Troubleshooting: HPLC sample preparation and introduction

A major advantage of HPLC is its widespread application to the majority of organic compounds as well as to many inorganic compounds. HPLC has four modes of separation: liquid-liquid, liquid-solid, ion exchange, and size exclusion. Using these available modes of separation, an analyst can handle almost any sample submitted.

As in most analytical problems, however, samples are usually not submitted in simple known matrices. Even with known systems, one or more interfering species are usually present. This is where chemical know-how and the knowledge of chromatographic theory are important to the chromatographer in order to choose the proper mode of separation. Approaches to selecting the separation mode are discussed elsewhere (1–4). Syringe injection techniques are described in the book *Maintaining and Troubleshooting HPLC Systems*, from which this material is condensed, but will not be covered in this article.

SAMPLE PREPARATION

Solid samples: Solid samples must be dissolved before being introduced into an HPLC system. The choice of solvent is critical. Dissolving the sample in the mobile phase has several advantages. The sample solvent peak at the void volume will be minimized, which is critical with unknown samples in which an impurity or peak of interest can be masked by the sample solvent peak. Precipitation on the column also will be avoided. Sample dissolution in a quickly prepared, known solvent may speed up sample preparation, but later may cause endless hours of troubleshooting and misinterpreted data as a result of the sample or a component of the sample precipitating on the column. This occurs when the sample slug dissolves and diffuses in the mobile phase. As the sample solute is dispersed, those components not readily soluble in the mobile phase can precipitate out. If the component of interest does not precipitate, interference from a precipitate may not be noticed until subsequent injections.

In a binary reversed-phase system of water/methanol, for example, it is common to dissolve the sample in straight methanol for ease of preparation or for cases in which the sample will not dissolve directly in the mobile phase. If precipitation takes place before or on the column, it is possible to observe unknown and randomly eluting peaks with later injections. These peaks do not maintain the same k' and can elute on or partially cover the peak(s) of interest. This can occur for two reasons. The precipitated material redissolves in the methanol slug from the next injection and is carried partially or totally off the column. The same effect can happen, but more slowly, with continuous washings of mobile phase. Precipitation can also occur at the head of the column, clogging the inlet, increasing the column back pressure, and restricting the flow. In addition, split peaks may be produced.

All of these symptoms hinder quantitative analysis. For fast qualitative studies it is possible to use the quick method of preparation; however, these potential problems must be kept in mind. If the sample preparation is done in a solvent different from the mobile phase, column cleaning with a suitable solvent followed by reconditioning with the mobile phase is recommended. This can be done with repeated 1-ml injections of a good solvent — straight methanol for example — or by running methanol through the HPLC system via the solvent pump. This must be followed by flushing and reconditioning with the mobile phase, preferably overnight. Be sure that whatever solvent is used is compatible with the column.

Samples that do not readily dissolve in the mobile phase can either be coaxed to or can be dissolved in a solvent system that will approximate the mobile phase. For binary or ternary mobile phase systems, dissolve the sample first in the component that will dissolve the sample most readily. Follow this by making to volume with the remaining solvent component(s), while keeping the ratio as close as possible to the mobile phase. This may take time, but the few additional minutes can be well worth it. The use of ultrasonics can also aid in sample dissolution, but caution is needed to avoid heat buildup and possible thermal degradation of the sample.

Preparing the sample in the mobile phase also maintains system compatibility. As mentioned in the April issue of *LC*, the mobile phase ratio can change after several hours of use. Continual sample makeup with the mobile phase as is can ensure little difference in the void volume peak. This can be important when the peak of interest has a low k' relative to the void volume.

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These guidelines are particularly important in the liquid-solid and liquid-liquid modes. The same general rules can apply to ion exchange or size exclusion, but are usually not as critical.

Liquid samples: Liquid samples provide the chromatographer with the option of injecting the sample directly; however, the solvent may or may not be compatible with the chromatographic system of choice. If the solvent is compatible (for example, a water sample with a reversed-phase, ion-exchange, or aqueous-exclusion system), direct introduction of the sample can be made. System modifications may be needed, depending on the complexity, interferences, and concentration levels of the sample.

For samples not in a desired solvent or not concentrated enough, two simple procedures can be used. One is to evaporate the sample to dryness and reconstitute the sample with the mobile phase or a more suitable solvent. The guidelines for solid samples discussed above apply. Partial evaporation can also be used to increase the concentration of a sample if the sample solvent is compatible with the chromatographic system.

Sample filtration: It is just as important, if not more so, to filter the sample prior to injection as it is to filter the mobile phase. The decision to filter a sample will be determined by the nature of the sample, its solubility, and interferences or contaminants.

Unlike the mobile phase, which may have a solvent reservoir filter and in-line pump filter(s) before the sample inlet and column, the sample's only filter may be the column itself. Whether it is insoluble material or lint from inside the sample container that remains after cleaning and drying the glassware, insoluble contamination buildup at the head of the column can lead to restriction of the mobile phase flow, thereby increasing the column back pressure, decreasing the column's efficiency, and producing split peaks.

To avoid these problems, the chromatographer can use classical filtration techniques, specialized equipment such as a syringe and a 5-μ filter pad in a Swinny adapter, or a commercial sample clarification kit. With any filter system it is advisable to determine whether the solute is absorbed by the filter media, particularly for quantitative analyses. This can be accomplished by spiking experiments with known samples of predetermined concentration. Failure to determine this can cause nonreproducible results.

Solvent degassing: It is advisable to prepare samples with degassed solvents in order to reduce the possibility of unwanted degassing occurring in the detector cell. For quantitative analysis, the sample solution should not be degassed. Degassing causes solvent evaporation, which will change the sample concentration. Degas the solvent before preparing the sample.

Internal standards: The use of an internal standard is preferred by many analysts for quantitative analysis. Its purpose is to minimize system and procedural variations, thus eliminating variations in precision as a function of sample size.

Several requirements should be met to ensure proper use of an internal standard (1,4):

- It should be completely resolved.
- It should not elute on or over another component, yet should have a k' as close to the peak of interest as possible.
- It should have similar chemical properties to eliminate or reduce differences in detector response between itself and the component of interest.
- It should be prepared at the same concentration level as the sample.
- It should be of good purity to prevent adding contamination and spurious peaks to the chromatogram.
- It should be chemically inert.

VALVES

High pressure valves for HPLC have become the most popular means of sample introduction as a result of the use of microparticle columns and solvent delivery systems operating most frequently in the 2000–6000 psi range. Valves, although relatively simple devices, can be a constant source of problems for the chromatographer. These problems can be avoided by following some simple guidelines.

FIGURE 1: HPLC valve inventory log.

Date Received	P.O. Number	Manufacturer	Purchased From	Valve Identification	Valve Type	Comments

FIGURE 2: HPLC individual valve log.

Valve Identification ____________________

Valve Location			Repairs				Number of Injections	Injection Technique	Comments
Date	Lab	Current Use	Repair Company	Date Sent	Date Returned	Repair Number(s)			

Operating pressure specifications: Know the operating pressure limit for the valve in use. The majority of valves used are rated for operating limits of 1500, 3000, 6000, and 7000 psi. If the pressure limits of your valves are in question, contact the manufacturer or supplier for this information. The limits are relatively accurate, and matching or exceeding these specifications will cause valve leakage around the rotor — resulting in excessive downtime for dismantling the valve, drying it out, and rebuilding.

Spare parts: Have plenty of dead volume fittings, ferrules, and rotors for valve repairs. It is common to replace the fittings and ferrules because of damage and changes made in the system, such as adding new loops or changing the valve's inlet/outlet tubing. Not all fittings and ferrules typically used in HPLC laboratories are interchangeable or useful with these valves. For the best nonleak fittings, use only parts supplied by the valve manufacturer or supplier.

Many valve manufacturers supply spare rotors. The rotor is the seal-handle assembly that is pressure-fitted into the stainless-steel valve body. The most common damage to a valve is crossport scratches on the rotor body, which produce leaks. This occurs because of particulates getting between the rotor and the stainless-steel valve body and scratching the rotor body by producing grooves between the valve port openings. When replacing a rotor, be sure to use the correct unit for the valve. Rotors are made from different materials to conform with the pressure limitation of the valve, so care must be taken when interchanging them. This is true even among different valves from the same manufacturer. When changing valve rotors, be sure to follow the manufacturer's recommended procedure carefully. One should also be aware of whether spring-loaded washers are used to seal the rotor.

Depending on the pressure limit for the valve, either all the washers will be oriented in one direction, or half the washers will be oriented in the opposite direction from the other half.

It is advisable to have several spare valves. Depending on usage and damage rate, repairs within the laboratory may not be possible. The damaged valve can be repaired by returning it to the manufacturer. The laboratory should have at least one spare valve for use and another back-up valve in case of problems that can occur while one valve is out for repairs.

When sealing stainless-steel tubing to the valve body, carefully butt the tube up against the inside surface of the valve body port. With the tube snugly in place, slide the ferrule over the tubing into the port and carefully tighten it down with the nut. Do not overtighten. Stop once a snug fit is made. It is better to run the system under pressure and retighten the nut than to overtighten. Using a wrench of the correct size will help. The wrong wrench, such as a large crescent wrench, can easily create too much torque, making it easy to overtighten and gall the fitting.

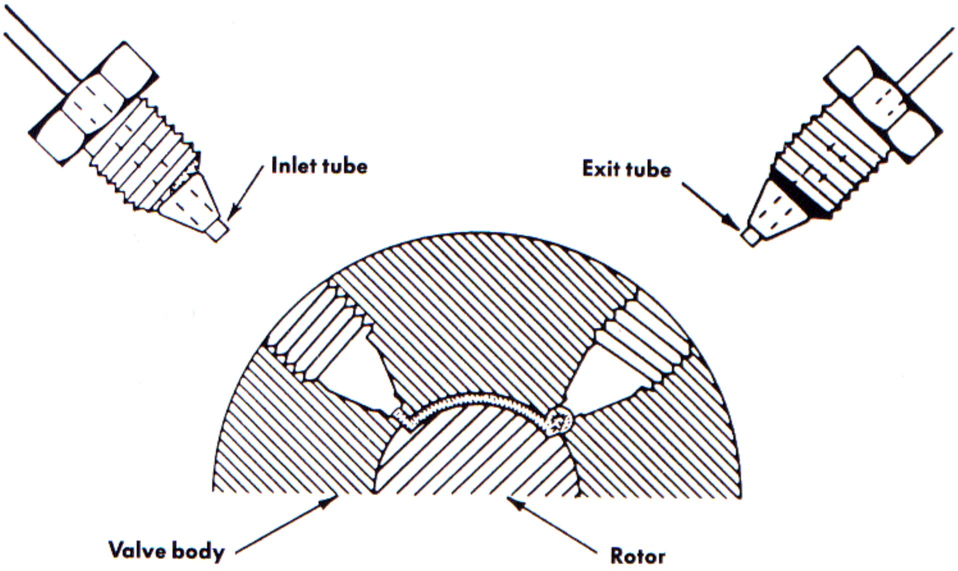

FIGURE 3: Zero dead volume fittings in an HPLC rotary sampling valve (courtesy of Valco Instruments Company).

For laboratories with many users, HPLC instruments, and valves, it is advisable to monitor the repair and location of all the valves. This can be done by carefully engraving a code on the outside of the stainless-steel body of the valves. The code should be sequential to accommodate the purchase of more valves. This information can be monitored by use of an HPLC inventory log book. Figures 1 and 2 illustrate examples of two forms for this purpose. The HPLC valve inventory log sheet (Figure 1) is used to code the valves with pertinent historical information as they are received. The individual valve log sheet (Figure 2) is used to monitor the location, repairs, and use of the valves.

Valve fittings: Leakage is commonly caused by mishandling the low dead volume valve fittings. These fittings, like all HPLC stainless-steel fittings, press a cone-shaped ferrule into a stainless-steel tube. This is tightened down into the valve body (Figure 3). When tightening the ferrule and fitting it in place, it is easy to apply more torque than is necessary to make the seal. These fittings, like all HPLC equipment, *must* be handled gently, and a herculean effort on a nut will not seal but only tend to distort the ferrule and cut a groove in the tubing surface.

Overtightening valve fittings — or any HPLC fittings — will produce several problems, such as a continuously leaking fitting. Once a valve fitting is overtightened, further torquing will not solve the problem. Loss of the nut, ferrule, tubing, and possibly the valve itself, which costs several hundred dollars, can also occur. Overtightening typically results in galled fittings where the threads are distorted. The threads in the valve body may be galled as well. Unless these can be rethreaded in your laboratory or shop, the valve body may be ruined or lost for repairs. Some fittings can be tightened so much that the hexagonal fitting heads may break off, leaving the male portion in the valve body. Unless this can be carefully drilled out, with an "easy out" from a local hardware store for example, this valve body may be lost. Finally, the user loses time. Downtime cannot be stressed enough as a loss to the chromatographer and the company or university.

Preparing valve tubing: When the small i.d. stainless-steel tubing frequently used with valves is first purchased, it will have a smooth, relatively flat end, with the i.d. base of the tubing relatively consistent with the remaining bore throughout the tubing. When the tube is cut by the user, however, deformation of the tube end can cause several problems.

The tubing should be butt centered and flat up against the inside of the port in the valve body for a good seal (Figure 3). A piece of tubing without a perpendicular flat end or with a bent and twisted end may never seal. One of the major causes of scratched rotors and valve bodies is metal particles produced from the tube cutting operation that get between the rotor and the valve body. Any metal particles left behind from cutting the tubing can wash into the valve with the mobile phase and scratch the assembly. New tubing should always be rinsed with mobile phase under operating pressure before being assembled onto any HPLC component.

When cutting tubing yourself, it is important to cut it flat without closing the i.d. bore of the tubing. Tube cutting can be done with a file, hacksaw, lathe, tube cut-off machine, or other commercial tubing cutter. Hacksawing tends to close the i.d. bore more than any other method. The most common technique is to score the tubing completely around the outer surface, and then carefully bend the tubing with short back and forth motions about the score mark. This will weaken the tubing, and it will break apart in the same way as a glass rod. If a vise is used to hold the tubing, be sure not to mar the surface where a ferrule seal will be made. Once the tube is apart, file the end flat, being sure not to close the i.d. bore of the tubing at that end. Next use a no. 69 drill bit (0.029 in.) to ream out the i.d. bore, smooth the opening, and remove metal particles. Rinse the tubing out with solvent before using. It is advisable to practice on a spare piece of tubing to learn the process. Problems occur more frequently with 0.009-in. i.d. tubing than with 0.020-in. or 0.040-in. i.d. tubing. Manufacturers traditionally use lathes or other on-line machine techniques to produce the fine ends on newly purchased tubing.

Crossport leaks: Most valves used are 6-port valves. More may be used depending on the system desired. Crossport leaks may start slowly and gradually increase in number and size. These leaks will ruin the precision of the injection and destroy any reproducibility and possibility for doing quantitative work. Obviously a lack of reproducible peak heights with a valve may be a result of crossport scratches.

Another way to identify crossport scratches early is to use teflon tubing for the exit line. A stainless-steel fitting and ferrule can be used gently on teflon, just as on stainless steel. The advantage of a teflon line is its transparency; if a crossport leak occurs, the mobile phase can be observed passing through the line when the valve is in the load or run position. The drain is for sample excess during sample loop loading and manual sample loop rinsing. If liquid runs out from this line while the instrument is in operation, there is a leak.

Lastly, the rotor can be dismantled and physically examined. The surface should be highly polished, smooth, and free from scratches.

Introducing samples: Samples are introduced by syringes. However, these are not pressure syringes as with the syringe/septum injections. A typical 1-ml glass luer-tip tuberculin syringe can be used with an appropriate Kel-F male luer adapter, mounted into the sample entrance port of the valve. For very small samples, 10-μl and 20-μl syringes are available with stainless-steel injection port fittings for injecting accurate, reproducible samples into valve sample loops where very little sample is available. This allows, for example, a 10-μl sample to be used to fill a 2-μl loop.

When injecting the sample into the loop, it is important to ensure that all the air is removed to prevent it from entering the system. This is done by flushing the loop with excess sample. When a teflon waste tube is used, air can be seen rinsing through to waste. All air should be expelled from the syringe.

Never leave the syringe, particularly the luer-tip type, in the valve overnight or for long periods of time. It is typical for the system sample port assembly to dry out and freeze up, making it difficult to remove the assembly without breaking it.

Always label the loop with its volume delivery and never apply a side force perpendicular to the rotor shaft when turning the valve rotor. This can place a side force on the rotor and valve body, possibly distorting the polymeric rotor body and creating leaks. Leaks can also occur if the rotor is left only partially actuated.

SAMPLE CONTAINERS

Because HPLC allows very small samples to be injected, evaporation can change the concentration of the sample while it is in use. It is therefore important when using a sample to keep it covered. This is particularly true when close tolerances on injection precision are required. Frequently 5- and 10-dram vials are used, and sloppy technique can ruin data and results. Vials with plastic retaining caps and rubber septa are also used.

Label the sample container with sample name, solvent, concentration, date, and an identification reference to some notebook where additional information such as the sample's preparation may be found. Although this is a simple point, many a day's work has been lost by not adequately labeling samples and standards.

REFERENCES

(1) E.L. Johnson and R. Stevenson, *Basic Liquid Chromatography* (Varian Associates, Palo Alto, California, 1978).

(2) D. Rodgers, *Developing an Analytical Method by High-Efficiency Liquid Chromatography* (Perkin-Elmer, Norwalk, Connecticut, 1974).

(3) K.J. Bombaugh, in *Modern Practice of Liquid Chromatography*, J.J. Kirkland, ed. (Wiley-Interscience, John Wiley & Sons, Inc., New York, 1971).

(4) L.R. Snyder and J.J. Kirkland, *Introduction to Modern Liquid Chromatography*, 2nd Ed. (Wiley-Interscience, John Wiley & Sons, New York, 1979). ■