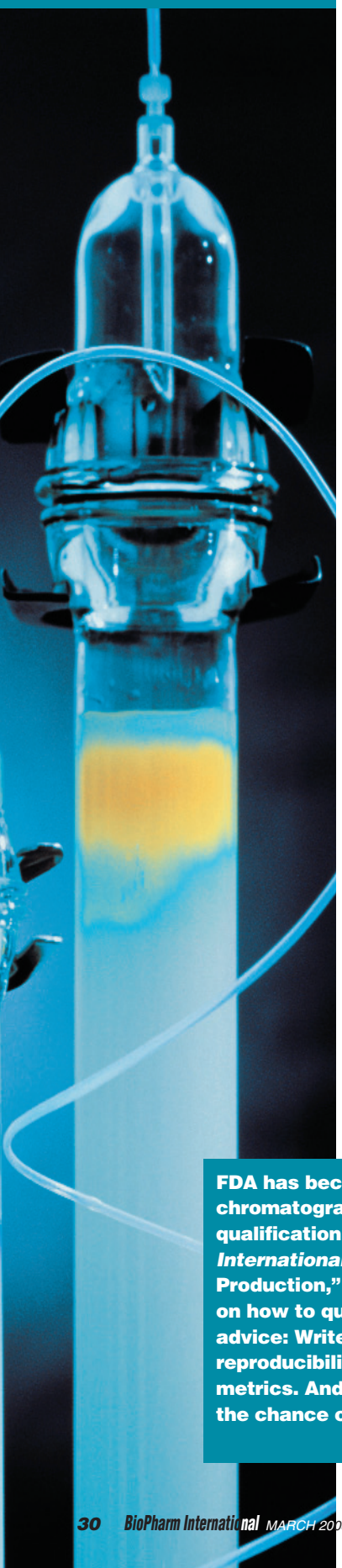


Qualification of a Chromatographic Column

Why and How to Do It

Anurag S. Rathore, Robert M. Kennedy, J. Kevin O'Donnell, Ivars Bemberis, and Oliver Kaltenbrunner

FDA has become more knowledgeable about process chromatography — and more demanding about column qualification. In the latest installment of *BioPharm International*'s "Elements of BioPharmaceutical Production," five industry experts share their insights on how to qualify a chromatographic column. Their advice: Write unambiguous SOPs. Focus on reproducibility of column packing. Choose appropriate metrics. And analyze your testing procedures to reduce the chance of erroneous results.

Preparative chromatography is the dominant purification technique in the production of biological compounds, especially for bioseparations. The column used is central to the performance of the chromatographic step. To ensure robust and reproducible column performance, columns need to be qualified in an acceptable fashion before each use.

Some of the critical elements of column qualification include identifying leachables and extractables from resins, product use, and column parts and accessories (such as residuals from manufacturing and shipping). Identity tests must be developed for chromatography resins, such as base matrices (FTIR spectroscopy, for example); functional groups and substitution levels (such as colorimetric titration); and porosity tests, or if necessary, function tests (such as dynamic capacity, selectivity, or real runs). Column qualification also involves writing protocols for training plant personnel and protocols for column hardware, packing, and operation. And qualification requires protocols for measuring bed integrity, such as background buffers, tracers, and injection volumes (see the "Measuring Column Bed Integrity" sidebar for further information and equations that help confirm the quality of chromatographic operations).

This *BioPharm International* series on the "Elements of Biopharmaceutical Production" presents the viewpoints of industry experts on issues commonly encountered in developing and manufacturing biopharmaceuticals. The series has previously covered process validation (1) and the optimization and scale-up of preparative chromatography (2). In this article, our series coordinator has brought together four other experts representing major chromatography media and equipment manufacturers and from biopharmaceutical companies to offer their viewpoints, insights, and experience on various facets of chromatography column qualification.

ROBERT M. KENNEDY

Qualifying Protocols and Hardware

Qualification is the part of process validation that ensures that the equipment and protocols are capable of fulfilling the specified requirements. Column qualification is documentation of work done to ensure that the column and the protocols written to direct operation of the column are appropriate for the purification step intended. Column qualification can be divided into two broad sub-

Measuring Column Bed Integrity

After a column is packed, integrity of the column bed needs to be measured to confirm the quality and consistency of the chromatographic operations. Several measures are commonly used for this purpose.

Number of Plates (*N*)

To calculate the number of theoretical plates for a column, use:

$$N = 16\left(\frac{t_r}{W_b}\right)^2$$

where t_r is the retention time of the probe molecule, and W_b is the width of the peak at the baseline.

Alternatively, use

$$N = 5.54\left(\frac{t_r}{W_{1/2}}\right)^2$$

in which $W_{1/2}$ is the width of the peak at one half the maximum height (figure a). Both of these equations are used widely for estimating *N* and yield very similar results.

For comparing two different columns, number of plates per meter can be calculated using:

$$\frac{plates}{meters} = \frac{N}{L}$$

where *L* is the total length of the column.

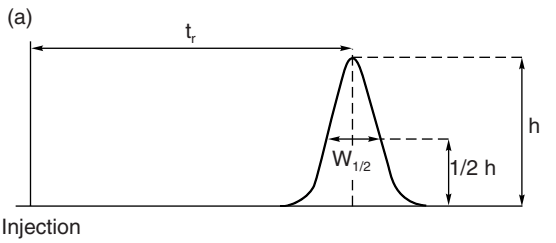
Height Equivalent to the Theoretical Plate (HETP)

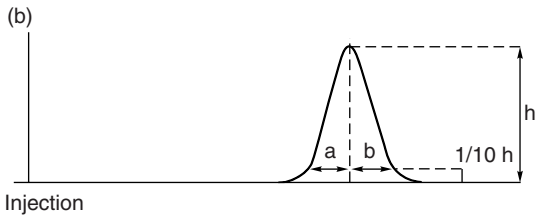
This equation also normalizes column performance to the length of the column, so it can be used to compare performance:

$$HETP = \frac{L}{N}$$

Another equation that uses a variant of HETP is reduced plate height *h*, which normalizes HETP for particle diameter d_p, and is defined as:

$$h = \frac{HETP}{d_p}$$





Tailing Factor (*T*)

The tailing factor of the peak can be defined as:

$$T = \frac{W_{0.05}}{2 \bullet f}$$

in which $W_{0.05}$ is the width of the probe peak at 5% of full height, and *f* is the distance from the leading edge to the midpoint of the peak.

Asymmetry (A_s)

Asymmetry is defined as:

$$A_s = \frac{b}{a}$$

where *a* is the distance from the leading edge of the peak to the midpoint of the peak, and *b* is the distance from the midpoint of the peak to the trailing edge (figure b). A_s is routinely determined at 5% or 10% of maximum peak height. A_s values between 0.8 and 1.4 are typically suitable. As shown in the *T* and A_s equations, A_s is not the same as peak tailing.

ject areas: column hardware qualification and column protocol qualification.

In **column hardware qualification**, the column size and manufacturing materials need to be documented. The intended packing material needs to be associated with a particular column. Resin qualification and the workflow associated with resin is another topic to be documented. The location in the plant needs to be established. A schematic needs to diagram the column and its associated peripheral equipment. Peripheral equipment, such as tubing and hoses, pressure gauges, clamps, and gaskets needs to be documented. Spare parts must be associated with a particular column as well.

A reference to the process workflow diagram should call out all the details of an individual column. The column can be defined by its serial number, its asset property number, or its unit operation number. The construction materials should be verified as part of the overall validation program, and records of those materials need to be kept in the validation record. Leachables and extractables from those materials need to be verified as well.

If the column is mobile; the process work diagram should show how the column travels, that is, where it is stored, where it is packed, how it is transported, where it is tested, and how it is removed from service and transported out of the operations area.

The schematic diagram shows all the associated lines into and out of the column. The process workflow diagram indicates the tanks that are connected to the inlet side and which tanks are connected to the outlet. Product is collected into one of the tanks on the outlet. People responsible for the process need to know the location and the condition of the product at all times. Qualification of these tanks and fluid path transfers is another part of hardware qualification. Included in the validation package for the column is the engineering drawing of the column. Spare parts are associated with an individual column, and engineers performing the scheduled maintenance on the column can be expected to require access to this part of the validation record to ensure that the appropriate spare parts are used in maintenance and reordered once they are consumed. Documentation of the gauges used to operate a column should include the calibration record of the gauge as well as the serial numbers or asset number to ensure that the correct gauge is in place during operation.

In **column protocol qualification**, process development work can contribute to the validation of the chromatography step by providing data on the stability of the chromatography resin under equilibration, operating, regenerating, cleaning and sanitizing, and storage conditions. To operate the column, an associated set of standard operation protocols (SOPs) needs to be written and tested: *SOPs must be unambiguous*. Regulatory requirements exist to ensure that operators are trained and regularly retrained to maintain their competence in performing the operation.

Each column in the purification process needs to have SOPs for column packing and testing. Once accepted for use, each column needs SOPs for equilibration, running (that is, applying feed to a collection of product), regenerating, and cleaning. SOPs are needed to direct the collection of data for use in ascertaining performance quality, to document how preventive maintenance should be done, and to instruct on how material should be stored.

Assistance with writing many of these SOPs is often available from the appropriate equipment and chemical suppliers. The supplier's technical department can be a valuable contributor to in-house writers of these protocols. Chromatography suppliers can contribute to SOPs on column packing, performance quality, maintenance, and storage, for example. Engineering companies can provide assistance with the process workflow diagrams and advice on data storage related to the physical column and the drawings and parts.

Purification by chromatography is at the heart of biopharmaceutical production. Column qualification is a critical activity in the assurance of quality in the production area.

—Robert M. Kennedy *is department director, separation sciences, Amersham Biosciences Corporation, 800 Centennial Avenue, Piscataway, NJ 08855, 732.457.8438, bob.kennedy@am.amershambiosciences.com.*

J. KEVIN O'DONNELL

The Science of Column Packing

Recent FDA inspections have shown an increased interest in the packing characteristics of process-scale, liquid chromatography columns. That is manifested by the agency's emphasis on reproducible packing of chromatography columns by pharmaceutical and biotechnology companies. Although process-scale column packing is still, in many cases, more of an art than a science, FDA — by its inquiries at inspections — is encouraging companies to move toward a column-packing science. A large part of that science is how a column is characterized or qualified after it is packed.

Resins. Many variables influence the science of column packing. The first variable is the chromatographic resin. A chromatographic resin is selected primarily for its ability to separate the molecule of interest from its impurities, not on how well it packs into a manufacturer's column. Many different resins are used in process chromatography, and all have individual column-packing characteristics.

Some resins are soft and cannot withstand even moderate pressures. As a result, when columns are packed with this group of resins, 20–30% more resin often has to be added to get the desired column volume at standard operating pressures. Semirigid resins, including those based on silica, are better able to withstand normal operating pressures and require only 5–15% extra resin to get to the specified volume. Silica resins are pressure resistant, so they can be used in columns with extremely high backpressures. Silica resins require little additional resin for efficient column packing.

Resin slurry concentration is often overlooked when packing process-scale columns. For unknown reasons, resin can pack differently when charged to the column at different concentrations. A slurry concentration that works in one column may not work as well in another manufacturer's column. Resin manufacturers generally have a good idea of what slurry concentration works best with different column technologies.

Column packing. Traditional columns with adjustable upper adaptors can be packed reproducibly with repeated practice. The caveat here is that it may take several attempts to get the first optimal pack. Once the technique is determined, subsequent packing can proceed more efficiently. Newer process-scale column designs, which include dynamic axial compression and self-packing technologies, are generally more user friendly and can be packed successfully on the first try. However these columns require more up-front training, and the hardware is usually more expensive because it includes additional equipment specially designed to assist in column packing. Expanded-bed columns may be the easiest to pack; however they also require more supervision while running.

Ideally, all resins should be defined before packing, eliminating any small fragments that can be generated by shipping and handling resin. Trace amounts of fines may not have any effect on the actual chromatography, but can eventually lead to occlusion of the bottom screens or frits. Such occlusion would ultimately create increased backpressure, which would require a reduced flow rate. As a result, the column would have a reduced throughput.

The mobile phase plays an important role in packing process columns. Generally the solvent that packs the resin most tightly is preferred. Placing and securing the upper adaptor directly on the top of the settled bed minimizes swelling when the mobile phase is changed. Exceptions to this method include some polymeric reversed-phase resins that can swell 15–25% in organic solvents. Under those conditions, a fixed adaptor or column housing could fail, with resulting obvious safety concerns. Allowing a void above the resin bed or using a dynamic axial compression column that moves with the shrinking and swelling of the resin prevents that.

Test result variations. Once a column is packed, a series of standard tests should be conducted to evaluate performance and qualify the column. The results of these tests do not predict success in the actual chromatography step; however, the results are useful for column packing reproducibility. It is this reproducibility that most interests FDA.

Troubleshooting Performance Evaluation

$A_s < 0.8$
- Overpacking the column
- Packing at too high of a pressure
- Column bed cracking

$A_s > 1.4$
- Column not packed "tight" enough
- Clogged screens or frits at top or bottom of the column
- Air pockets in column hardware void spaces
- Poor injection technique

High HETP
- Injection sample or detector too far from column
- Column not packed efficiently

Low HETP

Probe molecule retained on column because of interaction with functional group or backbone

Usually a small, unretained probe molecule is used for standard column tests. Most chromatographers use a UV-absorbing molecule such as p-aminobenzoic acid (PABA) or acetone. Alternatively, concentrated sodium chloride spikes are monitored using a conductivity detector. Many more companies use sodium chloride because it is almost always used in the mobile phase of applications that don't use reversed-phase chromatography (RPC).

Injection volume becomes more important as particle size decreases. A 0.1% column volume injection is a good starting point. The flow rate for such a test should be 60–80 cm/hr for best results. Data can be analyzed by computer if recorded digitally, or if not, data can be calculated by hand.

The location of the "injection loop" is important and should be located as close to the column inlet as possible. The detector at the bottom of the column should also be located as close to the column as possible. Sometimes neither suggestion is possible. In such cases, column performance will be noticeably less than what is observed at bench scale. That might not be interpreted as a negative result as long as the root cause is known, and the results are consistent. In future column-packing tests, similar results must be observed.

Fronting can occur if sodium chloride is injected onto an ion-exchange resin with a water mobile phase. Adding salt to the mobile phase

(0.1 M NaCl, for example) usually eliminates that problem. With hydrophobic-interaction chromatography (HIC) or RPC resins, using acetone or PABA can generate tailing or higher-than-normal height equivalent to a theoretical plate (HETPs) because of interaction with the functional group or resin backbone. Adding small amounts of organic solvent (10–20%) to the mobile phase eliminates that interaction (see Table 1 for troubleshooting performance evaluations).

Operators on the manufacturing floor should know how column performance affects the separation of the target molecule. In some cases, less-than-optimal column performance values may be tolerated because of the column's function. For instance, higher HETPs may be acceptable when the column is used for a simple capture step with a step elution. Tailings may also be acceptable if no closely eluting impurities are found and the extra volume is handled easily in subsequent unit operations.

FDA is now more knowledgeable about operations using process-scale column chromatography. It is now incumbent on pharmaceutical and biotechnology companies to address FDA inquiries with more appropriate scientific responses. One of the ways to do this is through reproducible column packing as indicated by comparing the performance of one column pack to another. That, in turn, helps to predict repeated success in purifying the target molecule.

—J. Kevin O'Donnell *is the technical service manager at Tosoh Biosciences LLC, 156 Keystone Drive, Montgomeryville, PA 18936, kevin.odonnell@tosohbiosep.com.*

IVARS BEMBERIS
Choosing the Right Metric

To ensure robust chromatographic performance, it is essential to have a proper packing and qualification protocol. Because chromatographic purification typically involves several columns, packing and qualifying columns is an important process validation concern. An effective packing and qualification protocol typically consists of a prepacking checklist, a description of the media's properties, choosing the right metric for column qualification, and qualifying the packing protocol.

Prepacking checklist. A column's mechanical robustness must be ensured before packing. That should include an inspection of all column seals, bed supports, and bolts to ensure they are fit for use. Hydrophobic bed supports, such as polyethylene, must be prewetted and checked for uniform wetting. Before packing, the column should be assembled and pressure tested with water-for-injection (WFI) to a pressure in excess of the highest operating pressure anticipated during the process.

Media properties. Understanding the flow and pressure characteristics is essential to reliable packing. Determining the compressibility of the media to be packed is most critical to ensuring that the proper amount of media is packed into the column. As always, the conditions of packing must exceed those to be experienced during the process to ensure bed stability and an extended cycle life for the packed column.

Choosing the metric. An appropriate metric must be chosen for qualifying the column. Conventional practice is to measure the HETP and the asymmetry (A_s). However, the usefulness of a method can be realized only if the method can be repeated with sufficient precision to allow it to be used to compare the packing performance over a period of time. HETP and A_s test results can be applied in many ways to aid in the overall control of the chromatographic process, from initial packing qualification to use as a periodic in-process check of bed stability. HETP and A_s tests are, with increasing frequency, used to verify bed integrity throughout the service life of a packed column. In the biotechnology industry, where processes are frequently campaigned, the use of HETP and A_s testing to requalify a column coming out of storage has particular merit.

Choosing the marker. Usually, pulse injection of a nonreactive marker is used to qualify a column. The marker must be chosen carefully to ensure that it is representative of the process and because it affects qualification. The marker can be an "internal" standard, selected to mimic the protein to be processed. Or the marker can be a generalized, "external" standard, such as acetone, NaCl, or NaOH.

Pulse injection is the favored packing method because it uses a small injection volume. However, because precise, nondiluted injections are needed, that small volume places a great burden on the test. The injection method must be refined to ensure it is precise, repeatable by many operators, and includes this desideratum: *It is strongly suggested that the qualification test be carried out using an automated skid.*

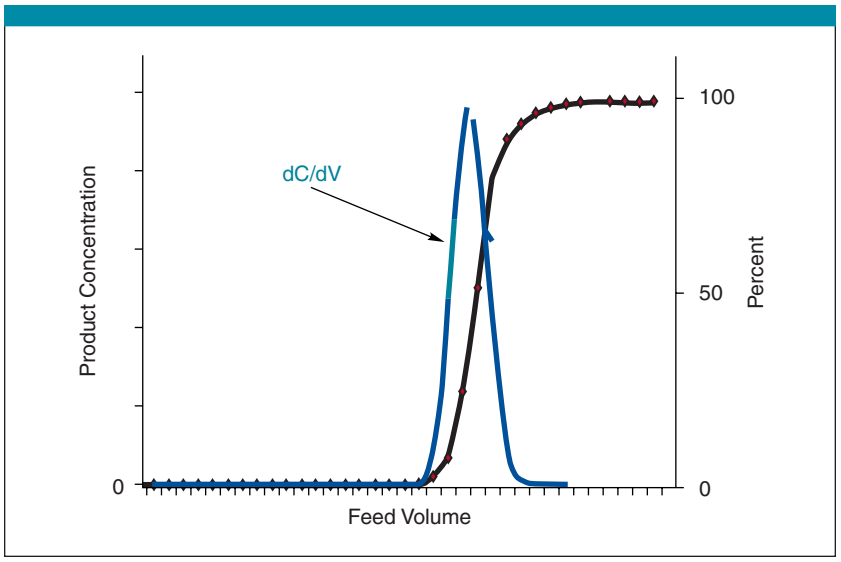


Figure 1. Frontal analysis curve and calculated peak

Pulse injection tests are intended for assessing the uniformity of the packed bed, not for assessing aspects of the hold-up volume of the HETP test rig, the inlet, or the flow cell. Therefore, the injection volume should be sufficiently large so that it remains undiluted by such hold-up volumes. Generally, media vendors suggest injection volumes of 1–2.5% of the packed-bed volume. For consistency, Millipore suggests that the volume be fixed at 1% of the total column volume. For a column with a tube height of 50 cm, the injection volume should be equivalent to the volume contained in 0.5 cm or 5 mm of the column to be tested. (For a column with a 45-cm diameter and a 50-cm height, the injection volume should be 750 mL.)

In its purest sense, the qualification procedure is really an "integrity test" of the packed bed. Postpacking testing is advised as a means to periodically validate column integrity. The repeatability of HETP and A_s data over the life of the packed column gives assurance that the packed bed remains stable and the column continues to be suitable for service.

An alternate metric for column qualification. In normal chromatographic operations, the column is exposed to a variety of buffers during equilibration, loading, washing, eluting, regenerating, and cleaning. During these steps, the column undergoes a number of buffer exchanges. Data from these steps can also be used for column qualification. The process is similar to the frontal analysis used for determining media capacity. However as a qualification technique, data from buffer exchanges have the advantage of requiring no process piping changes to run the test. So the potential for operator-induced variability is greatly reduced.

Frontal curves and pulse injection peaks don't look the same, but they can provide precisely the same data. A uniform frontal curve indicates that the packing is of high quality, the same as a narrow, symmetrical injection peak. The frontal peak data can be manipulated to compare it with a pulse injection peak. Taking the first derivative of the frontal curve produces the same curve as its analogous injection peak (see Figure 1).

Qualification of the packing protocol. A packing protocol must be rigorous to ensure good packing. Figure 2 shows the development of a packing protocol for Sephacryl S-200 HR resin — a gel filtration resin, which is a challenge to consistently pack well. The acceptance criteria required that Millipore demonstrate five repeat packings (in an IsoPak 630-mm column) meeting the performance specification: HETP $\leq$ 0.02 cm and A_s = 0.8–1.2. Client operators would then use the same packing protocol to pack the column to within the performance specification. The data in Figure 2 confirm that the seven packings all met the qualification (and process) specifications.

A rigorous packing protocol ensures good packing, which in turn ensures that columns can be well packed by any capable operator. Variability in packing methods must be understood, and the test method must be properly set to the qualification specifications. Some conservatism is suggested in finalizing the specifications because testing may be conducted on initial packing as well as throughout the service lifetime of the column and gel. Robust packing methods make qualification test results more consistent and reproducible.

—Ivars Bemberis *is technology manager, chromatography, Millipore Corporation, 80 Ashby Road, Bedford, MA 01730, 781.533.8452, fax 781.533.3110, ivars_bemberis@millipore.com.*

OLIVER KALTENBRUNNER
Preventing Erroneous Results

Chromatography column packing integrity is usually tested before process use of a column. Typically, a pulse of a noninteracting sample tracer is applied while flowing a column signal (which alters the initially rectangular tracer) under defined conditions. An analysis of the response signal of the pulse sample can be used to assess

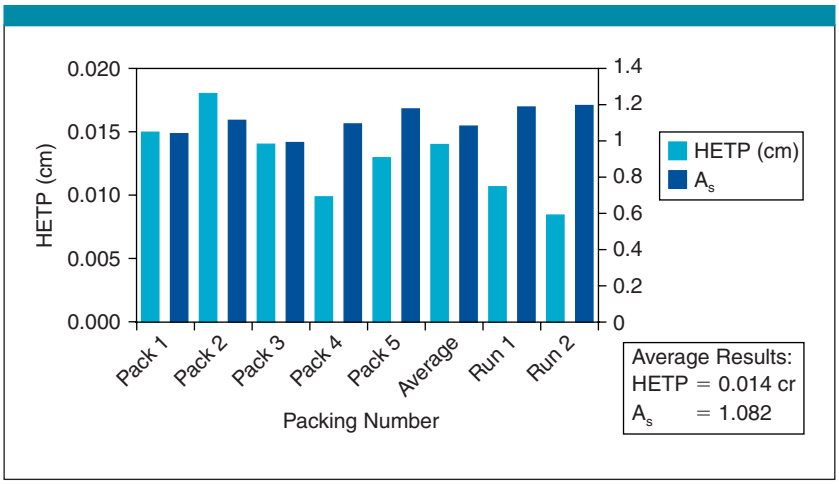


Figure 2. Summary of Sephacryl S-200 HR packing qualification

packing quality by determining the number of theoretical plates (N) of the column. N can be interpreted as the signal-to-noise ratio of the retention time, that is, the ratio between retention time and the peak width of the tracer pulse (see Figure 3). By relating N to column height, the HETP can be calculated and compared to the resin bead diameter to calculate the reduced plate height h. Chromatographic theory based on the van Deemter equation (2) provides a clear interpretation for the meaning of h as

$$h = A + \frac{B}{Pe} + \left(C \times Pe\right)$$

where h is influenced by the quality of the packing (A), diffusivity of the tracer molecule (B), and the mass transfer characteristics of the resin beads (C). For column qualification, test conditions have to be chosen to maximize the contribution of the packing quality and minimize the contribution of diffusivity and mass transfer resistance.

Making diffusivity invariable. To optimize column packing for qualification, the A term needs to be maximized by minimizing the B and C terms. Whereas in typical protein chromatography the B term can be ignored because the diffusion of macromolecules is insignificantly small compared to the other contributions, the same is not true for small molecule tracers. As demonstrated by Knox, h has its minimum value at $Pe \sim 5$. Because h grows quickly at $Pe < 5$ and increases gradually at $Pe > 5$, the flow rate region between $5 < Pe < 10$ gives the most stable results for HETP testing (4). The definition of the flow rate range based on the Péclet number indicates that, for reproducible and transferable test results, the diffusivity of the tracer molecule has to be invariable.

Additional information can be gathered when analyzing the A_s of the peak. Typically, a fronting peak indicates a compression that is too low (with an increased risk of column channeling), whereas peak tailing indicates excessive packing compression. However, interpretation of A_s is normally not that simple because most extra column equipment introduces exponential washout behavior that also manifests as peak tailing. Nevertheless, for a given column in a defined test setup, a change in peak A_s can typically be traced back to over or under compression of the packing.

Accounting for broadening. In large-scale chromatography, less-than-ideal theoretical HETP results are typically accepted because the chromatographic setup in a manufacturing plant is designed to handle larger volumes and flow rates than were used for the column qualification test. The broadening introduced by switching valves and the monitoring unit can be significant for an integrity test. To assess a specific equipment setup, a UV detector can be put before the monitoring assembly. The alteration of a UV transition signal on its pass through the assembly can be used for qualifying the monitoring setup. In our measurements, typically the apparent mixing volume of an assembly is between 20% and 40% of its actual volume. The volume of a $^3/_4$-inch OD monitoring assembly, including a column-switching valve, can easily exceed 200 mL with about 50 mL of apparent mixing volume. With a simple estimate of retention for a nonretained small tracer molecule of about 0.95 column volumes, and an ideal HETP of about 5 particle diameters, we can easily estimate the expected band broadening (σ) in the packed bed:

$$\sigma_{Column}^{\ 2} = \frac{V_R^{\ 2}}{N} = \frac{\left[\left(\frac{d^2\pi}{4}\right) \times L \times 0.95\right]^2}{\frac{L}{5\left(d_p\right)}}$$

Comparing this column broadening to external broadening of the system reveals that, in many cases, the monitoring equipment used for preparative runs significantly affects the column qualification result. Ignoring this aspect can cause problems when setting column acceptance criteria during process transfers. Difficulties are typically exacerbated when packing with a resin with a small particle size, for which the packing quality process requirements are higher and, at the same time, the relative negative effect of the

setup is also higher. Obviously the tracer application technique can have an even bigger effect on the test result, and a defined and reproducible sample application method or device needs to be established.

Preventing erroneous conclusions. Once reproducible test methods are in place, HETP results can be used to accept or reject columns for processing. Acceptance criteria should be set primarily by considering the best practice for column packing and the available equipment, then establishing criteria that are specific to that system. Consequently, there is not necessarily a close correlation between a column's qualification test result and its process performance. However, as in any acceptance test, there are two possible erroneous conclusions.

The most likely error is that a column fails the test criteria but would not fail to meet process requirements. This is the typical error to expect if the testing operation failed to follow the required best practices. It causes an operational problem of producing extra work and unnecessary repacking, but it does not set the process at risk. Setting appropriate acceptance criteria, reproducible test methods, and operator training can minimize the risk.

The second type of erroneous result is to accept a column that is not fit for the process. This obviously sets the process at risk and needs to be avoided. Reasons for this error can be column failure occurring at a particular flow rate, or viscosity of a process feed while the test conditions allow good packing integrity.

Bed stability is not only a function of the bed height and compression, but also of the height-to-diameter ratio of the packing. Identical linear velocities cause increasing bed compression with increasing column diameter. This leads to decreasing maximum operating linear velocities, with increasing column diameter (5). If the process flow rates are developed using small column diameters without consideration of potential flow rate limitations at large column diameters, bed instabilities can occur that are not evident during typical column qualification conditions. Consequently, test conditions have to be evaluated column by column, considering the process conditions at the respective scale.

—Oliver Kaltenbrunner *is in process engineering, MS: 30W-2-A, Amgen Inc., One Amgen Center Drive, Thousands Oaks, CA 91320, 805.447.2641, fax 805.499.5008, oliverk@amgen.com*

Corresponding author and series coordinator ***Anurag S. Rathore*** *is at Pharmacia Corporation, GG3K, 700 Chesterfield Parkway North, Chesterfield, MO 63017, anurag.s.rathore@pharmacia.com;* ***Robert M. Kennedy*** *is department director in separation sciences at Amersham Biosciences Corporation,* ***J. Kevin O'Donnell*** *is technical service manager at Tosoh Biosciences LLC,* ***Ivars Bemberis*** *is technology manager at Millipore Corporation, and* ***Oliver Kaltenbrunner*** *is in process engineering at Amgen Inc.*

ANURAG RATHORE
From the Series Coordinator

BioPharm International and I hope that the insightful comments from these authors are useful for biochemists and engineers practicing preparative chromatography in the biopharmaceutical industry. We conclude this article with a caution about some common pitfalls found in preparative chromatography:

- Packing procedures need to be optimized for each different type of resin to get reproducible packing;
- Measures of bed integrity need to be carefully chosen along with their specifications to guarantee robust column performance;
- The tracer used for estimating HETP or A_s can interact with column packing, yielding erroneous results;
- Flow rate or particle size effects must be eliminated when qualifying column packing to achieve consistent results;
- Function tests might be necessary, and lot-to-lot variations in chromatography media may be unacceptable for some applications if the process is very sensitive; and
- Resin qualification procedures need to be carefully chosen because excessive testing can be expensive and should be reduced gradually through reexamination.

—Anurag Rathore, *series coordinator, is at Pharmacia Corporation, 700 Chesterfield Parkway North, Chesterfield, MO 63017, 636.737.6790, fax 636.737.7281, anurag.s.rathore@pharmacia.com.* ***BPI***

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