

## COMMUNICATION

# Mobile Phase Containing Arginine Provides More Reliable SEC Condition for Aggregation Analysis

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**ABSTRACT:** Loss of protein aggregates due to matrix protein interaction during SEC often causes underestimation of aggregate content in the quality control of pharmaceutical proteins. Since new columns especially show stronger tendency to bind proteins, an effort should be made to suppress protein adsorption. We examined the effects of arginine on protein binding to SEC columns and found that loss of aggregates was reduced in the presence of arginine, leading to a reliable estimate of aggregate content even with newly used column and small protein load. This is not due to increased ionic strength, as NaCl resulted in increased protein loss. © 2009 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 99:618–620, 2010

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Size exclusion chromatography (SEC) is the workhorse technique for quantification of aggregates in development of pharmaceutical proteins.<sup>1</sup> However, loss and abnormal elution of loaded protein often occur in SEC analysis due to non-specific protein adsorption to the column matrix.<sup>2</sup> This is of particular concern for the analysis of aggregate content, as aggregated proteins normally show greater tendency to bind to the column.<sup>3</sup> For this reason, regulatory requirement for the analysis of therapeutic proteins asks for crossvalidation of aggregate content analysis done by SEC.<sup>4</sup> A number of factors need to be considered for developing the SEC method for aggregation analysis.<sup>5</sup> A new column has a greater tendency to

bind proteins, and hence an ideal SEC method should lead to an identical result no matter how long the column has been used. In addition, the protein recovery should be independent of the amount of proteins loaded. An optimal elution buffer should give quantitative recovery independent of the protein load. We have shown before that arginine improves performance of SEC on several proteins with a greater protein recovery and a better resolution.<sup>6</sup> This report investigates the effects of arginine on performances of SEC in conjunction of these two aspects.

SEC was run using a Hitachi HPLC system equipped with an L-6300 HPLC pump, an L-7405 UV detector and an L-2200 autosampler (Hitachi High-Technologies Corporation, Tokyo, Japan). UV absorbance data were analyzed by an EZ Chrom Elite analyzer (GL Sciences, Inc., Tokyo, Japan). Two new SEC columns (TSKgel G3000SW<sub>XL</sub>, 7.8 mm × 300 mm: Tosoh Corp., Tokyo, Japan) were used. These columns use the same lot of the

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packing material and were equilibrated with 0.1 M sodium phosphate at pH 6.8 containing either 0.2 M NaCl (NaCl-buffer) or 0.2 M arginine HCl (arginine-buffer). The flow rate was set at 0.8 mL/min throughout this study. After 2 h equilibration, both columns were loaded with 5  $\mu$ g (in 10  $\mu$ L) of mouse monoclonal antibody (mIgG1) formulated in phosphate-buffered saline at 0.5 mg/mL, which had been shown to contain 4–5% of soluble aggregates by analytical ultracentrifugation (sedimentation velocity). Elution was monitored by absorbance at 280 nm. Injection and elution were repeated 10 times. The aggregate content for each injection was determined from the peak area. For comparison of different loading amount, one TSKgel G3000SW<sub>XL</sub> column was used.

Figure 1 compares NaCl-buffer and arginine-buffer in the recovery of mIgG1 aggregates using the new columns. In both buffer systems, the mIgG1 was fully soluble. Note that the peak area of monomeric mIgG1 changed little between injections and in different buffers, suggesting that the elution of monomeric mIgG1 is not affected by repeated injections or different buffers. SEC analysis showed a similar or even higher resolution of aggregate peak in arginine-buffer, that is, the typical chromatographic resolution ( $R_s$ ) between monomeric and dimeric mIgG1 was 1.36 in arginine-buffer, and 1.25 in NaCl-buffer. The chromatographic resolution is defined by:  $R_s = (V_{\text{monomericIgG}} - V_{\text{dimericIgG}}) / 0.5 \times (W_{\text{monomericIgG}} + W_{\text{dimericIgG}})$ . Here,  $V$  is the retention volume and  $W$  is the peak width of monomeric

or dimeric mIgG1. On casual observation, a clear difference exists between the two elution solvents. The overall aggregate content was significantly higher for arginine-buffer than NaCl-buffer. The first injection showed that this sample contains 2.7% aggregate with 0.2 M NaCl and 3.5% aggregate with 0.2 M arginine used as an elution buffer. Although both numbers underestimate the aggregate content of  $\sim$ 4% in the later injection, 0.2 M arginine resulted in a more reliable value. A more detailed examination of NaCl-buffer data in Figure 1 indicates that while aggregate content gradually increased with the repeated injection, the earlier four injections gave a significantly lower aggregate content than the later six injections by  $t$ -test (5% confidence). Although the aggregate content appeared to plateau in the later injections, the level was still below 4% that was observed with arginine-buffer as shown later. After these 10 injections, the run was stopped for 14 h and resumed. A similar result as above was observed, that is, a consistently lower recovery, in particular in earlier runs.

An entirely different picture emerges with arginine-buffer. As described above, the first injection already gave about 3.5% aggregate, similar to the level achieved in the later injections of NaCl-buffer data. The next two consecutive injections gave a number close to 3.7–3.9%, already approaching the recovery observed in the later injections. More importantly, the overall recovery of aggregate in arginine-buffer was consistently higher, that is, 3.9–4.0%, than in NaCl-buffer. Similarly to NaCl-buffer, chromatographic

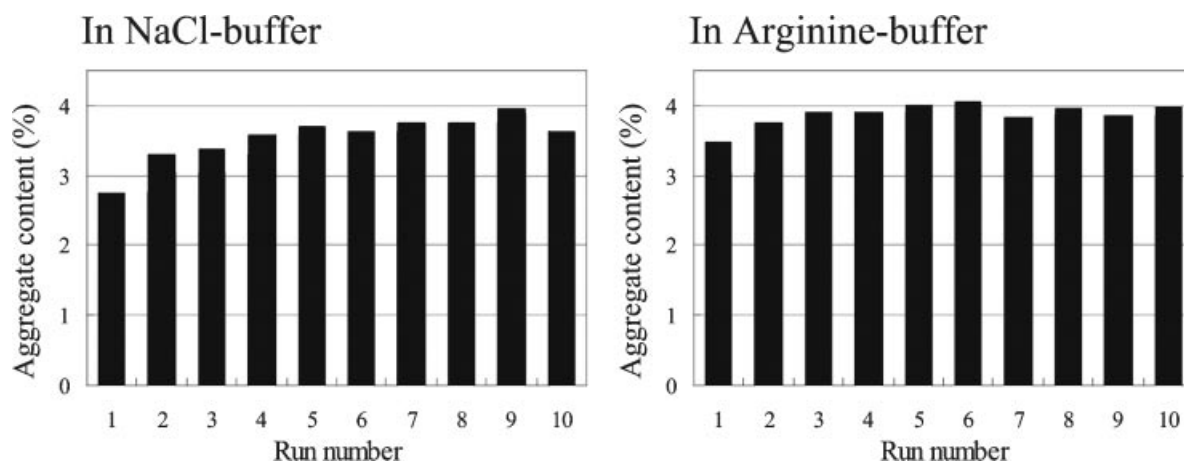


**Figure 1.** Effects of repeated injection on the recovery of aggregates. SEC buffer used was 0.1 M phosphate, pH 6.8, supplemented with either 0.2 M NaCl or 0.2 M arginine. Aggregate content is plotted against the run number (10 consecutive injections of mIgG1 sample).

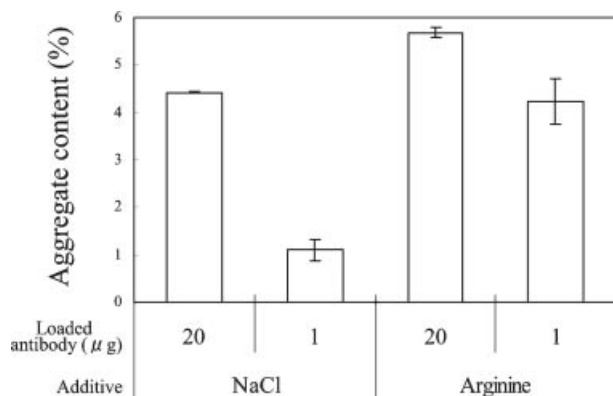


**Figure 2.** Effects of loading amount on the recovery of aggregates. The same SEC buffer as in Figure 1 was used. Aggregate content (average value  $\pm$  standard deviation) is plotted against loaded amount of mIgG1 sample.

run was stopped for 14 h and then resumed. The results were consistent with those in the first 10 injections; that is, aggregate content was nearly constant throughout the injections. It is thus evident that one would arrive at a different conclusion depending on the elution buffer used, that is,  $\sim 3.7\%$  versus  $\sim 4\%$ , which means a significantly lower estimate in NaCl-buffer. In the previous article,<sup>6</sup> we have shown that SEC column does underestimate the aggregate content using phosphate buffer and arginine-buffer improves the recovery of aggregates.

The effect of loaded amount was examined by injecting 20-fold different amount of another mIgG1 sample protein. TSKgel G3000SW<sub>XL</sub> was equilibrated with NaCl-buffer and loaded with 20  $\mu\text{g}$  of the sample. Ten preliminary runs were repeated, which allowed a constant yield of aggregate. Following the 10 injections, three consecutive runs were done to give an average aggregate content for 20  $\mu\text{g}$  injection. The aggregate content was about 4.4%. When only 1  $\mu\text{g}$  was injected, the aggregate content decreased to 1.1%. Thus, a majority of aggregate was lost during chromatography. Arginine-buffer greatly improved such dependence of recovery on injected amount of the sample protein. When 20  $\mu\text{g}$  were injected, elution with arginine-buffer gave an aggregate content of

5.7%, while the aggregate content was decreased to 4.1% with this buffer when the loading amount was reduced to 1  $\mu\text{g}$ . Thus, an overly underestimated aggregate content can be avoided using arginine-buffer, when lesser amount of sample was injected.

Figure 2 shows that NaCl-buffer is unsatisfactory even after the column has been used multiple times, in particular when the loaded amount is small; namely it leads to underestimated aggregate content. This drawback is enhanced with a new column as is evident in Figure 1. It takes multiple injections to reach a plateau level of aggregate content and even the plateau value is lower than what is observed with arginine-buffer. We have obtained similar results using multiple column lots to the results shown in Figures 1 and 2. Arginine-buffer is superior in terms of more consistent results when a new column is used and when loading amount is reduced.

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