HPLC

A Practical User's Guide

Marvin C. McMaster

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Marvin C. McMaster, Ph.D.
2070 Cordoba Drive
Florissant, MO 63033

Technical illustrations by Christopher A. McMaster.

This book is printed on acid-free paper. ♾

Library of Congress Cataloging-in-Publication Data

McMaster, Marvin C.
HPLC, a practical user's guide / Marvin C. McMaster.
p. cm.
Includes bibliographical references and index.
ISBN 1-56081-636-8 (acid-free)
1. High performance liquid chromatography I. Title.
QD79.C454M36 1994
543'.0894—dc20 93-42139
CIP

Printed in the United States of America

ISBN 1-56081-636-8 VCH Publishers

Printing History:
10 9 8 7 6 5 4 3 2 1

Published jointly by

VCH Publishers, Inc.
220 East 23rd Street
New York, New York 10010

VCH Verlagsgesellschaft mbH
P.O. Box 10 11 61
69451 Weinheim, Germany

VCH Publishers (UK) Ltd.
8 Wellington Court
Cambridge CB1 1HZ
United Kingdom

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CHAPTER

3

Running Your Chromatograph

This chapter is designed to help you get your HPLC up and running. We will walk through making tubing fittings, putting the hardware together, preparing solvents and sample, initialization of the column, making an injection, and, then, getting information from the chromatogram produced. Let us begin with connecting the hardware and work our way toward acquiring information.

3.1 Setup and Start-up

When your chromatograph arrives someone will have to put it together. If you bought it as a system, a service representative from the company may do this for you. No matter who will put it together, you should immediately unpack it and check for missing components and for shipping damage.

If you bought only components or if you are inheriting a system from someone else, you will have to put it together yourself. More than likely, you will need, at a minimum, a 10-ft coil each of 0.010-in. (ten-thousandths) and 0.020-in. (twenty-thousandths) tubing, compression fittings appropriate to your system, cables to connect detectors to recorder/integrators and pumps to controller, and tools. Our model will be a simple, isocratic system: a single pump, a flush valve, an injector, a C_{18} analytical column, a fixed wavelength UV detector, and a recorder (Fig. 1.4). The first thing we need to do is to get the system plumbed up or connected with small internal diameter tubing. For now, check the columns to make sure they were shipped or were left with the ends capped. We will put them aside until later.

3.1.1 Hardware Plumbing 101: Tubing and Fittings

We will need $\frac{1}{8}$-in. stainless steel HPLC tubing with 0.020-in. i.d. going from the outlet check valve of the pump to the flush valve and on to the injector inlet. Three types of tubing are used in making HPLC fittings: 0.04, 0.02, and 0.01 in. i.d.; the latter two types are easily confused. If you look at the ends of all three types, 0.04 in. looks like a sewer pipe, more hole than tube. Look at the tubing end; if you can see a very small hole and think that it is 0.01 in., it is probably 0.02 in. If you look at the end of the tubing, and, at first glance, think it is a solid rod and then look again and can barely see the hole, it is 0.01-in. tubing. From the injector to the column and from the column on to the detector we will use 4-in. pieces of this 0.010-in. tubing.

It is critically important to understand this last point. There are two tubing volumes that can dramatically affect the appearance of your separation: the ones coming from the injector to the column and from the column to the detector flow cell. It is important to keep this volume as small as possible. The smaller the column diameter and the smaller the packing material diameter, the more effect these tubing volumes will have on the separation's appearance (peak sharpness).

A case in point is a troubleshooting experience that I had. We were visiting a customer who had just replaced a column in the system. The brand new column was giving short, broad, overlapping peaks. It looked much worse than the discarded column, but retention times looked approximately correct. Since the customer was replacing a competitive column with one that we sold, I was very concerned. I asked her if she had connected it to the old tubing coming from the injector and she replied that the old one did not fit. She had used a piece of tubing out of the drawer that already had a fitting on it that would fit. This is *always* dangerous, since fittings need to be prepared where they will be used or they may not fit properly. They can open dead volumes that serve as mixing spaces. I had her remove the column and looked at the tubing. Not only was the end of the tubing protruding beyond the ferrule too short, the tubing was 0.04 in. i.d. This is like trying to do separations in a sewer pipe. We replaced it with 0.01-in. tubing, made new new fittings in the holes they were to connect with, and reconnected the column. The next run gave needle-sharp, baseline-resolved peaks!

To make fittings we need to be able to cut stainless steel tubing. Do not cut tubing with wire cutters; that is an act of vandalism. Tubing is cut like glass. It is scored around its circumference with a file or a microtubing cutter. The best apparatus for this is called a Terry Tool and is available from many chromatography suppliers. If adjusted for the inner diameter of the tubing, it almost always gives cuts without burrs. If you do not have such a tool, score around the diameter with a file. Grasp the tube on both sides of the score with blunt nosed pliers and gently flex the piece to be discarded until the tubing separates. Scoring usually causes the tubing to flare at the cut. A flat file is used to smooth around the circumference. Then, the face of the cut is filed at alternating 90° angles until the hole appears as a dot directly in the center of a perfect circle.

The ferrule should then slide easily onto the tubing. Be sure not to leave filings in the hole; connect the other end to the pumping system and use solvent pressure from the pump to wash them out.

The tubing is connected to the pump's outlet check valve by a compression fitting. The fitting is made up of two parts: a screw with a hex head and a conical shaped ferrule (Fig. 3.1a). The top of the outlet valve housing has been drilled and treaded to accept the fitting.

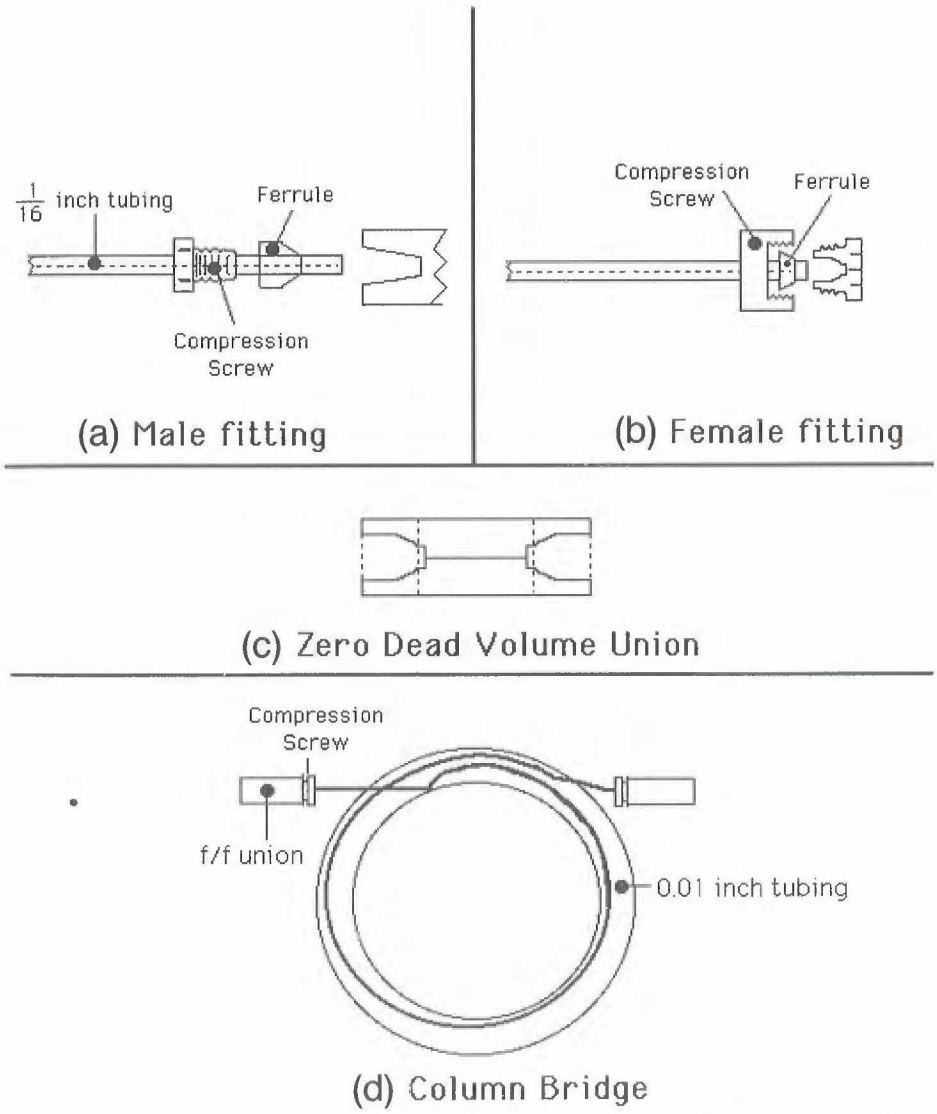

Figure 3.1. Compression fittings. (a) Male fitting; (b) Female fitting; (c) Zero dead volume union; (d) Column bridge.

First the compression screw then the ferrule are pushed on to the tubing; the narrow end of the ferrule and the treads of the screw point toward the tubing's end. The end of the tubing is pushed snugly into the threaded hole on the check valve. Slide the ferrule down the tube into the hole, followed by the compression screw. Using your fingers, tighten the screw until it is as snug as possible; then use a wrench to tighten it another quarter turn. As the screw goes forward, it forces the ferrule against the thread and squeezes it down on to the tubing, forming a permanent male compression fitting. The fitting can be removed from the hole, but the ferrule will stay on the tubing. The tubing must be cut to remove the ferrule.

It is important not to overtighten the fitting. It should be just tight enough to prevent leakage under pressure. Try it out. If it leaks, tighten it enough to stop the leak. By leaving compliance in the fitting, you will considerably increase its working life time. Many people overtighten fittings. If you work at it, it is even possible to shear the head off the fitting. But please, do not.

There is a second basic type of compression fitting, the female fitting (Fig. 3.1b), that you will see on occasion. Some column ends have a protruding, threaded connector tube and will require this type of fitting. This fitting is made from a threaded cap with a hole in the center. It slides over the tubing with its threads pointed toward the tubing end. A ferrule is added exactly as above and the tubing and the ferrule are inserted into the end of the protruding column tube with external threads. Tightening the compression cap again squeezes the ferrule into the tapered end of the tube and down onto the tubing, forming a permanent fitting. The third type of device for use with compression fittings is the zero dead volume union (Fig. 3.1c). A union allows you to connect two male connection fittings.

You will find that stainless steel fittings will cause a number of headaches over your working career. An easier solution in many cases is the polymeric "finger tight" fittings sold by many suppliers such as Upchurch and SSI. These fittings slide over the tubing and are tightened like stainless steel fittings, but are not permanently "swagged" onto the tubing and can be reused. They are designed to give a better zero dead volume fitting, but they have pressure and solvent limits. They are also more expensive, but only in the short run.

3.1.2 Connecting Components

New pumps are generally shipped with isopropanol or a similar solvent in the pump head and this will need to be washed out. Always try and determine the history of a pump before starting it up. Systems that have not been run for a while may have dried out. If buffer was left in the pump, it may have dried and crystallized. In any event, running a dry pump can damage seals, plungers, and check valves.

First we will need to hook up the pump inlet line. This consists of a length of large-diameter Teflon® tubing with a combination sinker/filter pushed into one end and a compression fitting that will screw into the inlet fitting at the

bottom of the pump head on the other end. Drop the sinker into the solvent reservoir and screw the other end into the inlet check valve housing.

The next step is to use compression fittings to hook the pump outlet check valve to the flush valve with a length of 0.02-in.-i.d. tubing. A flush valve is a small needle valve used to prime the pump by diverting solvent away from the column when rapidly flushing the pump to atmospheric pressure. Open the valve and the line is vented to the atmosphere. This removes back pressure from the column, a major obstacle when trying to push solvent into a plumbed system.

From the flush valve we can connect with fittings and 0.02-in. tubing onto the injector inlet port. The back of the injector usually has ports for an inlet and an outlet line, two ports for the injection loop, and a couple of wash ports. If a sample loop is not in place, connect it, then make a short piece of 0.01-in.-i.d. tubing with fittings to be used in connecting the column. Use the column end to prepare the compression fitting that will fit into it. At the outlet end of the column, hook up with compression fittings a piece of 0.01-in. tubing that connects to the detector flow cell inlet line. When this is done remove and recap the column and set it aside.

Next we are going to create a very useful tool for working with the HPLC system. I call it a "column bridge" (Fig. 3.1d). It bridges over the place in the system where we would normally connect the column. It is very valuable for running, diagnosing, and cleaning a "columnless system." It is made up of a 5-ft piece of 0.01-in. tubing with a male compression fitting on each end screwed into zero dead volume unions (female/female). Our column bridge now has two ends simulating the end fittings on the column.

Connect one end of our column bridge to the tubing from the injector outlet; the other end is connected to the line leading to the detector flow cell. We have one more line to connect to complete our fluidics. A piece of 0.02-in. tubing can be fitted to the detector flow cell outlet port to carry waste solvent to a container. In some systems, this line will be replaced with small-diameter Teflon® tubing.

In either case, the line should end in a backpressure regulator, an adjustable flow resistance device designed to keep about 40–70 psi backpressure on the flow cell to prevent bubble formation that will interfere with the detector signal. Air present in the solvent is forced into solution during the pressurization in the pump. The column acts as a depressurizer. By the time our flow stream reaches the detector cell, the only pressure in the system is provided by the outlet line. If this is too low, bubbles can form in the flow cell and break loose, resulting in sharp spikes in the baseline. The backpressure regulator prevents this from happening.

The final connections are electrical. A power cable needs to be connected to the pump. Check the manuals to see if fuses need to be installed and do so if required. Finally, connect the 0- to 10-mV analog signal connectors on the back of the detector to similar posts on the strip chart recorder. Connect red to red, black to black. If a third ground wire is present in the cable connect it only

at one end, either the detector or the recorder end. (*Note:* The ground wire connects to the cable shield, which is wrapped around the other two wires in the cable. If no ground is connected, no shielding of the signal occurs. If both ends of a ground are connected, the shield becomes an antenna; this is worse than no shield at all.)

Now our system is ready to run. We will need to prepare solvent, flush out each component, then connect, flush out, and equilibrate the column before we are ready to make our first injection of standard.

3.1.3 Solvent Cleanup

Before we tackle the column, let us look at how to prepare solvents for our system. I have found that 90% of all system problems turn out to be column problems. Many of these can be traced to the solvents used, especially water.

Organic solvents for HPLC are generally very good. There are four rules to remember: always use HPLC grade solvents, buy from a reliable supplier, filter your solvents, and check them periodically with your HPLC. Most manufacturers do both GLC and HPLC quality control on their solvents; some do a better job than others. The best way to find good solvents is to talk to other chromatographers.

Even the best solvents need to be filtered. I have received solvents, from what I considered to be the best manufacturer of that time, that left black residue on a 0.54-μm filter. There is a second reason to filter solvents. Vacuum filtration through a 0.54-μm filter on a sintered glass support is an excellent way to do a rough degassing of your solvents. Because of their filter and check valve arrangements, some pumps cavitate and have problems running solvents containing dissolved gases.

There are numerous filter types available for solvent filtration. Cellulose acetate filters should be used with aqueous samples with less than 10% organic solvents. With much more organic in the solvent, the filter will begin to dissolve and contaminate your sample. Teflon® filters are used for organic solvent with less than 75% water. The two types are easily told apart; the Teflon® tends to wrinkle very easily, while the cellulose is more rigid. If you are using the Teflon® with high percentages of water in the solvent, wet the filter first with the pure organic solvent, then with the aqueous solvent before beginning filtration. If you fail to do this it will take hours to filter a liter of 25% acetonitrile in water. Recently, nylon filters for solvent filtration have appeared that can be used with either aqueous or organic solvents. They work very well as a universal filter, but use with very acidic or basic solutions should be avoided.

If you are still having problems after vacuum filtration, try placing the filtrate in an ultrasonication bath for 15 min (organic solvents) or 35 min (aqueous solvents). Ultrasonic baths large enough to accept a 1-liter flask are in common use in biochemistry laboratories and are very suitable for HPLC solvent degassing. Stay away from the insertion probe type of sonicator; they throw solvent and simply make a mess. Ultrasonication is much better than heating

for removing gases from mixed solvents. There is much less chance of fractional distillation with solvent compositional change when placing mixtures in an ultrasonic bath. One manufacturer actually designed an HPLC system that was designed to remove dissolved gas by heating under a partial vacuum. Obviously they never used rotary vacuum flash evaporators in their laboratories, at least not intentionally!

Other techniques recommended for solvent degassing involve bubbling gases (nitrogen or helium) through the solvent. Helium sparging is partially effective, but expensive when used continuously. It is required in some low-pressure mixing gradient systems as will be described later. The only other time I use any of these degassing techniques is in deoxygenating solvent for use with amine or anionic-exchange columns, which tend to oxidize (see Fig. 6.3).

Water is the major offender for column contamination problems. I have diagnosed many problems, which customers initially blamed on detectors or pumps or injectors, that turned out to be due to water impurities. Complex gradient separations are especially susceptible to water contamination effects.

In one case, a customer was running a PTH amino acid separation, a complex gradient run on a reverse-phase column. He would wash his column with acetonitrile, then water, and run standards. Everything looked fine. Five or six injections later his unknown results began to look weird. He ran his standards again only to find the last two compounds were gone. He blamed the problem on the detector. I said it looked like bad water. He exploded, and told me that his water was triple distilled and good enough for enzyme reactions. It was good enough for HPLC, he said. Over the following 6 months we replaced every component in that system. Eventually, the customer borrowed HPLC grade water from another institution, and washed his column with acetonitrile, then with water. The problem disappeared and never came back—until he went back to his own water. Nonpolar impurities codistilling with the water were accumulating at the head of the column and retaining the late runners in the column.

While HPLC grade water is commercially available, I have found it to be expensive and to have limited shelf life. The best technique for purifying water seems to be to pass it though a bed of either reverse-phase packing material or of activated charcoal, as in a Milli-Q system. Even triple distillation tends to codistil volatile impurities unless done using a fractionation apparatus.

I have used an HPLC and an analytical C_{18} column at 1.0 ml/min overnight to purify a liter of distilled water for the next day's demonstration run. The next morning, I simply washed the column with acetonitrile, then with water, equilibrated with mobile phase, and ran my separation. It might be a good idea to reserve a column strictly for water purification.

An even better solution is to use vacuum filtration through a bed of reverse-phase packing. Numerous small C_{18} SFE cartridges are available that are used for sample cleanup and for trace enrichment. They are a tremendous boon to the chromatographer for sample preparation, but also can be of help in water cleanup. These SFE cartridges are a dry pack of C_{18} packing and must be wetted

before use with organic solvent, then with water, or an aqueous solution. You wash first with 2 ml of methanol or acetonitrile and then with 2 ml of water before applying sample. If you forget and try to pass water or an aqueous solution through them, you will get high resistance and nonpolars will not stick. SFE cartridges contain from 0.5 to 1.0 g of packing and will hold approximately 25–50 mg of nonpolar impurities. If care is taken not to break their bed, they can be washed with acetonitrile and water for reuse. Eventually, long eluting impurities will build up and the SFE must be discarded. I have used them about six times, cleaning about a liter of single distilled water on each pass. If larger quantities of water are required, there are commercially available vacuum cartridge systems using large-pore, reverse-phase packing designed to purify gallons of water at a time.

The most common choice for large laboratories is mixed-bed, activated charcoal and ion-exchange systems that produce water on demand. These systems usually have a couple of ion-exchange cartridges and one activated charcoal filter in series. They work very well, but I prefer to have the charcoal as the last filter in the purification bank. After all, we are trying to remove organics. I find that the ion-exchange resins break down after about 6 months and begin to appear in the water. The system uses an ion conductivity sensor as an indicator of water purity, but water that passes this test often is still unsuitable for HPLC use.

3.1.4 Water Purity Test

The final step is to check the purity of the solvents. Again I have found the C_{18} column to be an excellent tool for this purpose. Select either 254 nm or the UV wavelength you will be using for the chromatogram. Wash the column with acetonitrile until a flat UV baseline is established and then pump water though the column at 1.0 ml/min for 30 min. This allows nonpolar impurities to accumulate on the column. The final step is to switch back to acetonitrile. I prefer to do this by running a gradient to 100% acetonitrile over 20 min. If no peaks appear after 5 min at final conditions, the water is good. The chromatogram (Fig. 3.2) gives you an idea of the expected baseline appearance.

Peaks that appear during the first acetonitrile washout are ignored as impurities already on the column. Watch the baseline on switching to water. At 254 nm, the baseline should gradually elevate. If instead it drops, you may have impurities in your acetonitrile. If the baseline makes a very sharp step up before leveling off, you may have a large amount of polar impurities in the water. Polar impurities probably will not bother you on reverse-phase columns, but might have some long-term accumulation effects. Peaks appearing during the acetonitrile gradient come from nonpolar impurities in the water that accumulated on the column and are now eluting.

I have done this with water from a Milli-Q system in need of regeneration. Even though their indicator glow light shows no evidence of charged material being released from the ion exchanger, peaks that will affect reverse-phase chro-

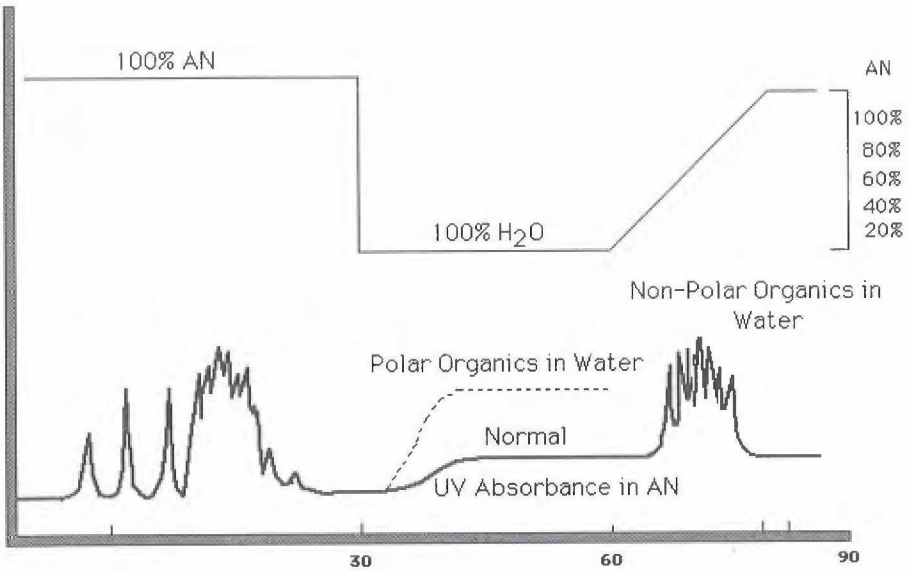

Figure 3.2. Water purity test.

matography show up at around the 70% acetonitrile portion of the gradient run.

If your water passes this test at the wavelength you will be using for your chromatography, you are ready to use it to equilibrate the column. The next step is to flush out the dry system and prepare to add the column.

3.1.5 Start-up System Flushing

Fill the solvent reservoir with degassed, filtered solvent by pouring it down the wall of the flask to avoid remixing air into it. I usually start pumps up with 40–50% methanol in water. Even if the pump was shut down and allowed to stand and dry out in buffer, there is a good chance this will clear it. It is also a good idea to loosen the compression fitting holding the tubing in the outlet check valve at the top of the pump head to relieve any system backpressure. This is an especially important step to use if the column is still connected. When running with a column bridge, as we are, it is less important.

The first step is to ensure that the pump is primed. This may mean pushing solvent from an inlet manifold valve through the inlet valve and into the pumping chamber. A few pumps on the market, like the old Waters M6000, use spring-loaded check valves, so you may have to really work to get solvent into the chamber. With other pumps, you open a flush valve and use a large priming syringe to pull solvent through the pumphead. The next step is either to turn the pump flow to maximum speed or use the priming function of the pump, which does the same thing.

As soon as the pump begins to pump solvent by itself, tighten the outlet compression fitting and drop the flow rate to about 1 ml/min. The pump is ready to run and should be allowed to pump into a breaker for a few minutes to wash out any machining oils, if new, or soluble residues or dissolved buffers if old.

Before we move on, let us talk about shutting down a pump. The pump seal around the plunger is lubricated by the contents of the pumping chamber. There is always a microevaporation through this seal/plunger combination, whether the pump is running or not. Buffers and other mobile phases containing dissolved solids should not be left in a pump when it is to be turned off overnight. This evaporation causes crystallization on the sapphire plunger and can result in either plunger breakage or seal damage on starting up the pump. Solvents containing dissolved solids should always be washed out before the pump is shut down. I prefer to wash out and leave a pump in 25–50% methanol/water to prevent bacteria growth in the fluidics system.

Occasionally, I have had to leave buffer in a pump overnight. When I do that I leave the pump running slowly (0.1 ml/min) and leave enough solvent in the reservoir so that it can run all night. This has an additional value of washing the column overnight. If the column is clean and does not require further washing, you can throw the detector outlet into your inlet reservoir and recycle the solvent, ensuring that you will not run out.

Now we can move past the flush valve to the next major system component, the injector. Whichever position you find the injector handle in, leave it there! *Never turn the handle on a dry injector.* The injector seal is hardened Teflon® facing a metal surface and can tear if not lubricated with solvent. Once solvent is flowing through the injector to lubricate the seal, turn the handle to the inject position so that the sample loop is washed. Watch the pressure gauge on the pump; a plugged sample loop will cause the pressure to jump. If this happens go to the troubleshooting section in Appendix C.

3.1.6 Column Preparation and Equilibration

The next step is to hook up the column. Stop the pump flow. I assume you have a C_{18} column compatible with 40% methanol/water (otherwise, select a solvent appropriate for your column). Disconnect the column bridge, remove the column fittings from both ends of the stored column, and connect the inlet end to the line coming from the injector. The inlet end of a column is almost always marked; check for an arrow or a tag pointing in the direction of flow. I have always preferred to hook up a column with some solvent running. Turn the flow rate on the pump to 0.2 ml/min. Fill the end of the column with solvent and screw in the compression fitting at the end of the injector line. Place a beaker at the outlet end of the column to catch washout solvent. Flush the column with start-up solvent if it is an old column that might have been stored in buffer. (This is a very bad technique, but you never know if you were not the last person to use the column! It is a good idea to label a column with the last solvent used before you store it.)

Next, change the solvent in the reservoir to 70% acetonitrile in water, turn the pump on, and flush it with the new solvent. Turn the flow rate up to 1.0 ml/min while catching the column effluent in a beaker. Check back up line for leaks; if you see any, tighten the appropriate fittings until the leaks just stop. You will always have leaks! If you do not you are probably overtightening your fittings. Leaks are messy, but are probably a sign of successful technique (leaks, not streams).

Check the pump pressure. The pump pressure gauge and the baseline trace are the two major tools for diagnosing system problems. If the column was shipped in isopropanol or methanol it should start high (3000–4000 psi) then slowly drop to around 2000–3000 psi.

Stop the pump flow and connect the column outlet with the short piece of 0.10-in. tubing connected to the inlet of the detector flow cell. Resume flow to the column. Turn the detector on and start the recorder chart speed at 0.5 cm/min. You should have a flat baseline. If the baseline continues to drift up or down, the column still has not finished its washout and equilibration, or the detector is not warmed up.

By the way, I must hasten to add that we really have not reached a true equilibration at this point. I have been informed by the experts that it takes about 24 hr to reach a true equilibration on reverse-phase packings. However, after six column volumes we have generally reached a reproducible equilibration point good enough for our purposes.

We are now ready to prepare for injecting a sample. Let us turn our flow rate down to 0.1 ml/min and get our sample ready.

3.2 Sample Preparation and Column Calibration

The worst thing a chromatographer can do is to grab a column out of its box, slap it into an HPLC, and shoot a sample. Before we begin, it is important to make sure the sample is clean. We will talk about removing soluble contaminants later. Here we are going to be dealing with suspended solids or particulates. Second, we need to know the initial condition of the column, so that we may return to it when we begin to develop problems. In other words, we need to do column quality assurance, or QA.

3.2.1 Sample Cleanup

The generally recommended procedure for cleaning samples is to filter them through a 0.54-μm filter in a Sweeny filter holder or a disposable plastic filter cartridge. The same types of filter materials are available as those that were discussed in the section on solvent filtration: Teflon®, nylon, and cellulose. In-line filters are now available that fasten between the syringe barrel and the injection needle. These are useful if you are not sample limited or are doing repeat injections of the same material. I have found that most chromatographers will not

bother with the time, cost, and sample loss that this entails, although I am finding an increase in the use of syringe in-line filters.

Sample clarification is, however, important! The column frit pore size is usually 2.0 μm; anything larger builds up and plugs the frit. Being a lazy chromatographer, but not a stupid one, I decided to use a different clarification procedure. I place the sample in a microcentrifuge tube and sediment solids by spinning at maximum speed in a clinical centrifuge (1000g) for 1–2 min. I pull a sample carefully from the supernatant and shoot that as my sample. It has the advantage of spinning down most of the solids, can be used on a number of samples at the same time, works even with very small samples, and is fast and inexpensive, if you already have the centrifuge. While it may not be as efficient as filtration, most chromatographers are willing to use it on every sample. It greatly extends column life between cleanups.

A third alternative combines the two techniques. A commercially available filter/reservoir fits in a microcentrifuge tube. The sample in the reservoir is filtered by spinning the unit. It is more efficient than simple centrifugalization, but takes longer to assemble and costs more.

Like the oil filter advertisement says, "you can pay me now, or pay me later." If you don't take time to remove particulates, you will spend much more time and effort cleaning the column. The choice is yours.

3.2.2 Plate Counts

Once the shipping solvent is washed out of the column, it is important to determine whether the column bed survived shipping and to determine its running conditions. Most good chromatography laboratories have established a quality control test for entering columns. A stable test mixture of known running characteristics has been prepared and stored to test new columns.

One commercially available standard used for testing C_{18} columns is a solution of acetophenone, nitrobenzene, benzene, and toluene in methanol. (Many chromatographers like to add a basic component, such as aniline, as a check against tailing problems, but these standards degrade on standing in oxygen-containing solvents.) To adjust for extinction coefficient differences, add 10 μg of each of the first two ingredients and 30 μg of the last two compounds in 2 ml of MeOH. Inject 20 μl of the mixture into the column equilibrated with 70% MeOH in water and read at 254 nm on the UV detector. This is a convenient mixture since separations between pairs of peaks double as you go to larger retention volumes. Be sure to keep this mixture tightly stoppered. The last two compounds will selectively evaporate from the mixture on access to air. For use at low UV wavelengths, dissolve these same four ingredients in acetonitrile and run in 60% acetonitrile in water.

Using this or similar mixtures, inject a sample into an equilibrated column, elute the resolved bands, and record them on the strip chart recorder. Calculate plate counts for the first and last peak using the "5σ" method discussed in section 4.1.1. Log these numbers in the form V4/V1 = 1.1/6.5; N4/N1 = 7500/

3600. When we see changes in a separation we have been running, we can reequilibrate in 70% MeOH/water and rerun our standards. Changes in these ratios will be useful in troubleshooting column problems later on.

Obviously, this mixture will not be useful on other types of columns, although I have used this mixture on C_8 columns. Each column type should have its own standards. They should be stable against both chemical and bacterial changes. With them you always have a touchstone to return to in case of problems.

3.3 Your First Chromatogram

Now that we have our system set up and the column equilibrated and standardized, we are ready to carry out an HPLC separation on a real sample. We might add an internal standard (if necessary, to correct for injection variations), then dilute our sample to a usable concentration and prepare it for injection. After injection, we will record the chromatogram, making sure that it stays on scale. Then, from the trace we obtain, we will calculate elution volumes either by measuring peak heights or by calculating peak areas by triangulation.

We can compare these values of areas or peak heights with known values for standard compounds. From elution volumes or retention times, we can begin to identify compounds. Comparing peak areas or heights to those derived from standard concentrations, we can calculate amounts of material under each peak.

3.3.1 Reproducible Injection Techniques

From the last section, it becomes obvious that we must first make a decision about what we are trying to accomplish. Are we doing scouting, trying to identify compounds by their retention times? Or are we trying to quantitate peaks by comparison to standards?

In scouting, we may be running very expensive samples and have simply to guess at the amount to inject. In this case, I would pull up >10 μl of the supernatant in a 25-μl syringe, turn the syringe point up, and pull the barrel back far enough so I could see the meniscus just below the needle. I would check for bubbles at the face end of the barrel, on the inside wall, and at the meniscus. Small bubbles generally can be dislodged by gently snapping the outside wall with your finger. Slowly push the barrel forward to the 10 μl mark, then quickly wipe the outside of the barrel past the tip with a tissue. Place the syringe into the injector syringe port, make sure the injector handle is in the LOAD position, and slowly push the sample into the loop to ensure that the sample goes in as a plug.

If the syringe is new or dry, you may find a large, tenacious bubble clinging to the barrel face. This can often be avoided by rinsing the barrel with the stronger solvent and then with the sample solvent. It can be dislodged by rap-

idly expelling the sample from the syringe back into the tube (try not to resuspend the pellet) and then slowly pulling up a new sample. Repeat the check for bubbles, expel the excess sample, and wipe before injecting. Do not let the tissue linger at the tip; it can wick up solution from the needle and give irreproducible sampling.

When working with sample we do not mind wasting, the simplest way to achieve reproducible injections is to overfill the loop. With a 20-μl loop we need to flush with at least 30 μl of sample solution to ensure complete removal of mobile phase. Almost all autosamplers operate on the principle of overfilling the sample loop to achieve a reproducible injection.

It is possible to inject partially a loop below its capacity. It is important not to add more than 75% of loop capacity and to inject the sample slowly. Rapid injection can lead to a phenomenon known as "viscous fingering" in which the sample does not cleanly displace solvent out the other end of the loop as a plug, but some of the sample overruns the loop and is lost.

Quantitative sample injection is handled a little differently. We usually know the expected concentration level and retention times. After clarification, we add a known amount of the sample solution and an internal standard to a volumetric flask and dilute. The sample is pulled into the syringe for injection as above.

Internal standards are used for many reasons in chemistry. Here we are using it to correct for differences in sampling volumes. It takes much practice for a person to deliver accurately the same size sample every time if they are partially injecting a loop. It is nearly impossible for two people to deliver accurately the same sample each time. If we add a known amount of internal standard to both our sample and our known standard mixture, we can calculate peak heights or areas relative to that of the internal standard. Variations in the injection size of the sample do not affect these relative areas.

To make the injection, we turn the handle of the injector to the LOAD position (see Fig. 9.9). Push the syringe needle into the needle port and slowly push the barrel forward so the sample goes in as a plug. Leave the needle in the injection port to prevent syphoning of the sample out the waste port. The handle is thrown quickly to the INJECT position. This last step is done quickly to prevent pressure buildup while the ports are blocked in shifting from one position to the other. Remember: load slowly, inject quickly.

Mark the injection point on the chromatogram. Some detectors or integrators will do this automatically if they are connected to a contact sensor on the injector or autosampler. It is good laboratory practice to annotate the chromatogram at the first injection point of the day with the operator's initials, time, date, sample ID number and injection volume, mobile phase composition, flow rate, detector wavelength and attenuation, and chart speed. If a gradient is being run, mark the starting composition, gradient start and end, and final composition. You can annotate later injections only with condition changes, such as sample i.d. and injection size. If you tend to cut your chromatograms apart, however, you may lose critical information if you do not

annotate every run with full information. There are commercially available rubber ink pad stamps that provide spaces for all the necessary information. Do not rely on your memory to come up with the data at some future time.

3.3.2 Simple Scouting for a Mobile Phase

The first step is to determine a starting point. If I am handed a mixture of a completely unknown nature, I will probably first try to get more information. I will try to determine the mixture's solubility in organic solvents, the effect of acid on the solubility, and something about the molecular weights and isoelectric points, if it is a mixture of proteins.

If this information is not available, I will try to separate the mixture using a C_{18} column in acetonitrile and water. Something like 70% of the separations in the literature are now made on a C_{18} column. Acetonitrile is my solvent of choice because of its low wavelength transparency, its polarity, and its intermediate position between methanol and tetrahydrofuran. Generally, I will use 254 nm for the detector because the majority of the literature separations can be made at that wavelength.

If I know that the compound is not soluble in aqueous solvents, I will probably select a silica column and a chloroform/hexane mobile phase. Separations of proteins will take me first to a TSK-3000sw column and a 100 mM Tris-phosphate pH 7.2 mobile phase unless I am separating soluble enzymes; then I use a TSK-2000sw column.

For illustration purposes, we will take the most common case. We will start with a 15-cm-long C_{18} column, 254 nm, and acetonitrile/water in a scouting gradient. Scouting gradients are run much more rapidly than analytical gradients. A mixture of the compounds to be separated is dissolved in 25% acetonitrile in water. A sample is injected into an HPLC equilibrated in the same mobile phase and a 20 min gradient is run to 100% acetonitrile.

Examination of the chromatogram while the separation is occurring lets us select conditions for a starting isocratic run. Since we were running very rapidly, conditions inside the column were not at equilibration. We use the gradient position of the first peak maximum as a guide to an isocratic mobile phase. Find the solvent composition from the controller %B output corresponding to the first peak and drop back to 10% less acetonitrile for a 25-cm column (7% less for our 15-cm column). Using the gradient controller to dial-a-mix the solvent, we reequilibrate the column for 15 min at this acetonitrile concentration and reinject our standards.

If all the peaks are accounted for and separated, we have our conditions. If not, we can do k' development, control pH by buffering, or change the stronger solvent or the type of column to produce an α change. We have a starting point, and that is half the battle.

If you do not have a gradient, I have developed a fast isocratic scouting technique. You select the same column and detector wavelength, but equilibrate in the column in 80% acetonitrile in water for our first injection. Strong solvent

is selected to blow everything off quickly. Look at the peaks; if they are resolved, quit. If they are still unresolved, mix the mobile phase with an equal volume of water making 40%, reequilibrate, and shoot again. This time the peaks should be much farther apart. If not, do another equal volume dilution to 20%, reequilibrate the column, and reinject the sample.

If the first peak from the 40% run takes more than 20 min or the peaks are too far apart, wash everything off with 100% acetonitrile. Mix mobile phase 80 and 40% in equal volumes to get 60%, reequilibrate, and shoot again. I usually find that I have my conditions by the third run or I need to make a solvent alpha change.

Silica columns are run the same way. Start gradients at 25% chloroform/hexane and run to 100% in 20 min. For isocratic scouting, start at 80% chloroform/hexane and make dilutions with hexane.

3.3.3 The Chromatogram: What Do You Look for?

I usually run scouting samples at an initial UV attenuation of 0.2 AUFS (absorbance units full scale) or refractive index attenuation of 8×. This way I can increase attenuation if the peaks start to go off scale or decrease attenuation if they are too small. An integrator will see everything from the baseline up to full attenuation, but you have to be reasonably close if you are using a strip chart recorder or you will lose peak information.

I would rather blow my first sample off scale and have to dilute the second one. At least I know I got the sample in and what the next step should be. If I shoot too little, I wait and wait for something to happen and waste a lot of valuable time. Besides, I have found that the first shot of the day is usually a "column tranquilizer." It seldom agrees with other sample of the day. The second and third agree, but not necessarily with number one. I have discussed this problem with other chromatographers and many have observed the same thing. If this bothers you, remember that chromatography is still art as well as science. Shoot the first sample and go and have some coffee. Then, you can get down to work.

I am often asked if peak heights or peak areas give more accurate results. The answer to this question is yes. When working with mixtures of pure compounds with very little overlap, peak areas give more accurate results. However, my clinical friends, who must quantitate on peaks from complex mixtures with overlapping peaks, insist that peak heights are more accurate.

3.3.4 Basic Calculation of Results

In peak height measurements, we measure the vertical displacement from the baseline and compare that to the peak height of a known standard amount. Peak area calculations are a little more complicated. They are usually done by triangulation; assume a right triangle and multiply the peak height times the half peak width. The areas of each peak are summed to give a total area. Divid-

ing this into the area of each peak gives a relative area percentage for each peak. Like peak heights, peak areas can be compared to peak areas for known compounds to allow calculation of the amounts of compound present.

Another more accurate method is to copy the chromatogram, cut out the peaks, and weigh them. Of course, if you have an integrator, it will do the job for you. They usually can be set to do either peak heights or areas. They also can be calibrated for standard runs and will calculate actual amounts relative to these earlier runs. Some also can be calibrated with compound names to provide annotated output.

Integrators are designed to make the chromatographer's life easier, but they can complicate it if not properly used. They usually have an autozero function that, when selected, looks at the baseline before injection and sets various baseline parameters. This is designed to prevent integration of very small or extraneous peaks, or of baseline noise. On most integrators, autozero must be requested by the operator and should be used every time a detector attenuation change is made. Be aware that you are letting a machine make decisions for you. It is possible to override the machines, and, sometimes, it is possible for you to produce a more accurate repetitive analysis by doing so. We will touch on integrator optimization in Chapter 14 on data collection.

Once we have returned to the baseline from one chromatogram, we are ready to make our next injection. When we have finished for the day, shut off the detector (lamps have finite lifetimes) and the strip chart recorder paper drive. If we are pumping solvents containing solids, they must be washed out before shutting down the pump. The system can be stored overnight or over a weekend with solvent containing more than 50% organic in the mobile phase. If you will be storing longer than a weekend, wash the system out with acetonitrile, remove and cap the column, and store it in its box labeled with the solvent and the last sample run in it.