive ingredient without insulin. To analyze insulin of Humulin N, the sample was acidified using 1 N HCl to a final concentration of 0.01 N before injection, as the isoelectric point of insulin is 7.0 and insoluble in neutral pH and both insulin and excipients are completely soluble at pH 2–3. Insulin powder (Sigma) was dissolved in 0.01 N HCl at 10 mg/mL and subsequently diluted to 1 mg/mL using the PBS. Rat IgG powder was diluted to 1 mg/mL using PBS before injection. All monoclonal antibodies formulated in product specific inactive excipients, which includes, buffer, salt, and or bulking agents such as Trehalose or Sucrose providing stability to the protein was used without any further dilutions. Appropriate µg protein was loaded without further sample preparation and the samples were kept at 4°C autosampler.

Liquid Chromatography

In the final method, 2 units of insulin or 30 µg antibody were injected. Insulin. and Rat IgG were assayed with TSKgel G2000SWXL (7.8 mm × 30 cm, 5 µ) columns (Tosoh Bioscience, Grove City, OH) using a mobile phase containing 100 mM sodium acetate pH 6.0 and 100 mM Sodium sulfate with or without 1–10% hydroxypropyl cyclodextrin with a flow rate 1 mL/min (30 min run). The responses were monitored at 280 nm using a DAD detector. The monoclonal antibodies Humira and Actemra were analyzed using TSKgel G3000SWXL (7.8 mm × 30 cm, 5 µ) columns (Tosoh Bioscience, Grove City, OH) using a mobile phase containing 100 mM sodium acetate pH 6.0 and 100 mM Sodium sulfate with or without 2% hydroxypropyl cyclodextrin with a flow rate 1 mL/min (15 min run). The responses were monitored at 280 nm using a DAD detector. The 55 KDa protein (50µg injection) was analyzed using Waters Protein Pak 300SW column and 25 mM Sodium Phosphate, 75 mM Sodium sulfate, at pH 5.0, 6.0, and 7.0 was used as

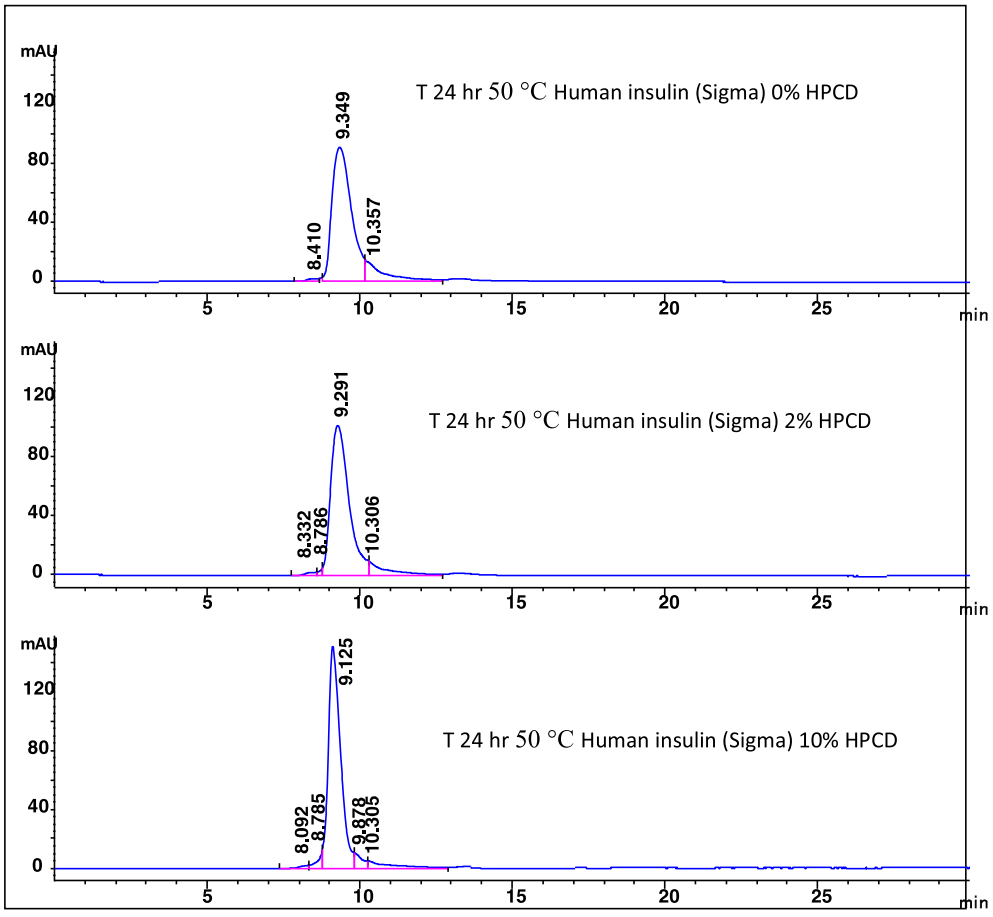

Fig. 2 Overlay chromatogram of 0.5 units equivalent of Human insulin (Sigma) after thermal stressed at 50°C for 24 h, with the elution buffer containing 100 mM sodium acetate and 100 mM sodium sulfate at pH 6.0 with 0, 2 and 10% HPCD.

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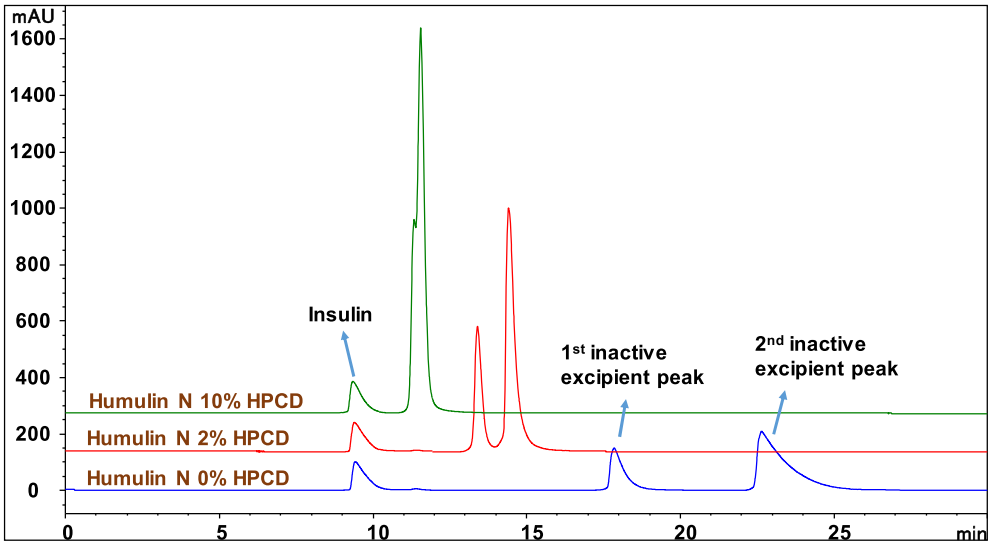

Fig. 3 Chromatogram of 2 units of Humulin N with the elution buffer containing 100 mM sodium acetate and 100 mM sodium sulfate at pH 6.0 with 0, 2 and 10% HPCD. Note the severe interaction of excipients to column. The retention time of insulin is changed very little. However, the retention time of excipients are changed drastically when 2 and 10% HPCD is used and at very high concentration of cyclodextrin, the two excipients peaks are almost merged.

mobile phase with a flow rate of 0.5 mL/min for 50min. The responses were monitored at 214 nm using a DAD detector. All the integrations were done using the optimal auto integration parameters set by the Agilent chemstation software.

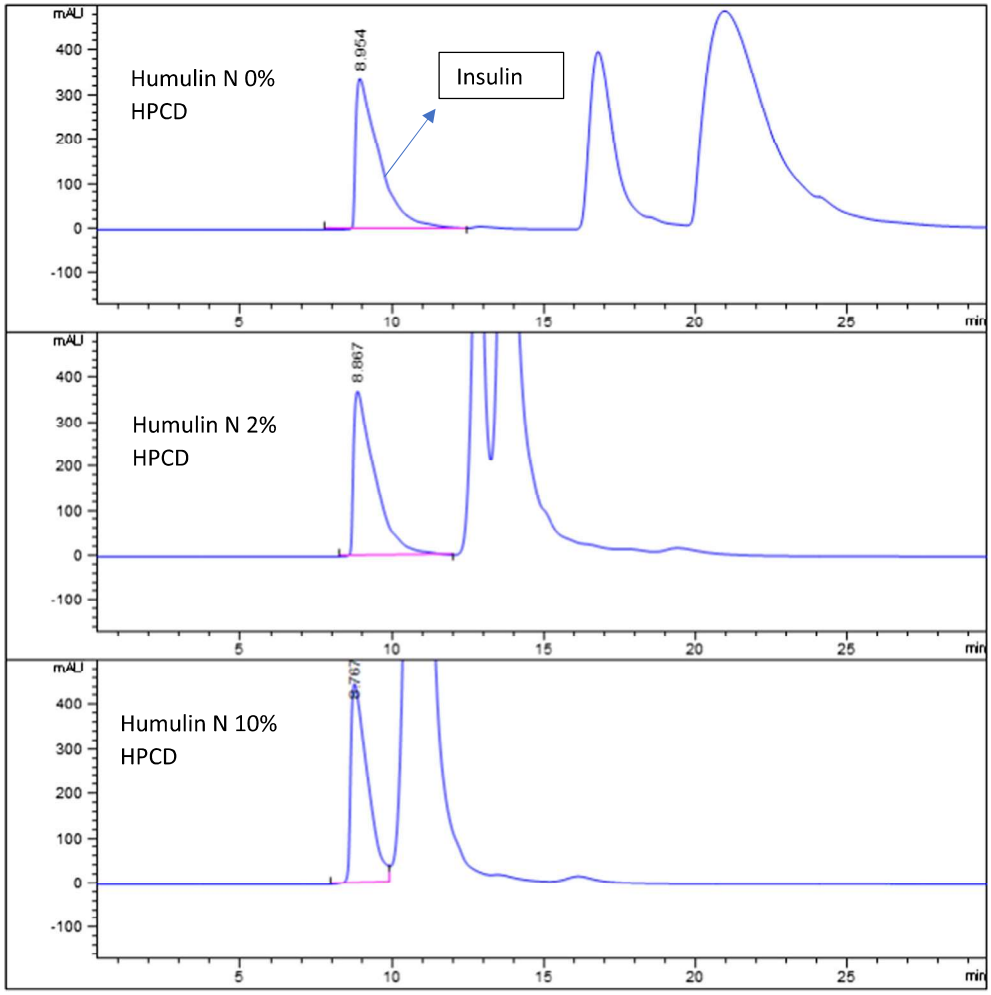

Fig. 4 Zoomed chromatogram of 2 units of Humulin N with the elution buffer containing 100 mM sodium acetate and 100 mM sodium sulfate at pH 6.0 with 0.0, 0. 2 and 10% HPCD.

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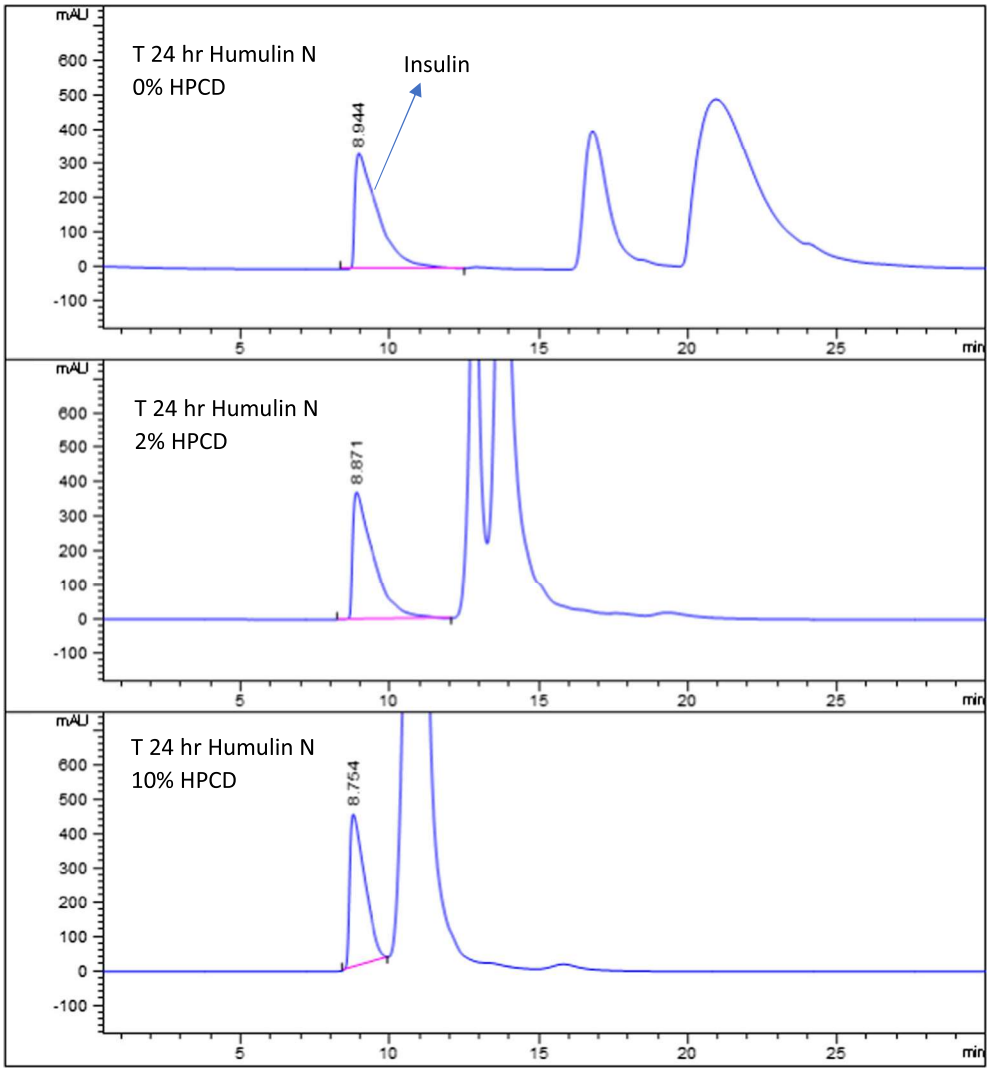

Fig. 5 Zoomed chromatogram of 2 units of Humulin N incubated at 50°C for 24 h eluted with the elution buffer containing 100 mM sodium acetate and 100 mM sodium sulfate at pH 6.0 with 0, 2 and 10% HPCD.

RESULTS

The paper presents experiments carried out using different molecules with molecular weight ranging from an insulin Peptide 5.8 kDa, a 55 kDa protein- different Antibodies ~150kDa and it's aggregates ~ 300 kDa.

Insulin and Humulin N

It is known that insulin, and insulin analogs, exhibit a complex self-assembly process to produce hexamers. In the current investigation insulin was studied with and without heat stress. Heat stress was introduced to generate aggregates. The insulin was dissolved in 0.01 N HCl at 10 mg/mL and further diluted to 1 mg/mL using PBS was evaluated using different concentrations of HPCD. The data indicates that the insulin peak performance is improved with reference to peak height, peak width, plates, the peak shape, and the retention times, when 0–10% cyclodextrin is used in the eluent (Table I). The plate refers theoretical plate number (N), which is an index that indicates column efficiency. The larger the theoretical plate number means the sharper the peaks with less peak width. In the absence of cyclodextrin, the aggregates are underestimated. Inclusion of cyclodextrin in the samples and absence of cyclodextrin in the mobile phase resulted slight improvement in peak performance of a 14 kDa peptide including RT, peak height, resolution and plates, though there is no significant effect on aggregation (Data not shown). Similarly, Right Angle Light Scattering data also suggest that there is no aggregation of biologics in presence of 10% cyclodextrin (data not shown). This tempted us to include in the mobile phase so that different samples in different formulations can be analyzed at the same time and prevent the silanol (present in the column) interaction. Inclusion of cyclodextrin in protein or peptide does not increase the aggregates as cited in literatures as well as orthogonal method such as Right Angle Light scattering (RALS) (Data not shown). Other orthogonal methods such as Analytical ultra-centrifugation

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Table II Chromatographic Peak Performance of Insulin and Protamine Buffer of Humulin N

Conditions	Area mAU	Peak height mAU	RT min	Plates	Peak Width min
0% HPCD 1st inactive excipient peak	4805	163	17.97	11,086	0.40
1% HPCD 1st inactive excipient peak	6958	417	14.73	25,735	0.22
2% HPCD 1st inactive excipient peak	7600	538	13.38	25,972	0.20
4% HPCD 1st inactive excipient peak	8173	658	12.83	26,756	0.18
6% HPCD 1st inactive excipient peak	8393	733	11.79	26,280	0.17
8% HPCD 1st inactive excipient peak	7925	797	11.56	26,837	0.17
10% HPCD 1st inactive excipient peak	8026	872	11.36	28,415	0.16
0% HPCD 2nd inactive excipient peak	13,635	205	22.97	3540	0.91
1% HPCD 2nd inactive excipient peak	17,597	417	16.49	17,453	0.29
2% HPCD 2nd inactive excipient peak	18,619	1081	14.38	25,530	0.21
4% HPCD 2nd inactive excipient peak	19,284	1343	12.83	25,434	0.19
6% HPCD 2nd inactive excipient peak	19,450	1475	12.14	24,810	0.18
8% HPCD 2nd inactive excipient peak	19,819	1585	11.78	24,975	0.18
10% HPCD 2nd inactive excipient peak	20,955	1684	11.58	23,998	0.18
0% HPCD Insulin	18,962	300	8.95	696	0.79
2% HPCD Insulin	18,323	372	8.87	830	0.72
10% HPCD Insulin	17,555	450	8.77	1166	0.60
0% HPCD Insulin T 24 h	18,669	337	8.95	693	0.80
2% HPCD Insulin T 24 h	18,434	368	8.87	802	0.74
10% HPCD Insulin T 24 h	17,967	458	8.77	1161	0.60

(AUC) and light scattering data also showed that SEC, light scattering, and AUC quantified aggregates to a similar extent for a monoclonal antibody (25). The thermal stressed sample, which generated higher aggregates was also analyzed with and without cyclodextrin in the mobile phase. The HPLC profile data indicated higher aggregates are generated and the

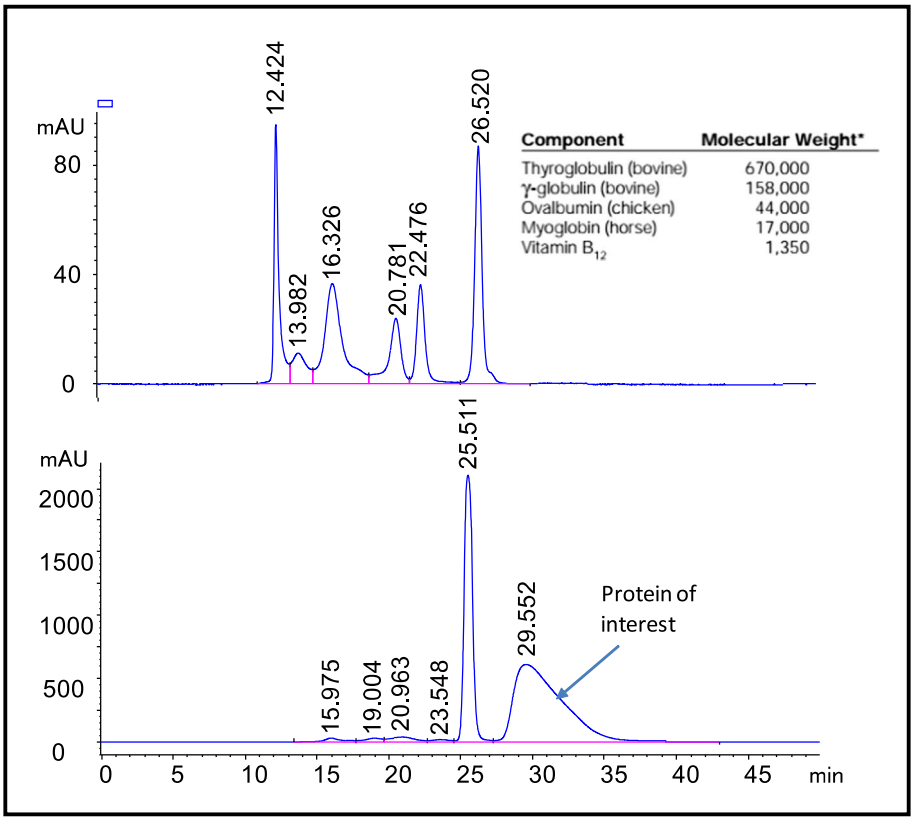

Fig. 6 Chromatographic profile of Bio-Rad molecular weight marker and 55 KDa protein response at 210 nm on Waters Protein Pak 300SW column using 25 mM Sodium Phosphate, 75 mM Sodium sulfate, pH 6.1 as mobile phase (50 μg injection).

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interaction of aggregates to the column was mitigated and the peak performance was improved significantly in presence of 10% HPCD. (Figs. 1 and 2 and Table I). The Table I shows that the aggregate quantitation in presence of 10% HPCD is 25% higher, suggesting that the aggregates binds to the column in the absence of HPCD and hence an underestimation of aggregates without the HPCD.

This method was also evaluated for Humulin N, which is formulated in 0.35 mg of protamine sulfate, 16 mg of glycerin, 3.78 mg of dibasic sodium phosphate, 1.6 mg of meta-cresol, 0.65 mg of phenol, and 0.025 mg zinc ion to provide prolonged duration of action (26). The SEC-HPLC is challenging because the molecular weight of insulin (Humulin N) is 5808 (27) and the molecular weight of Protamine sulfate is 4500 (28), which is rich in Arginine polymer. Although, a good separation is attained with the eluent 100 mM sodium acetate and 100 mM sodium sulfate at pH 6.0 with the flow rate of 1 mL/min using the Tosoh TSK G 2000 SWXL 5 µ 7.8 X30 mm column, two broad inactive ingredient peaks were eluted after the column inclusion volume, suggesting the strong interaction of inactive ingredients such as protamine, phenol and cresol with the column. This was mitigated by having HPCD in the mobile phase. When 2% HPCD is used in the mobile phase, the inactive ingredient peak eluted within the inclusion volume, and inactive ingredients are separated as two peaks (Fig. 3). These two peaks overlap, when 4–10% HPCD is used in the eluent. The performance of the chromatographic profiles of insulin and excipients improved. The relationship of cyclodextrin and peak performance are presented in Table III.

To evaluate the insulin profile of Humulin N, the insulin suspension in protamine buffer was dissolved in 0.01 N HCl and 2-unit insulin equivalent was injected with eluent containing buffer with 0, 2, and 10% cyclodextrin. The data indicates that the insulin peak performance is improved with reference to peak height, peak width, plates, the peak shape, and the retention times, when 10% cyclodextrin is used in the eluent (Figs. 3, 4 and 5 and Table II). The plate refers theoretical plate number (N), which is an index that indicates column efficiency. The larger the theoretical plate number means the sharper the peaks with less peak width. These data suggest that optimum concentration of HPCD in the mobile phase will alleviate the column interaction of difficult peptide such as insulin and its inactive excipients. Although the heat stressed formulation did not show significant difference (Table II and Fig. 5), in presence of stabilizers such as protamine, there was an improvement on the peak performance in presence of 10% HPCD (Table II).

Column Interaction Mitigation for a 55 kDa Protein

The 55 kDa protein is highly interactive with Protein Pak 300SW column. The separation was evaluated using the mobile phase 25 mM Sodium Phosphate, 75 mM Sodium sulfate, pH 6.1. The data presented in Fig. 6 shows that the main protein is eluting after the buffer peak with the apparent molecular weight less than 1350. The protein streaked due to adsorption onto the column at all pH studied and was worst as the pH increased to pH 7.0 (Figs. 6 & 7). The peak performance of protein at different pH of the mobile phases are presented in Table V and the data indicated that the relative performance of the peak with reference to peak height, peak width, and plates are improved, when the mobile phases are

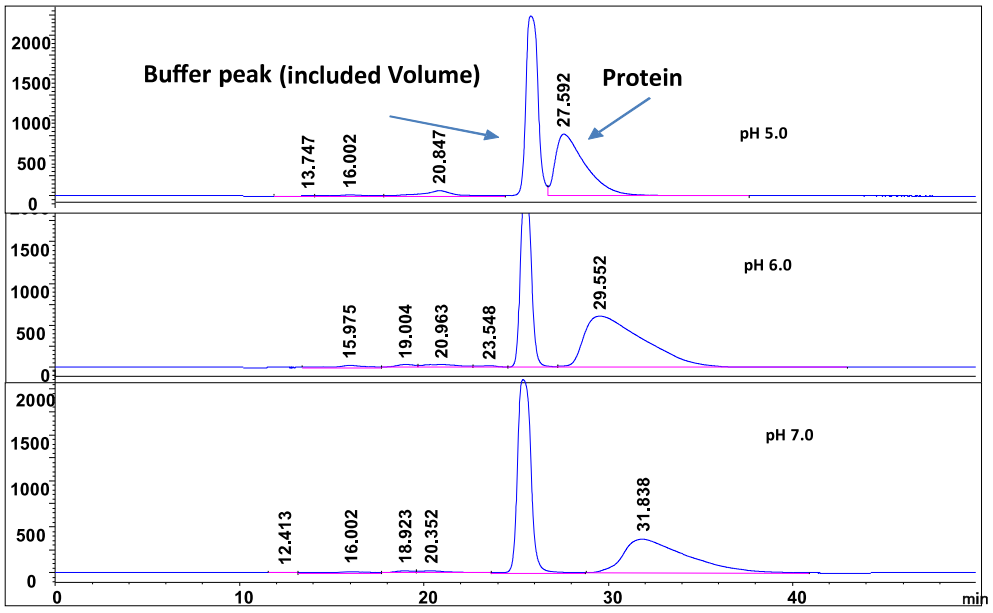

Fig. 7 Chromatographic profile of 55 KDa protein on Waters Protein Pak 300SW column using 25 mM Sodium Phosphate, 75 mM Sodium sulfate, at pH 5.0, 6.0, and 7.0. (50 µg injection).

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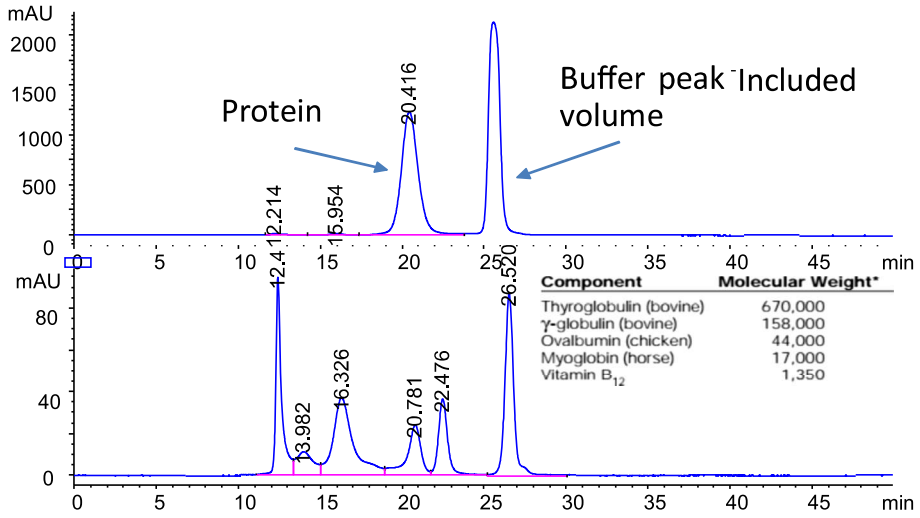

Fig. 8 Chromatographic profile of Biorad molecular weight marker and 55 KDa protein on Waters Protein Pak 300SW column using 25 mM Sodium Phosphate, 75 mM Sodium sulfate, pH 5.0 and 1% cyclodextrin as mobile phase (50 μg injection).

kept at pH 5.0 compared to pH 6.0 or 7.0 (Table V). However, the apparent molecular weight is less than 1350 eluting less than buffer peak for all pH of the mobile phases used. In contrast, with the inclusion of 1% cyclodextrin in the mobile phase the protein eluted before buffer peak and the peak showed symmetrical pattern, without any streaking and eliminating the column interaction (Fig. 8). The peak performance with reference to peak height, peak width, and plates are more improved in presence of 1% cyclodextrin (Table III) The apparent molecular weight calculated based on the retention time is very closer to the theoretical molecular weight (Table III) and the retention time changed from 28 min to 20 min in presence of cyclodextrin. Moreover, the aggregate peaks are well resolved from the main peak (Fig. 9). These data suggest that the optimum elution condition for this protein is 25 mM Sodium Phosphate, 75 mM Sodium sulfate, pH 5 and 1% cyclodextrin to improve the peak shape without adsorbing to column and elute at retention time closer to the theoretical molecular weight (Table III) and least column interaction (Fig. 7).

Polyclonal Rat IgG

It is known that most of the pharmaceutical industries use EP-specified eluent, 40 mM phosphate-0.2 M NaCl-0.005% NaN_3 buffer, pH 7.0 for therapeutic proteins such as antibodies and this method shows delayed elution of the peaks, broad peak shapes, significant peak tailing, and poor peak resolutions, in addition to the polymer (aggregate and tetramer combined) peaks of certain IgG products being partially retained by the column (7). Retention of proteins on the column and/or delayed elution due to interaction with the stationary phase has been widely reported in the literature (11–14). Thus, these results indicate interaction between the stationary phase and different molecular forms of IgG (7). It is also pointed out that aggregates and tetramer of certain IgG products are partially retained by the column when eluted with 0.2 M NaCl.

The data using the rat polyclonal antibody indicates that the antibody has no significant effect on chromatographic profile when 0.0%, 0.2% or 10% cyclodextrin were used as eluent. However, after the thermal stress, the elution profile of IgG, on the commercial column, (Tosoh 2000 SWXL) (Figs. 10 and 11 and Table IV) suggests a strong interaction with the column. This streaking resulted higher recovery of monomer AUC, because of increase in peak width and shift the RT. In addition, lower aggregate recovery was observed either be due to very high aggregate not entering the column or due to interaction with the column. The data suggests interaction with the column is eliminated in presence of 10% cyclodextrin (Figs. 10 and 11).

Table III Chromatographic Peak Performance of 55 kDa Protein on Waters Protein Pak 300SW Column Using 25 mM Sodium Phosphate, 75 mM Sodium Sulfate, pH 5.0, 6.0 and 7.0

Mobile phase pH	Peak height mAU	RT min	Plates	Peak Width min	Apparent molecular weight
5	58	27.63	1517	1.67	< 1350
6	32	29.21	487	3.11	< 1350
7	27	31.84	446	3.55	< 1350
pH 5 with 1% cyclodextrin	93	19.93	2576	1.18	49,083

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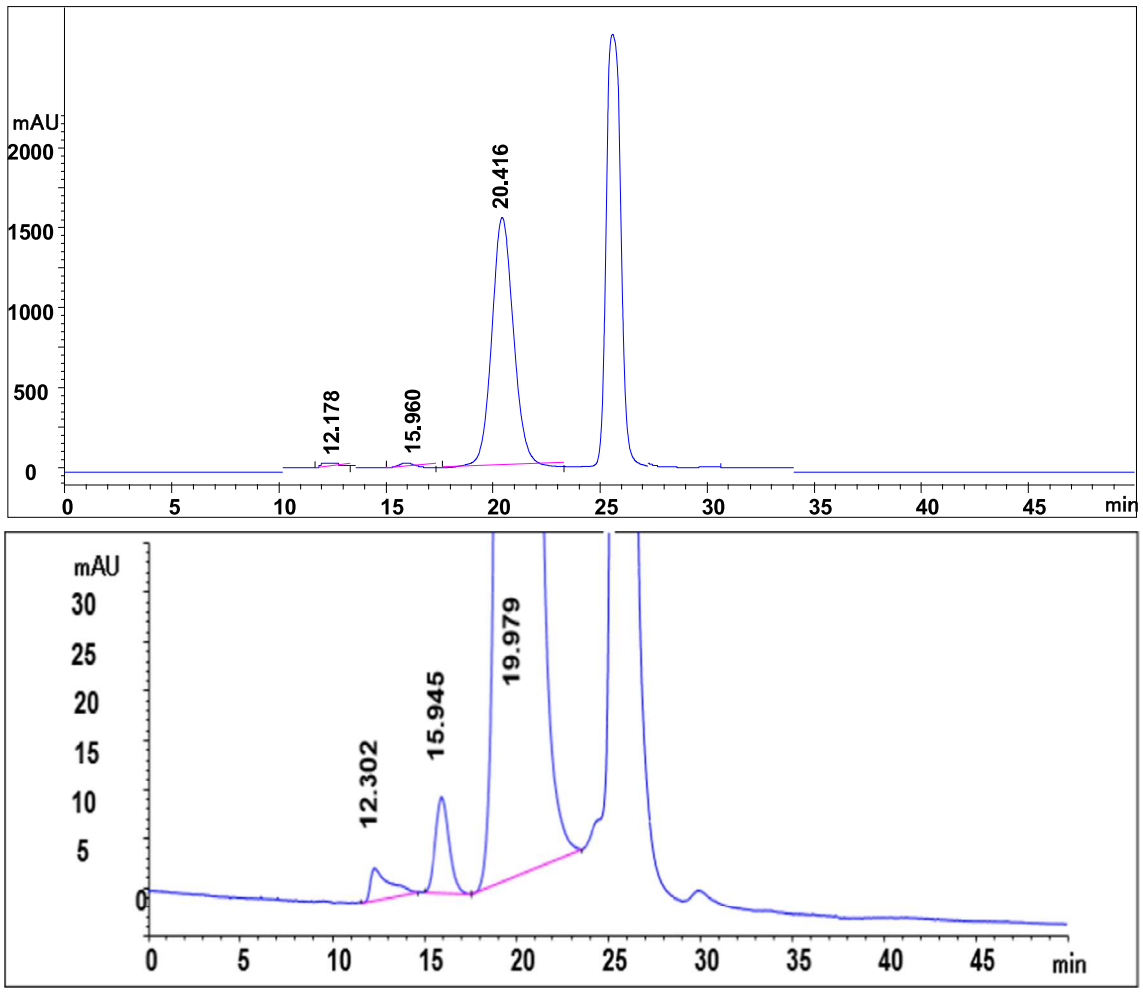
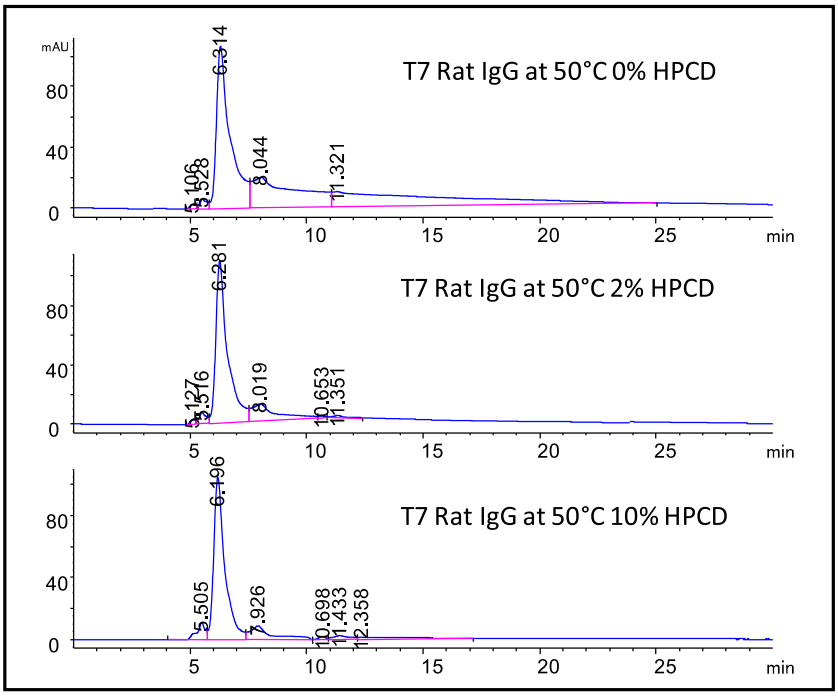


Fig. 9 Zoomed chromatographic profile of 55 kDa protein on Waters Protein Pak 300SW column using 25 mM Sodium Phosphate, 75 mM Sodium sulfate, pH 5.0 and 1% cyclodextrin as mobile phase (50 μg injection) to show the aggregates.

Fig. 10 Overlay chromatogram of 50 μg Rat IgG heat stressed at 50°C for 7 days eluted with buffer containing 100 mM sodium acetate and 100 mM sodium sulfate at pH 6.0 with different concentrations of HPCD (0–10%).



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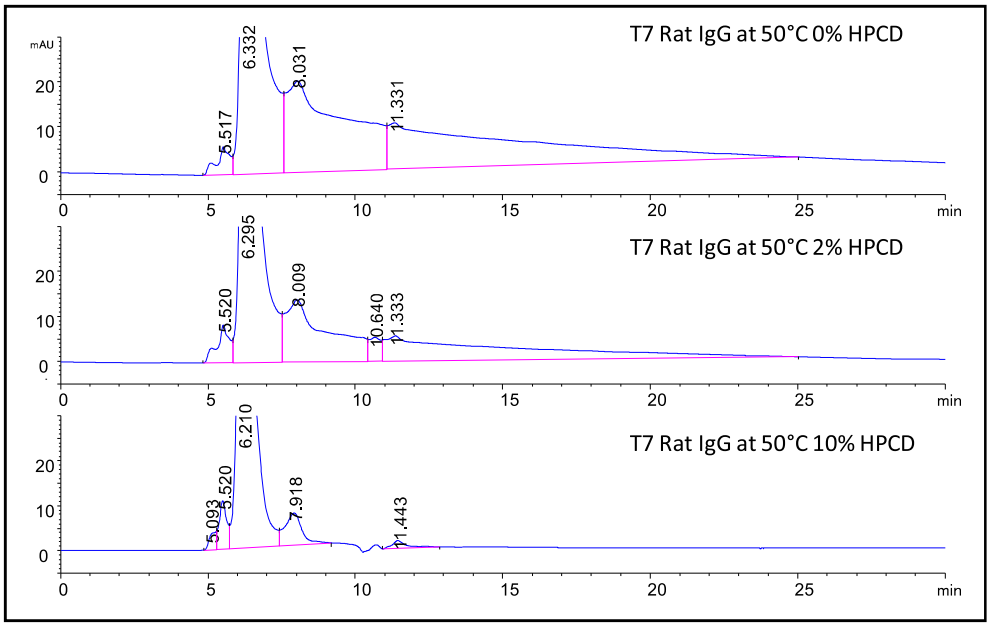

Fig. 11 Zoomed overlay chromatogram of 50 μg Rat IgG incubated at 50°C for 7 days eluted with buffer containing 100 mM sodium acetate and 100 mM sodium sulfate at pH 6.0 with different concentrations of HPCD (0–10%).

Monoclonal Antibody

In this study two commercial monoclonal antibodies Humira and Actemra were tested. The results obtained on monoclonal antibody Humira and monoclonal antibody Actemra with and without 2% cyclodextrin in the eluent containing 100 mM sodium acetate and 100 mM sodium sulfate pH 6.0, show that there is a slight shift in retention time of the two monoclonal antibodies tested and the aggregate peaks which eluted at 5.8 min are not retained in the column in presence of 2% cyclodextrins (Figs. 12-13 and Table V) for the stability samples. Though there is no significant difference in the HPLC profiles utilizing the mobile phase with and without cyclodextrin for the samples without additional stress, the HPLC profile of the thermally stressed samples, at 50°C for 24 h showed that the aggregated polymer hydrophobic peak of both Humira and Actemera with the RT of 5.8 min are bound to the column tightly and got eluted by 2% Cyclodextrin (Figs. 12-13).

Column interaction mitigation for other monoclonal antibodies.

The results obtained with and without 1% cyclodextrin in the eluent containing 100 mM sodium acetate pH 6.0 and 100 mM sodium sulfate pH 6.0, shows that there is slight shift in retention time of the two more antibodies tested and the aggregate peaks are not retained in the column in presence of 1% cyclodextrins Figs. 14-15 and Table VI.

DISCUSSION

The SEC-HPLC method is widely used to quantitate the aggregates, monomer and fragments to ensure product quality and safety of the therapeutic biologics, including monoclonal antibodies. Porous silica for SEC was explored since 1970 to improve performance by reducing particle size (1). However, it suffered from strong ionic interactions due to the acidic surface silanols. To overcome these interactions, a diol functional group is used, which has minimal hydrophobic interactions (1,29–31). However, residual silanols still remain, creating issues on column interaction of protein or peptides. To diminish interactions with these residual silanols, high ionic strength mobile phases, basic amino acids such as arginine, organic solvents (with a concentration low enough

Table IV Chromatographic Peak Performance of Rat IgG

Conditions	Area mAU	Peak height mAU	RT min	Plates	Peak Width min	% Aggregates
0% HPCD rat IgG	3364	113.87	6.299	1333	0.406	15.8
2% HPCD rat IgG	3263	115.9	6.279	1384	0.397	16.4
10% HPCD rat IgG	3371	111.01	6.209	1346	0.414	15.8
0% HPCD rat IgG heat stressed	4440	106.70	6.442	872	0.503	1.72
2% HPCD rat IgG heat stressed	4022	107.77	6.295	1080	0.450	3.29
10% HPCD rat IgG heat stressed	3440	103.54	6.210	1082	0.341	6.23

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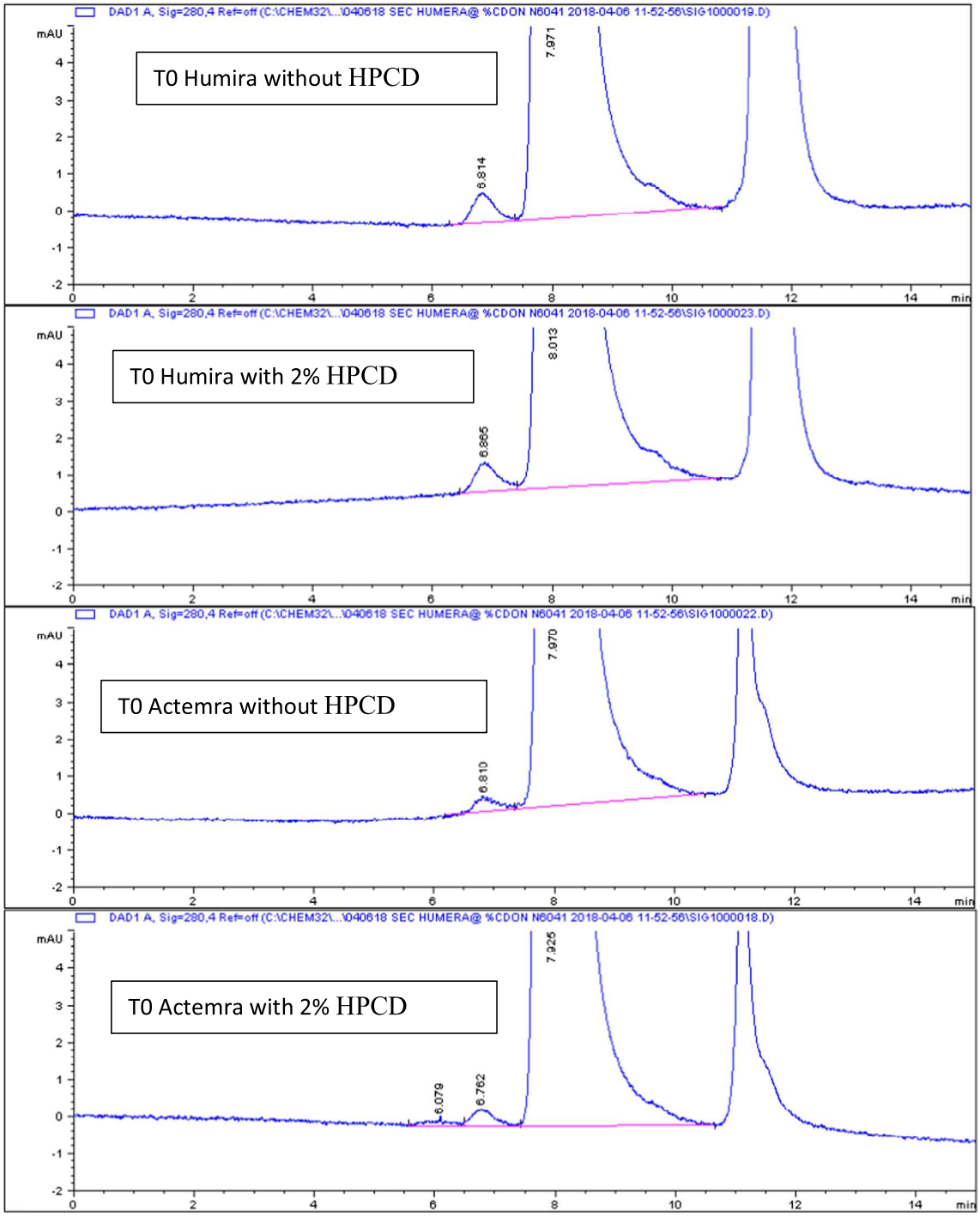

Fig. 12 Chromatographic profile T0 hr. Antibody Humira and Antibody Actemra Zoomed to same level for comparison.

not to precipitate protein) and ion pairing agents are typically used. This interaction is highly dependent on the molecules that are analyzed, in some cases the commonly used solutions works and in other cases it doesn't, affecting retention time, peak recovery and peak resolution, which could result in incorrect peak assignments and may even lead to alternate compliance decisions (14).

We report that inclusion of cyclodextrin in the eluent shows better performance characteristics, including better peak resolution, sharper peak shape without peak tailing for most of the proteins studied including the hydrophobic 5.8 kDa peptide, and 150 kDa antibodies. Though the world market of insulin is $ 20. 8 billion in 2012 (31), SEC-HPLC analysis of stability of insulin is a

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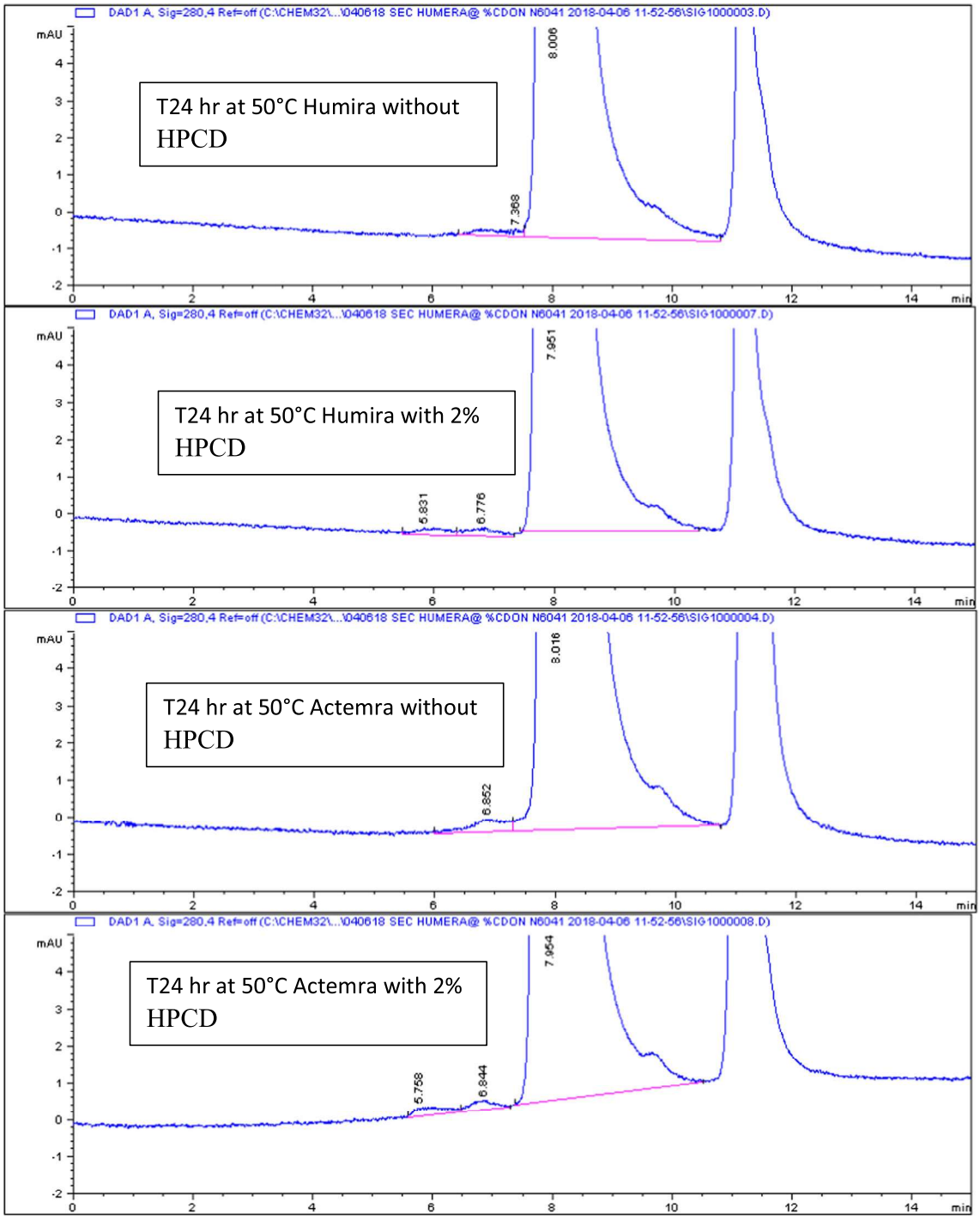

Fig. 13 Chromatographic profile of thermally stressed forT24 hr. at 50°C, Humira and Actemra Zoomed to same level for comparison.

great concern due to interaction of both insulin and inactive excipients such as protamine, phenol and or cresol to commercial columns. Teska *et al.* (32) observed significant degradation in the size-exclusion chromatography (SEC) signal over a moderate number of insulin sample injections, which generated concerns about the quality of the separations. They reported that EDTA, a zinc chelator, in the mobile phase helped to maintain column performance. Current data indicates that the column interaction of inactive ingredients of Humulin N is severe and the protamine peptide and meta - cresol buffer eluted after inclusion volume. The insulin peak performance also improved, when 10% cyclodextrin is used in the eluent. This column interaction is reduced or eliminated with appropriate concentration of cyclodextrin. In the absence of protamine, cresol

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Table V Retention Time and % HMW of Humira and Actemra

Conditions	% HMW	Monomer Retention time	Monomer AUC	Monomer Purity
Humira Antibody without HPCD	0.31	7.97	3661	99.68
Humira Antibody with 2%HPCD	0.47	7.92	3690	99.52
Actemera Antibody without HPCD	0.44	8.01	4521	99.55
Actemera Antibody with 2% HPCD	0.45	7.97	4760	99.54
Humira Antibody without HPCD after incubation at 50°C for 24 h	0.13	8.01	3606	99.86
Humira Antibody with 2% HPCD after incubation at 50°C for 24 h	0.46	7.95	3660	99.54
Actemera Antibody without HPCD after incubation at 50°C for 24 h	0.26	8.02	4623	99.73
Actemera Antibody with 2% HPCD after incubation at 50°C for 24 h	0.39	7.95	4719	99.61

and phenol, insulin formulated in PBS also showed better performance in presence of 10% cyclodextrin and the column interaction of aggregates are prevented in presence of 10% cyclodextrin.

Our results show that a significant portion of the aggregate peaks of antibodies are retained by the column in the absence of 1% cyclodextrin. The data obtained by FDA (7) also have shown that comparison of the results obtained from the elution of TSK Super SW3000 column with 40 mM phosphate-0.4 M $NaClO_4$ buffer, pH 6.8 and the EP-specified eluent, 40 mM phosphate-0.2 M NaCl-0.005% NaN_3 buffer, pH 7.0 shows delayed elution of the peaks, broad peak shapes, significant peak tailing, and poor peak resolutions, in addition to the polymer (aggregate and tetramer combined) peaks of certain IgG products being partially retained by the column on elution by the latter eluent. Thus, the percent of aggregates obtained by the EP method are significantly lower than those present in certain IgG products (7). The data obtained in presence of 1% cyclodextrin show that our SEC-HPLC method for analysis of IgG is better than the method described in the literature.

As aggregates have been attributed to play a critical role in adverse reactions our data indicates that inclusion of 1–10% cyclodextrin will mitigate the problem on column interaction of impurities, which are responsible not only immunogenicity but also potency. Addition of 1% cyclodextrin, which is equivalent to 7 mm ionic strength will not change ionic strength of the mobile phase suggesting that the prevention of column interaction is not due to ionic interaction of the protein or peptide to the column. Inclusion of cyclodextrin in protein or peptide does not increase the aggregates as evidenced by no increase in Right Angle Light scattering (RALS) after the addition of cyclodextrin up to 10% (Data not shown). Other orthogonal methods such as Analytical ultra-centrifugation (AUC) and light scattering data also showed that SEC, light scattering, and AUC quantified aggregates to a similar extent for a monoclonal antibody (25). Tong *et al.* (22) separated five small molecules by Reverse phase HPLC with hydroxypropyl-β-cyclodextrin (HP-β-CD) as chiral mobile phase additive and found that there was a negative correlation between the concentration of HP-β-CD in mobile phase and the

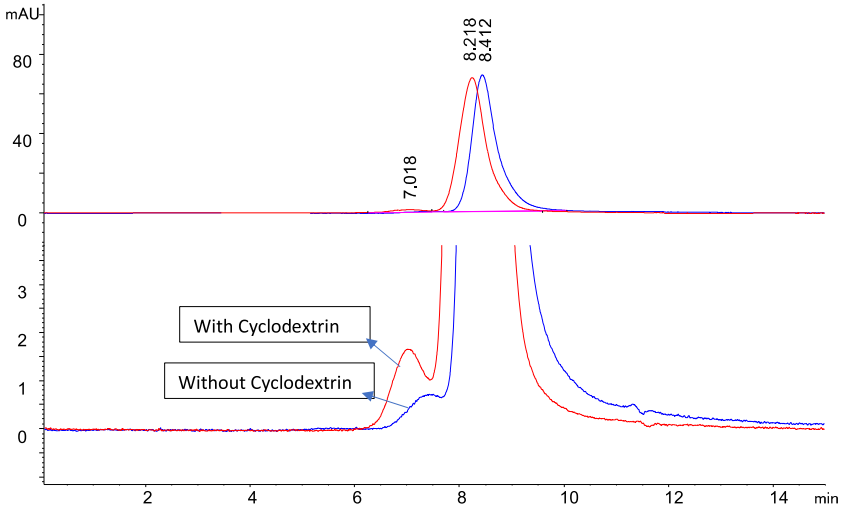

Fig. 14 Chromatographic profile of Antibody 1 on TSKgel G3000SWXL [7.8 mm × 30 cm, 5 μ] column using 0.1 M Sodium Acetate/0.1 M Sodium Sulfate at pH 6.0 with and without 1% cyclodextrin as mobile phase zoomed to show the dimer [5 μg injection].

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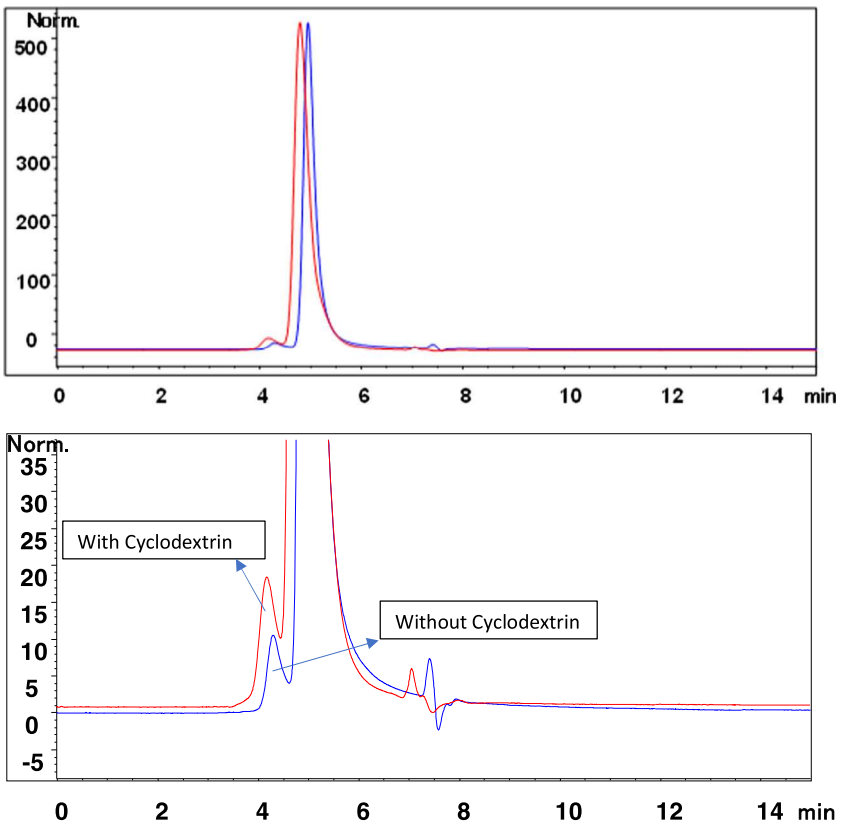

Fig. 15 Chromatographic profile of Antibody 2 on TSKgel G3000SWXL (7.8 mm × 30 cm, 5 μ) column using 0.1 M Sodium Acetate/0.1 M Sodium Sulfate at pH 6.0 with and without 1% cyclodextrin as mobile phase zoomed to show the dimer (30 μg injection).

retention time under constant pH value and column temperature. These data suggest that cyclodextrin potentially complex with silanols of RP-HPLC column also eluting the small molecule earlier.

It is known, that when cyclodextrin is used as an excipient for proteins and peptide, it keeps the protein in native form even after stress such as shear preventing the protein-protein interaction due to hydrophobic interface of protein or peptide (23,24). Cyclodextrin is generally used in the pharmaceutical industry to encapsulate the hydrophobic therapeutic small molecules to enhance the solubility of the small molecules. For example, Itraconazole is formulated with 40% cyclodextrin to increase the solubility of hydrophobic small molecule. As silanol is a hydrophobic molecule, cyclodextrin could potentially complexes with excess silanol exposed in the column, thereby, helping the peptide or protein not to interact with the silanols of commercial SEC-HPLC column. Addition of Arg-HCl in the SEC-HPLC elution buffer has been reported to give reliable results (2,33). However, the data presented by FDA reveals that the antibodies are known to interact with the column even in presence of 0.4 M Arginine. We also noted that higher Arg-HCl produce high back pressure for the SEC-columns due to its chaotropic property and possible crystallization of Arginine in the parts of HPLC systems. The suitability of usage of perchlorate have also been questioned in the literature as a chaotropic salt in the eluent for the analyses of proteins by SEC-HPLC. As perchlorate is an oxidizing agent (8) and it has been reported to dissociate protein multimers (9–11), perchlorate in the eluent may produce an artifact. The dissociation of the octameric enolase enzyme from *Streptococcus pyogenes* was observed, using $NaClO_4$, to perturb the quaternary structure and $NaClO_4$ dissociated the octamer into inactive monomers (11). According FDA that they could not find any literature report of oxidation of proteins by perchlorate at neutral pH (7). Although the oxidation potential of perchlorate is high in acidic pH ($E° = +1.42$ V), it is much smaller at the neutral and alkaline pH ($E° = +0.56$ V), our current study shows that lower pH is more optimum for preventing protein or peptide interaction to the column and the neutral pH

Table VI Retention time and % HMW of Antibody 1 and Antibody 2

Conditions	% HMW	Retention time
Antibody1 without CD	2.86	8.4
Antibody1 with 1% CD	3.06	8.2
Antibody2 without CD	1.29	8.4
Antibody2with 1% CD	2.64	8.2

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is not suitable for some proteins such as 55 kDa protein of current study. Perchlorate may potentially oxidize protein in slight acidic conditions.

As cyclodextrin does not contribute more ions, favoring ionic interaction of the protein or peptide is excluded. A bridged ethyl hybrid (BEH) particles, surface modified with diol groups resin was also evaluated as it is known for, its significant reduction in silanol activity. We observed that some hydrophobic protein/peptide could not be resolved without interaction to column in the absence of HPCD even when BEH column is used which is known to provide a significant reduction in silanol activity, thus requiring lesser amounts of salt additives to minimize the ionic interactions with proteins. (34) (Data not shown). Cyclodextrin is a complexing agent for hydrophobic molecules and are water-soluble, biocompatible in nature with hydrophilic outer surface and lipophilic cavity. They are of truncated cone or torus shape rather than cylinder because of the conformation of glucopyranose unit (35). The most common cyclodextrins are α, β, and γ consisting of 6, 7, and 8 glucopyranose units (36). β-cyclodextrin is more ideal for complexation of hydrophobic molecules (37). As these cyclodextrins are superior complexing abilities (37), they are considered as one of the favorite agent in elution buffer to mitigate, the interactions of proteins or peptide or their impurities with the residual silanols of commercial SEC-HPLC columns and improve the separation of proteins, peptides and impurities. To our knowledge, cyclodextrin is used for the first time in this study to mitigate the interaction of protein/peptides to SEC-HPLC columns. Usage of α, and γ cyclodextrins on column interaction of biologics deserves further study, though, it has same potential of complexing with the silanol.

ACKNOWLEDGMENTS AND DISCLOSURES

All authors are employee of CuriRx. The authors declare that they have no conflict of interest

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