

Assuming that columns ordered today will behave identically to those ordered previously can be unwise for analytical chemists dealing with protein pharmaceuticals

In biotechnological production of protein pharmaceuticals, analytical chemists must verify the correct structure of the recombinant products with respect to primary structure (amino acid sequence), three-dimensional structure (including correct -S-S- bonds), post-translational modifications, aggregation behavior, and immunological and biological activity. They also must detect and quantify any degradation products. In a typical characterization scheme the recombinant product must be separated and isolated, and most often an enzymatic degradation is performed. The fragments are separated, isolated, and characterized by amino acid sequence, mass, and amino acid analysis. All these procedures depend on some sort of LC, which is probably the most common individual tool in today's protein chemistry laboratory.

The column is the heart of the LC system. Although this statement often has been misused in commercial connections—column manufacturers claim that their specific stationary phase is far better than those of the competitors—we believe that the column is the single link requiring the most attention in the LC chain. Chromatographers must be able to repeat the separation they developed and optimized yesterday for use today, tomorrow, and the day after tomorrow. Registration of a protein pharmaceutical takes time, and changes in analytical methods for registered pharmaceutical products are laborious and expensive. In addition, analytical characterization of a drug compound should be robust and rugged, and regulatory demands do not seem likely to decrease. Consequently, the ability to repeat a documented analysis over a prolonged period of time is of utmost importance.

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As a result, analytical chemists must address issues such as column-to-column and batch-to-batch variations for commercially available stationary phases used in HPLC. These phenomena are rarely reported in the literature but, as we will show in this article with a variety of samples and separation techniques, they may present such a severe threat that they require appropriate action.

Reversed-phase HPLC

Aprotinin. Aprotinin is a 58-amino-acid polypeptide with a pI of 10.5. Separations at neutral and acid pH must address the unusual electrical charge of the molecule. For purity analysis, we selected a double-endcapped Vydac C₄ column (214BTP54) and developed a separation involving elution at pH 5 with an acetonitrile gradient in a buffer with high salt content (Figure 1a). The analysis is performed isocratically until the main component is eluted, and then the gradient is applied.

In 1994 we needed a new column and specifically asked the manufacturer for one from the same batch we had been using. When we used the new column, we immediately found that the separation had

been transformed; severe tailing, loss of efficiency, and different retention times were clearly evident (Figure 1b).

When we asked the manufacturer to explain this difference, we were informed that the packing procedure had been changed—distilled water was being used in place of tap water. The company also admitted that we were not the first customer to complain about the performance of this specific C₄ column after the change in procedure. At the company's recommendation, we obtained 10 samples of another Vydac C₄ column (214ATP54) that was specified as partially endcapped. Of these 10 columns, only one performed comparably with the standard operating procedure for aprotinin analyses we had developed based on earlier C₄ columns.

A recent technical note from Vydac dealing with the use of the partially endcapped C₄ column for estimating deamidated growth hormone, originally described by Riggan et al. (1), mentioned that the 214ATP54 columns occasionally exhibit tailing of the hGH (human growth hormone) peak. They recommended using a conditioning procedure for such columns (washing with TFA/acetonitrile [50/50] or running two blank acetonitrile gradients in TFA) before actual use.

The estimation of deamidated growth hormone with use of the Vydac 214ATP54 column appears in the European Pharmacopoeia and as a proposal for the U.S. Pharmacopoeia, and considerable robustness and ruggedness are normally expected for such analyses. However, variations in the stationary-phase material, and the fact that RP-HPLC analyses of proteins (as well as CE analyses) using silica-based stationary phases operated at neutral pH are much more sensitive toward stationary-phase variations than similar analyses performed at acid pH, indicate a substantial uncertainty factor for this Pharmacopoeia analysis.

Human growth hormone (hGH)

This hormone is a 191-amino-acid protein with a molecular weight of 22,125. A hitherto unknown derivative of bhGH (bio-synthetic human growth hormone) containing a trisulfide bridge instead of the usual disulfide bridge (i.e., an extra sulfur atom between Cys 182 and Cys 189; TS-bhGH), was recently described (2).

We developed an RP-HPLC analysis to

estimate the TS-bhGH content using an Asahipak C₄ column, a polymer-based column in which the skeleton of the stationary phase is made of polymerized vinyl alcohol. We had investigated more than 25 different commercially available C₄ columns, both silica- and polymer-based varieties; the Asahipak C₄ proved to be best suited for the task at hand. We eluted the column with an acetonitrile gradient at neutral pH and rapidly achieved a separation (Figure 2a). Similar separations could be achieved on Asahipak C₈ and Asahipak C₁₈ columns, but the differences in retention times between TS-bhGH and bhGH were only ~ 50% and 25%, respectively, of what could be achieved on the Asahipak C₄. In addition, interference with deamidated Met-O-bhGH was much more pronounced for the C₈ and C₁₈ columns than it was for the C₄ column.

The separation for the C₄ column was developed and optimized on three columns acquired in 1990. The columns had comparable performance; the only variation was in the degree of tailing, but this might be ascribed to their different histories. We purchased five new Asahipak C₄ columns in 1993 and found immediately that the separation was totally absent (Figure 2b).

After contacting the HPLC division of Asahi Chemical Co., we were advised to wash the columns in a manner similar to that described above by Vydac, but doing so produced no improvement in performance. Further new specimens of the Asahipak C₄ column were investigated, but the separation was lost for good. After we questioned the reason for the dramatic shift in growth hormone selectivity, Showa Denko informed us that they had taken over Asahipak's business in April 1993 and purchased the Asahipak inventory, including the stock of Asahipak C₄ columns.

The columns we acquired in 1990 and 1993 were all produced by Asahipak technical staff. Because the people involved in the manufacture of Asahipak columns at Asahi Chemical Co. had moved to new positions, the new owners of the company were unable to trace the cause of the batch-to-batch difference. Showa Denko also told us that the relevant lots of Asahipak C₄ had been manufactured before they took over the business.

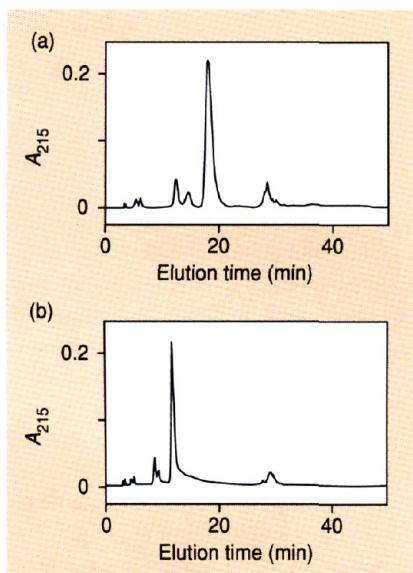


Figure 1. Reversed-phase liquid chromatograms of 30 µg aprotinin.

Chromatogram generated with (a) original 250 × 4.6 mm i.d. Vydac C₄ 214BTP54 column and (b) new column. Both columns were eluted at 1.0 mL/min with a CH₃CN gradient in 0.05 M Na₂HPO₄/0.225 M (NH₄)₂SO₄/12.8% CH₃CN, pH 5.0.

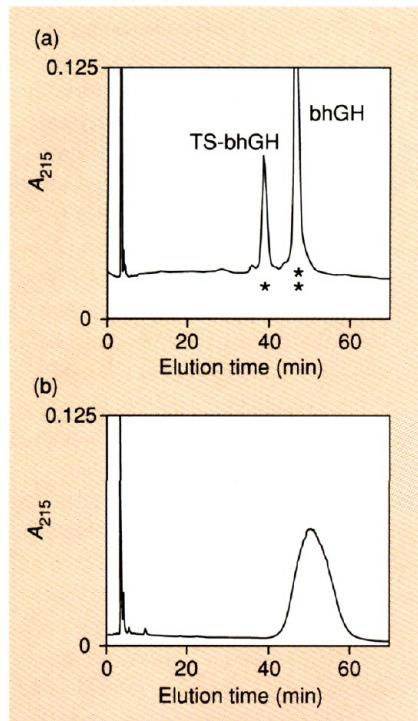


Figure 2. Reversed-phase liquid chromatograms of 40 µg of a mixture of bhGH and TS-bhGH.

Chromatogram obtained with 250 × 4.6 mm i.d. Asahipak C₄ columns purchased in (a) 1990 and (b) 1993. The columns were eluted at 0.5 mL/min with a CH₃CN gradient in 0.2 M (NH₄)₂HPO₄, pH 7.0, at 45 °C.

We also investigated the performance of Asahipak C₈ and C₁₈ columns produced in 1990 and 1993. Interestingly enough, the performance of the “old” and “new” Asahipak C₈ and C₁₈ columns was virtually identical—only the C₄ columns produced in 1990 and 1993 differed drastically in performance.

The Asahipak C₄ column was unique in another way. At neutral pH, the area in the chromatogram where TS-bhGH was found to elute (i.e., before the native bhGH) is quite crowded, occupied by such compounds as deamidated growth hormone and Met-O-growth hormone. The Asahipak C₄ was the only column of the dozens tested that would elute Met-O-bhGH derivatives after growth hormone in a much less crowded area of the chromatogram. This ability is important if analysts need to minimize interference from other bhGH derivatives.

This situation raises two questions: Why is a single stationary phase (out of dozens of similar types investigated)

unique for a given polypeptide separation? Which parameters should be measured and which standards should be run in which mobile phases?

To the best of our knowledge, no exhaustive answers can be given. To a considerable extent, we are left with the empirical approach. It is a pity that the know-how behind the “old” quality of Asahipak C₄ columns—columns that were unique in more than a single aspect—seems to be lost for good.

Gel permeation chromatography

The polymer content in protein pharmaceuticals is a critical parameter that is estimated in many cases by using GPC. For glucagon, a 29-amino-acid polypeptide, the analysis can be performed using a high-performance size exclusion column (Waters Protein Pak 125) eluted with acetic acid/isopropanol/water.

In early 1994 we asked for another column from a batch we had found usable. The UV spectrum obtained from an old column of that batch is shown in Figure 3a. The performance of the new column we received (Figure 3b) was rather surprising. We then received three columns packed with another batch of Protein Pak 125 stationary phase, and of these (Figures 3c–e) only one (Figure 3e) met the system suitability criteria (i.e., selectivity for monomer/polymer theoretical plate height) in the standard operating procedure developed with the older columns. In this case we seem to have encountered problems with column-to-column as well as batch-to-batch variations. We also experienced similar problems with estimations of high molecular weight insulin derivatives (e.g., proinsulin, insulin dimers) in insulin preparations when using the same stationary phase.

Hydrophobic interaction chromatography

The hormones bhGH and TS-bhGH can be separated much more efficiently with HIC (Figure 4) than with RP-HPLC. We eluted a PolyLC HIC column with a decreasing ammonium sulfate gradient at neutral pH and found that the addition of 2% acetonitrile was necessary to obtain sufficiently sharp peaks. Having optimized the separation on a 300 Å pore size polyaspartamide column from PolyLC Inc., we ordered four columns that had been processed together as a group during the packing procedure.

In use, we found that the columns varied considerably with respect to the mobile-phase additive needed for performing the separation in a comparable way. One of the columns needed addition of 2% acetonitrile, two needed addition of 5% acetonitrile, and another needed addition of 10% acetonitrile to the mobile phase. Because the addition of organics to HIC mobile phases is a two-edged sword, we were interested in understanding the reasons for these differences in behavior. PolyLC Inc. suggested that although the 300 Å pore size version of the column should be considered optimum for growth hormone analyses, our analyses using the rather thick coating on the polyaspartamide columns might benefit from the use of columns with larger pore sizes. Indeed, after a shift to columns with the same bonded chemistry but based on silica with 1000-Å pore size, we found that all performed in a comparable and sufficiently effective manner if they were eluted with 5% acetonitrile in the buffer.

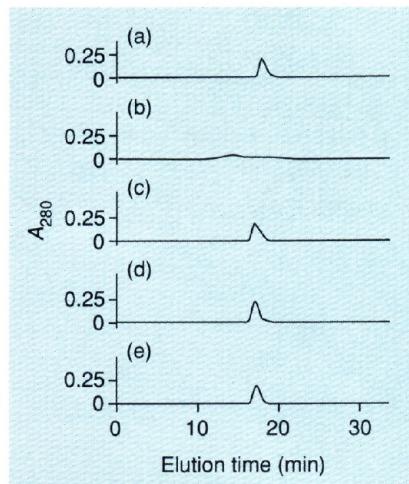


Figure 3. Gel permeation chromatograms of glucagon using five different Waters Protein Pak 125 columns.

Chromatograms in (a) and (b) were obtained from two different columns packed with the same batch of stationary phase; those in (c)–(e) were obtained from three different columns packed with another stationary-phase batch. The columns were eluted with acetic acid/isopropanol/water (30/10/60) at 0.5 mL/min.

Another consequence of the thick layer of coating on the polyaspartamide columns is the time required for the column to stabilize. It has been pointed out that it may take some time before this layer achieves its final three-dimensional structure when it is exposed to the mobile phase in HIC, which contains large amounts of salts in an aqueous milieu.

We flushed a new polyaspartamide column with water and salt buffer and started to perform growth hormone analyses. Each analysis runs for 50 min, followed by a 35-min equilibration time, a total of about 1.5 h; the retention times for TS-bhGH obtained for 150 ongoing analyses are shown in Figure 5. It is clear that after 150 analyses the column is still not stable; TS-bhGH elutes 3–4 s earlier (and reproducibly) than in the previous chromatogram. After 500 analyses, the retention time drifts \sim 5 s per 4–5 analyses, still reproducibly and still toward lower values. It seems impossible to reach the situation familiar in optimized RP-HPLC analyses, in which the retention times may vary slightly but normally are scattered around an average value. Such variations may not necessarily be fatal for a particular HIC analysis, but they must be taken into account before the system suit-

ability parameters are established in the standard operating procedure.

What can be done?

If researchers take for granted that the HPLC columns ordered today will behave identically to those ordered yesterday, they may face trouble, as we have demonstrated here for reversed-phase columns, size exclusion columns, and columns for HIC. How are these difficulties most effectively obviated?

The answer depends on the potential use of the particular column. If a given separation is to be performed only once or twice, column reproducibility can be ignored. If it is desirable to perform the separation continuously on an analytical scale and only a single usable column exists, researchers may want to scale down from the most commonly used 4.6-mm i.d. columns to 0.8-mm i.d. columns or to capillary columns. A single 250×4.6 mm i.d. column contains sufficient stationary phase for packing more than 20 individual 250×0.32 mm i.d. columns. Our experience has been that the separation of bhGH and TS-bhGH, performed on a 300×0.8 mm i.d. column packed with stationary-phase material taken from an emptied 250×4.6 mm Asahipak C₄ column pro-

duced in 1990, is quite similar to that obtained on the original 4.6-mm column.

If this solution is unsatisfactory, the chromatographer must initiate a critical dialogue with the column manufacturer. Questions to be asked should include the following: How long has this type of column been produced? Will the production of this particular stationary phase be continued? Are there any plans for introducing "basically the same column, but improved in this or that aspect" in the future? (In this situation, one should remember that "old" and "new" columns rarely perform in a comparable manner.) Can I obtain columns for testing purposes from different batches and from different production periods? How is the column tested? Is it tested at acid or neutral pH? Is the test substance relevant for my applications? (Virtually nothing can be predicted about the behavior of proteins from an RP-HPLC test chromatogram with aromates.) Is the stationary phase available in different pore sizes and geometries, or with different ligands? Can you provide a list of references on actual use of the stationary phase? Can I see a list of users of the actual stationary phase?

The manufacturer's ability to document column production, as well as knowledge and experience concerning regulatory issues for the pharmaceutical industry, should be questioned. Validation, GMP, SOP, and QA/QC should be much more than abstract ideas for the column manufacturer, who should be treated as a contract laboratory. The results originating from the manufacturer's products will, in the end, be crucial for users' patent applications, registrations, and drug applications. If researchers are to rely on a stationary phase for analytical and/or preparative use in biotechnological production of protein pharmaceuticals, the capability of the column manufacturer must be demonstrated unambiguously.

If the production of HPLC columns is (over)simplified to the application of bonded-phase chemistry to derivatize basic silica, followed by packing, a testing scheme should include at least three different sets of columns: those from the same packing procedure; those having similar basic silica, derivatized at different times; and those having different basic silica, derivatized at different times. If you can gener-

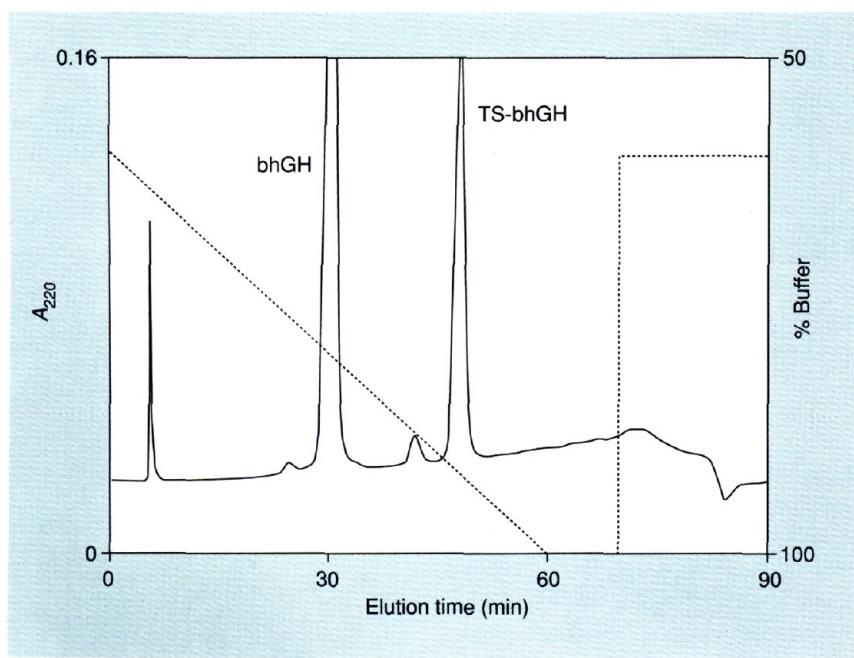


Figure 4. HIC separation of a mixture of bhGH and TS-bhGH.

The separation used a 200×4.6 mm, 300-Å polyaspartamide column (PolyLC Inc.) eluted at $0.5 \text{ mL}/\text{min}$ with a decreasing $(\text{NH}_4)_2\text{SO}_4$ gradient in $0.1 \text{ M Na}_2\text{HPO}_4/2\% \text{CH}_3\text{CN}$, pH 6.5, at 30°C . T_R for bhGH was ~ 30 min, and T_R for TS-bhGH was ~ 50 min.

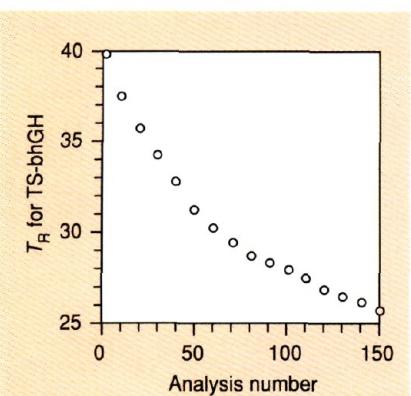


Figure 5. Variation in retention time for TS-bhGH during the initial 150 HIC injections.

A new 200×4.6 mm i.d., $1000\text{-}\text{\AA}$ polyaspartamide column (PolyLC Inc.) eluted with a decreasing $(\text{NH}_4)_2\text{SO}_4$ gradient in 0.1 M $\text{Na}_2\text{HPO}_4/5\%$ CH_3CN , pH 6.5, was used for continuous analysis of $40\text{ }\mu\text{g}$ of a mixture of bhGH and TS-bhGH. Flow rate: 0.5 mL/min ; separation temperature $30\text{ }^\circ\text{C}$. For clarity, only every tenth analysis appears on the plot.

ate similar chromatograms with your compounds of interest, the column manufacturer has a good QC system for his silica supplier, a reproducible bonded-phase chemistry, and knowledge of how to pack the columns.

The question of whether column production is an art, a technique, or a science has been debated. In our opinion, it is probably a bit of each. If chromatographers find a column manufacturer whom they trust to be a scientist in this tricky field, they should maintain the relationship. Life for analytical chemists dealing with recombinantly produced protein pharmaceuticals is already full of pitfalls, and in the area of column reproducibility it seems unwise to take unnecessary chances.

References

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- (2) Jespersen, A. M.; Christensen, T.; Klausen, N. K.; Nielsen, P. F.; Sørensen, H. H. *Eur. J. Biochem.* **1994**, *219*, 365–73.

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