Size Exclusion Chromatography Method for the Isoform Separation of a Novel Tri-specific Antibody

Piyasmita Das, Mohan Kumar, Karthikeyan Subbiramani, Amaresh Panigrahy, Chaitra Bhargava, Pradeep Kabadi, Anindita Das, Madhava Ram Paranandi

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Introduction:

Size variants determination is a critical quality attribute for a therapeutic recombinant antibody, since they can impact the drug product safety, potency, and efficacy. Size exclusion chromatography is the most frequently employed technique for estimation of size variant and SE-HPLC principally separates out variants based on size, shape and molecular weight.

A novel tri-specific antibody is recombinantly produced in Chinese hamster ovary (CHO) cells. Our studies exemplify the effectiveness of native SE-HPLC for separation of isoform variant (*P1) of Novel Tris-specific antibody. The study involves development of SE-HPLC method for Tri-specific antibody to differentiate the HMWP and the isoform variant of Tri-specific antibody.

SEC-MALS and functional assay by ELISA results demonstrated that *P1 peak observed in SE-HPLC contains structural isoform variant with molecular weight similar to main peak, and the enriched *P1 peak fraction had an equal potency to the main peak, confirming this isoform variant has similar access to the antigen-binding site.

Objective:

To identify and demonstrate the identity of *P1 peak observed in SE-HPLC. To demonstrate the potency of *P1 peak in comparison to main peak.

Approaches:

Separation of isoforms for tri-specific antibody using different analytical columns → **Samples enriched with Main peak and HMWPs** → **Determination of isomer mass by SEC-MALS** → **Evaluation of isolated isoforms for potency**

Method detail:

Mobile Phase Details: 10mM Phosphate Buffer + 500mM NaCl, pH 7.0

Column Details:

- **Column 1:** Waters Xbridge Premier SEC 250A 2.5um 7.8*300nm
- **Column 2:** Waters Xbridge Protein BEH SEC 200A 3.5um 7.8*300nm

Chromatographic conditions:

- **Flow rate:** 0.5 mL/min
- **Column Temperature**: 25±5°C
- **Sampler Temperature:** 5±5°C
- **Detection:** PDA (210-400nm); 280nm

Experiments & Results discussion:

✓ Using various analytical columns, variations in the %HMWP profile and content were noted during the development of the SE-HPLC method for Tri-specific antibody. Refer to **Figure 01** and **Table 1**. The *P1 peak in particular was identified to be an additional species that contributed to the greater content of %Total HMWP.

✓ The presence of *P1 peak also confirmed by other makes columns where a fronting was observed and corroborated the %total HMWP content differences.

✓ In SE-HPLC, the molecule separation is majorly based on weight where the higher molecular weight species/aggregates elute early whereas the lower molecular weight species elute later. In general, the HMWP/aggregates species and content should remain comparable between SE-HPLC columns.

✓ The nonspecific interaction is a common observation in SE-HPLC. This leads to analysis-induced artifact. The same was eliminated by analysis using different strength of salt concentration in mobile phase.

✓ To establish the identity of *P1 peak, the enriched fraction from the downstream purification process was chosen for peak enrichment. In addition, HMWP peak 2 to peak 4 was also enriched to establish the identity and impact on potency.

✓ **Figure 02** depicts the SE-HPLC profile of the Main peak peak along with enriched fractions. The SEC-MALS and potency analysis were performed on this enhanced fraction.

✓ SEC-MALS is one of the most popular characterization tools for conformation of HMWP peak under native conditions which uses the identical SE-HPLC condition (native state) in conjunction with LS detection.

✓ As evident in Figure 02 and Table 02, the additional peak (*P1) is seen to have a molecular weight comparable to that of the Main peak by the SEC-MALS analysis indicating that the *P1 may be an isoform of a tri-specific antibody.

✓ Furthermore, the molecular weight of peak 1 and 2 is also comparable with Main peak indicating the identity as an isoform variant. The SEC-MALS data confirms, that the peak 2 to peak 4 species are regarded as HMWP/higher aggregates since they have a molecular weight greater than the Main peak.

✓ Additionally, the functional experiment verifies that this isoforms has no effect on the Tri-specific antibody's efficacy.

Figure-01

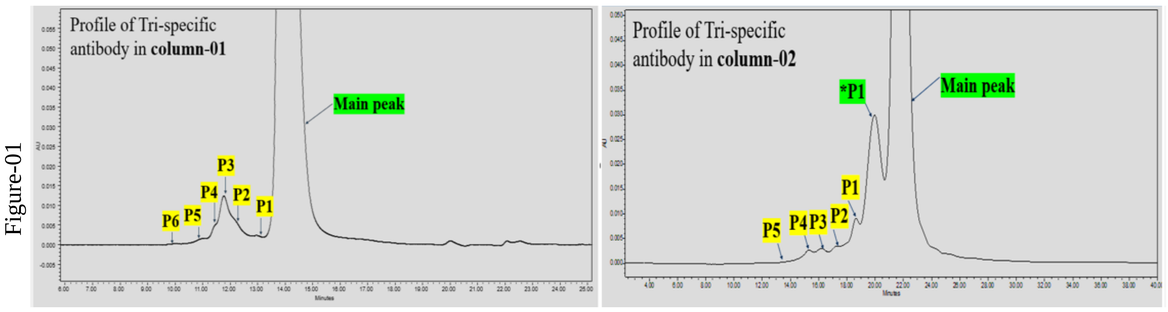

Table-01

Column details	Column label	%Total HMWP	%Main peak	%Total LMWP
Waters Xbridge Premier SEC 250A 2.5um 7.8*300nm	Column 1	3.11	96.08	0.80
Waters Xbridge Protein BEH SEC 200A 3.5um 7.8*300nm	Column 2	14.91	82.19	2.90

❖Variation in Percentage of dimer found between two different columns for relative quantification of dimer/aggregation by SE-HPLC.

Figure-02

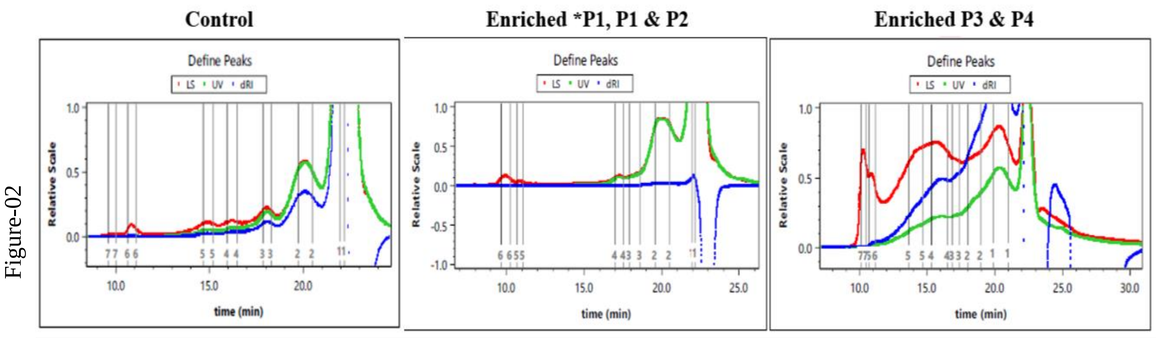

Table-02

Sample Details	Molecular Weight (kDa) by SEC-MALS					
	Main Peak	*P1	Peak 1	Peak 2	Peak 3	Peak 4
Control	147.6	138.9	160.1	265.3	322	NA
Enriched *P1, P1 & P2	132.0	136.2	134.1	140.3	NA	NA
Enriched P3 & P4	NA	157.6	205.8	297.1	374.5	553.6

❖Functional assay by ELISA has been performed to confirm the potency of isoform variant of the tri-specific antibody (**Refer Table -03 & Figure-03**).

Figure-03

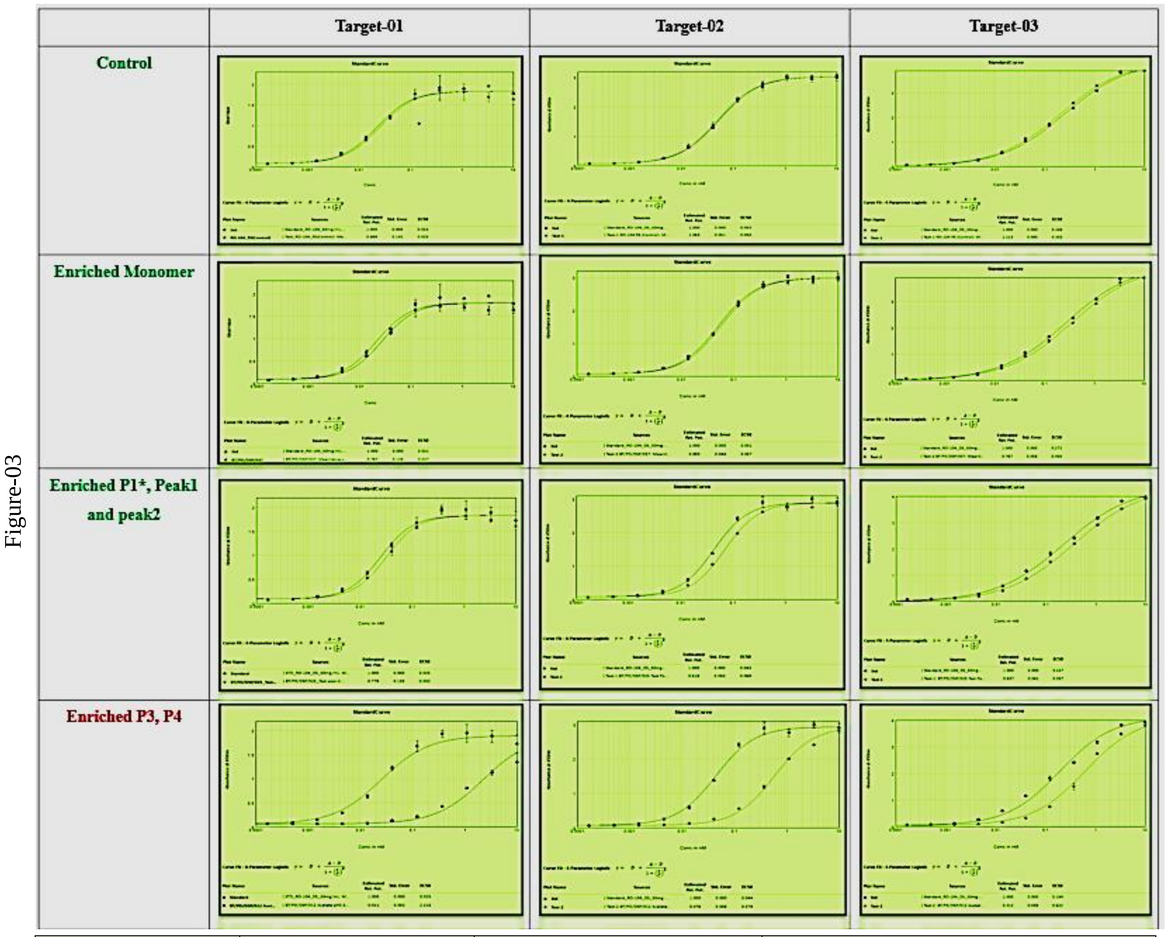

Table-03

S.No	Sample Name	Receptors	Relative Potency
1	Control	Target 1	**0.889**
		Target 2	**1.063**
		Target 3	**1.213**
2	Enriched Main peak	Target 1	**0.787**
		Target 2	**0.893**
		Target 3	**0.767**
3	Enriched *P1, Peak1 and peak2	Target 1	**0.779**
		Target 2	**0.618**
		Target 3	**0.637**
4	Enriched P3, P4	Target 1	0.011
		Target 2	0.076
		Target 3	0.312

Conclusion:

❖ The enriched Main peak and enriched *P1 + P1 + P2 were shown to have biological activity comparable to that of the control. On the other hand, there is no biological activity or potency in Enriched P3 and P4.

❖ Confirmed by the SEC-MALS result, the peak left to Main peak peak (*P1) is not an HMW impurity peak and identified as main peak's conformational isoforms. The differential elution of *P1 isoforms could be attributed to differential size/shape in comparison to main peak

❖ Further confirmation on isoforms will be carried out by HOS techniques like, NMR and HDX-MS