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High purity BioXPro™ Arginine improves column chromatography performance for protein samples

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Abstract

Column chromatography methods provide vital tools for both protein purification and analysis. A range of techniques including affinity chromatography and ion-exchange chromatography (IEX) are commonly used during downstream processing of monoclonal antibodies (mAbs), the most established format in biologics. In addition, other methods such as size exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC) are used to characterise and purify protein species. In all of these methods protein interacts with the stationary phase of the column *via* specific or non-specific interactions. The nature of these interactions and the ability to disrupt them using buffers impacts the quality and efficiency of the chromatography step. Here, the use of high purity **BioXPro™ Arginine** in the mobile phase is shown to improve recovery and separation of protein species during affinity and size-exclusion chromatography.

The amino acid arginine (Arg) is frequently used as an additive to stabilise proteins against aggregation. The mechanism of action is debated but it is known that, in a similar fashion to guanidine hydrochloride, Arg interacts favourably with most amino acid side chains and peptide bonds but its binding to the protein surface is limited meaning that it acts to suppress aggregation but not to destabilise the proteins. [1] Therefore, it can be highly effective, especially when added at high concentrations, in reducing both undesirable protein-protein interactions and preventing proteins from binding to other molecules and surfaces. These properties make Arg a beneficial reagent for downstream processing applications including column chromatography.

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Improved elution for protein A affinity chromatography

Protein A affinity columns have greatly facilitated the use of antibody formats as pharmaceuticals as they provide a highly specific and effective platform purification step used during downstream processing. The protein A ligand binds particularly strongly to the Fc domain of antibodies allowing capture of these therapeutically relevant biologics whilst contaminants are removed by washing. A subsequent elution step permits collection of the desired antibody product. The affinity of the specific interaction between protein A and the Fc domain is dependent on the exact ligand used plus the species and subtype of antibody but it is typically very tight (for instance, the K_D for binding to human IgG1 has been reported as ~10 nM). [2] This means that elution can usually only be achieved with relatively harsh conditions, typically *via* use of a low pH buffer such as sodium citrate at pH 3.5. However, many proteins display reduced stability under these conditions potentially leading to partial unfolding and aggregation meaning that elution at milder pH would be advantageous.

The elution of a mAb, lebrikizumab, captured on a 1 mL protein A affinity column (HiTrap™ Protein A HP, Cytiva) under mild conditions was assessed. The effectiveness of elution with Croda's **BioXPro™ Arginine Hydrochloride** buffer at pH 4.4 was compared to that of sodium citrate at pH 4.3 during a series of runs. In each case, 1 mg of lebrikizumab in 10 mM His pH 6.0 was injected onto a protein A column in PBS running buffer at 1 mL/min. After capture of the mAb, test elution buffers at mild pH (~pH 4.3 - 4.4) were loaded over the column at 1 mL/min for 15 column volumes (CV). Finally, a low pH buffer (0.1 M sodium citrate, pH 3.5) was run through the column to ensure that any residual mAb was eluted and so that the efficiency of the test elution buffer could be assessed.

The chromatographic traces (FIG 1A) demonstrate that elution of mAb from the protein A column using 2.0 M **BioXPro™ Arginine Hydrochloride** (pH 4.4) is more efficient than that using a pH-adjusted version of a typical elution buffer (0.1 mM sodium citrate (pH 4.3)). The elution peak with 2.0 M Arg.HCl (pH 4.4) is sharp and protein is removed from the column immediately after switching to elution buffer. The recovery is good as the elution peak accounts for the total 1 mg of lebrikizumab injected and the entire eluate is collected in a single 2 mL fraction. Subsequent switching to a harsh low pH buffer, typical of that used in protein A affinity chromatography (0.1 M sodium citrate (pH 3.4)), does not remove any further protein, confirming that 2.0 M Arg.HCl is effective at completely disrupting the interaction of protein A with mAb. A further test run using equivalent concentration of a comparator Arg.HCl from an alternative supplier demonstrated very similar results.

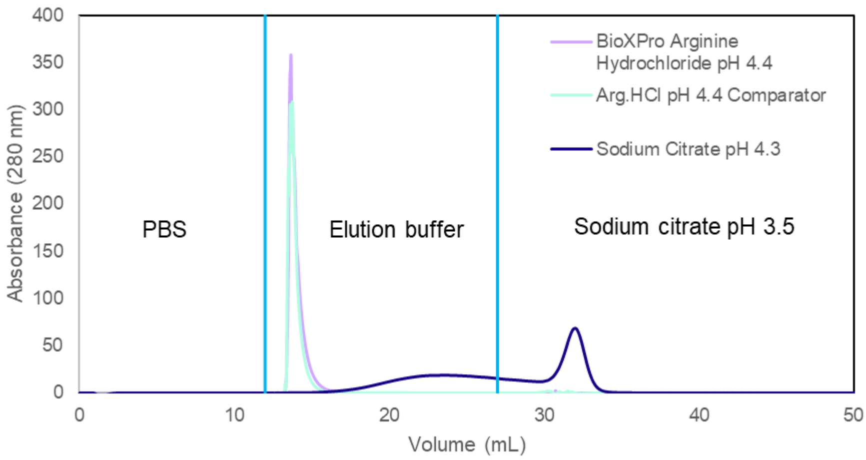

FIG 1A: Protein A affinity chromatography traces displaying elution profiles for mild pH buffers. With buffers used during the loading (PBS), elution (BioXPro Arginine Hydrochloride pH 4.4) (Arg.HCl 4.4 Comparator) (Sodium Citrate pH 4.3), and washing (Sodium Citrate pH 3.5) stages.

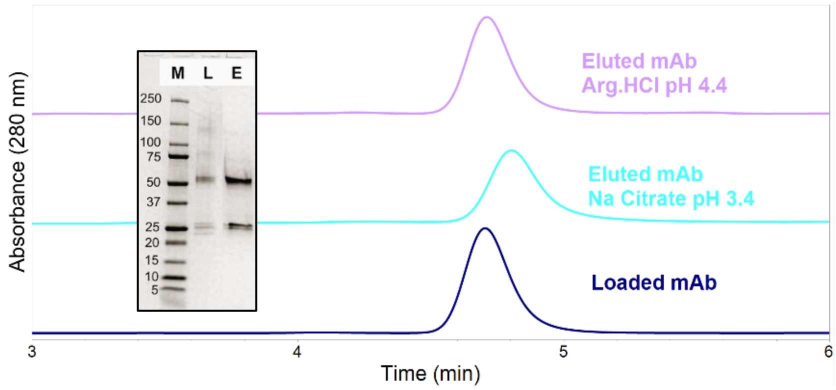

FIG 1B: Stacked SEC-HPLC traces of eluted mAb stored in various elution buffers for 21 days. INSET: Reducing SDS-PAGE of loaded sample (L) with protein eluted in Arg.HCl (E).

In contrast, use of 0.1 M sodium citrate (pH 4.3) results in a delayed protein elution with a resultant broad peak stretching out over the remainder of the 15 CV used (~13 mL). Upon switching to low pH buffer an additional peak is observed suggesting that residual protein remained on the column. Integration of the peaks suggests that over 15 CV, 58.4 % of the loaded mAb was eluted in 0.1 M sodium citrate (pH 4.3).

Characterisation of the loaded protein and the sample eluted in **BioXPro™ Arginine Hydrochloride** by SDS-PAGE demonstrated that the recovered protein was comparable to that loaded (FIG 1B). In addition, the eluted samples were stored in elution buffers for 21 days prior to analysis on an AdvanceBio SEC HPLC column (Agilent) using sodium phosphate as the mobile phase (FIG 1B). The data demonstrate that lebrikizumab eluted and stored in Arg.HCl retains the same retention time as the loaded sample, whereas a sample eluted and stored in sodium citrate at pH 3.4 demonstrates a shift.

In conclusion, Croda's **BioXPro™ Arginine Hydrochloride** makes an effective buffer for antibody elution from protein A resin under mild pH conditions. As Arg acts to disrupt protein interactions it can also be applied as an elution reagent for other types of affinity column. [3, 4, 5]

Improved sensitivity for size exclusion chromatography

SEC-HPLC is commonly used as an analytical tool to assess the quality of protein samples by characterising the size distribution of the species present. It can characterise populations of degradants in protein samples, such as high molecular weight species (HMW) including oligomers and aggregates or low molecular weight species (LMW) such as fragments. Indeed, the detection and quantification of these species is of high importance during development of protein therapeutics.

The technique depends on a column packed with porous beads, where molecules in solution flow through the column. Smaller molecules enter the pores taking a meandering, longer path than larger molecules resulting in a distribution of retention times based on molecule size. The resulting retention time can be affected by interactions of the protein with the column matrix or by interactions with other protein species present. Therefore, the result can depend on the choice of mobile phase, which may influence these interactions.

The impact of addition of Croda's **BioXPro™ Arginine Hydrochloride** to the mobile phase (sodium phosphate) during SEC of a mAb, lebrikizumab, was assessed. To determine the effect on storage stability studies the mAb was stored both under recommended conditions and under stress conditions intended to degrade the protein. Lebrikizumab (1 mg/mL) in 10 mM His (pH 5.9) was divided into two samples and stored for 21 days, the first aliquot was stored at 2-8 °C in quiescent conditions and the second was stored at 40 °C with agitation from occasional vortexing. Each sample was loaded onto an AdvanceBio SEC HPLC column (Agilent) in two different running buffers: 0.1 M sodium phosphate (pH 6.8) and 0.1 M sodium phosphate, 0.2 M Arg.HCl (pH 6.7) at 0.35 mL/min.

The lebrikuzimab sample stored under recommended conditions (2-8 °C) demonstrates equivalent results for each running buffer, albeit with a small shift in retention time. The sample is dominated by a sharp peak, corresponding to monomeric antibody with a minor peak at a shorter retention time, indicative of a small contribution from HMW. The longer retention times observed in the absence of Arg.HCl are likely due to non-specific interactions of the protein species with column matrix. These retarded retention times may affect the use of this technique to estimate molecular weight of these proteins when performed with this mobile phase.

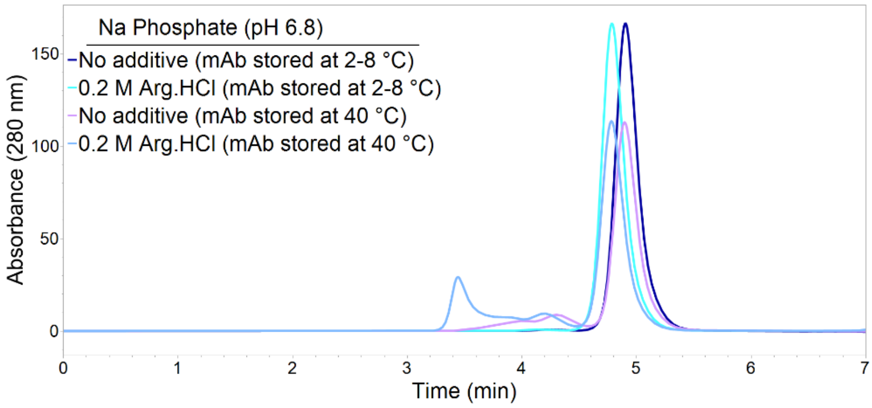

FIG 2B: SEC-HPLC traces of monomeric and degraded mAb samples run in sodium phosphate in the presence and absence of Arg.HCl.

The mAb subjected to forced degradation conditions maintains a large monomer peak, but evidence of HMW species is much increased, where the size of peak observed for HMW depends upon the mobile phase used. The 0.1 M sodium phosphate (pH 6.8) is a typical buffer used for SEC chromatography and demonstrates a small HMW peak (7.2 % height of monomer), whereas the presence of **BioXPro™ Arginine Hydrochloride** results in a higher sensitivity to HMW (28.9 % height of monomer). As aggregates or unfolded proteins are more likely to interact with the column matrix than monomeric natively-folded species their presence may be underestimated in the sample run in mobile phase without arginine hydrochloride.

The addition of 0.2 M **BioXPro™ Arginine Hydrochloride** to the mobile phase increased the recovery of soluble oligomers from a SEC-HPLC column. This is likely due to the effects of Arg on suppression of non-specific protein adsorption allied to its inability to dissociate protein oligomers, particularly at the concentrations used here. [6] These properties mean that its addition to the mobile phase is highly effective for improving the sensitivity of SEC-HPLC analyses and producing a more accurate reflection of the species present in the sample. In addition, it provides the means to increase the yield of proteins purified using SEC and to decrease the requirement for column cleaning steps as fouling may be reduced

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