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WHAT THEY DON'T TEACH YOU IN MEDICAL SCHOOL

Opportunities in postgraduate medical education through the clinical academic training route

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High performance liquid chromatography

Methods in Laboratory Science: What They Don't Teach You In Medical School

This month we publish the first in a series of articles that explain basic scientific methodology. What is their relevance in a predominantly clinical journal of medicine? Many doctors will undertake a period of postgraduate research, in which they are thrust back into the world of basic science after a hiatus of often 5–10 years since the material was covered at medical school. Even for those not actively engaged in laboratory studies, the articles will be of value for understanding molecular research papers relevant to their field of clinical practice.

Each article will explore the principles underlying individual techniques, their applications and limitations, and how to troubleshoot in the (sadly almost inevitable) scenario that the user runs into difficulty. This inaugural article considers high performance liquid chromatography, a method used to separate, identify and quantitate components of a mixture. This lends itself in medicine to the detection of a host of compounds in blood and other body fluids, isolation and analysis of mediators from complex biological samples, and development of pharmaceuticals. It is hoped that this collection of articles will constitute a valuable primer for doctors re-engaging with basic science and laboratory research.

Daniel JB Marks, Philip J Smith

Introduction

High performance liquid chromatography is a chromatographic technique that is used to separate, identify and quantify components of a mixture, for example the separation of chemical compounds or identification of constituents of a biological sample. The glossary overleaf explains all terms used.

A typical high performance liquid chromatography system comprises a stationary phase, a mobile phase of varying polarity and an ultraviolet detector (*Figure 1*). The mobile phase is typically composed of two solvents of different polarities, used in a gradient so that the overall polarity can be increased or decreased as desired. The stationary phase is commonly a solid column casing of a few centimetres in length housing silica support particles and a bonded substance immobilized on them which effects the separation of components of the analyte based on their relative polarities. Different compounds interact to varying degrees with the stationary phase, meaning that the polarity of the solvent mixture needed (and therefore the time it takes) to remove them from the column, or 'retention time', can be used as a characteristic identifier for that compound (under the same conditions, i.e. composition, gradient and flow rate of the mobile phase).

In normal phase chromatography, unmodified silica is used along with a gradient moving from non-polar to polar solvents. The more polar components of the analyte adsorb more strongly to the stationary phase and therefore require a more polar mobile phase to displace them and elute them from the column. Conversely, non-polar components have a weak affinity for the stationary phase and therefore elute with a shorter retention time.

Reverse phase chromatography uses 8-carbon or 18-carbon chains bonded to the silica, and a gradient moving from polar to non-polar solvent. The more lipophilic components of the analyte adsorb more readily and are eluted from the column when the polarity of the solvent is decreased. Proper experimental set-up is crucial when using high performance liquid chromatography to ensure accurate analysis; when used properly, high performance liquid chromatography is an incredibly powerful tool and is considered to be a key technique in analytical investigation.

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GLOSSARY

Analyte – the sample to be analysed

Back-pressure – resistance to the flow of the mobile phase through the column

Carryover – undesired presence of peaks from previous samples in a given spectrum

Chromatogram – spectrum obtained from high performance liquid chromatography analysis (representation of the separation achieved by the column)

Column – houses the stationary phase, through which the analyte passes

Eluent – the solvent that carries the analyte

Equilibration – passing mobile phase through the column to achieve a stable and equal distribution of mobile phase

Ghost peak – undesired peak of analyte retained from previous injection

Hydrogen bonding – strong electromagnetic interaction formed between a hydrogen atom and an electronegative atom (such as oxygen or nitrogen)

Isocratic – mobile phase of constant composition (i.e. no gradient)

Mobile phase – mixture of solvents and analyte passed through the column

Packing material – support particles bonded to stationary phase within the column. In normal phase chromatography (where polar stationary phase and non-polar mobile phase are used) porous silica particles are commonly used as support particles as they can withstand high pressures and are compatible with most organic and aqueous mobile phase solvents

Polarity – a separation of electric charge leading to a molecule having an electric dipole moment

Preparative chromatography – larger scale, enabling isolation of analyte components in addition to analysis

Protic solvent – a solvent that has a hydrogen atom bound to an oxygen or a nitrogen atom such that the molecules of such solvents readily donate protons to reagents

Retention factor – ratio of time an analyte is retained in the stationary phase to the time it is retained in the mobile phase, or migration rate of the analyte on the column

Retention time – time at which an analyte peak elutes from the column

Reverse phase chromatography – non-polar stationary phase (usually C8 or C18 carbon chains) and polar mobile phase are used

Silanols – the terminal –Si-OH groups found at the surface of the silica particles in the packing material which are attachment sites for the chosen material of the stationary phase

Sonication – applying sound energy to agitate particles in a sample, commonly using an ultrasonic water bath

Sorbent – material used to adsorb the mobile phase and its contents

Stationary phase – silica support particles and the substance immobilized on them within the column which effects the separation of components of the analyte based on their relative polarities

High performance liquid chromatography has many applications in the fields of biochemistry, clinical science, forensics, environmental science, nutraceuticals and pharmaceuticals. For example, in clinical research, high performance liquid chromatography may be used for analysis in drug trials or in the quantitation of biological components in a given patient sample.

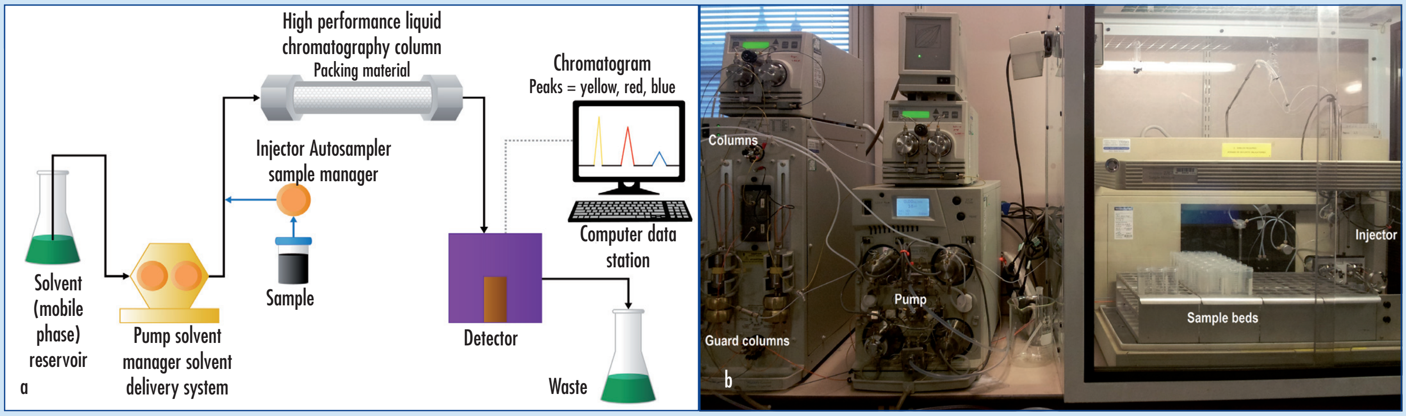
This article discusses some of the common pitfalls associated with high performance liquid chromatography across many of these major areas, including sample preparation, column equilibration, carryover and system pressure. The article will concentrate on useful practical tips and some troubleshooting advice to ensure smooth running of samples. It is not intended to be an exhaustive list of problems that one may encounter when using a high performance liquid chromatography system; for complex issues not addressed contacting your service provider is recommended.

Common pitfalls: what they are and how to deal with them

Sample preparation

Preparative high performance liquid chromatography is a useful alternative to normal or reverse phase flash column chromatography, avoiding the column preparation and sample loading stages for every new

Figure 1. a. A reservoir holds the mobile phase. A high-pressure pump is used to generate and meter a specified flow rate of mobile phase, typically millilitres per minute. An injector introduces the sample into the continuously flowing mobile phase stream that carries the sample into the high performance liquid chromatography column. The column contains the packing material (stationary phase, held in place by the column hardware) needed to effect the separation. A detector is needed to see the separated compound bands as they elute from the high performance liquid chromatography column. b. A typical high performance liquid chromatography setup, including guard columns which contain the same packing material as the main columns and are used to trap any solids or unwanted material from reaching and blocking the main columns.



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cartridge. However, low recovery can be a frustrating problem, often caused by poor scaling of the chosen method from analytical to preparative columns. While a method may be adequate for analytical purposes, sample loss on preparative scale may be too great for the method to be useful. The most common reason for low recovery in reverse phase chromatography is incomplete adsorption of the analyte onto the sorbent, causing the analyte to be lost. This is most likely to be caused by lack of activation of the sorbent; in reverse phase columns this must be activated with organic solvent such as methanol and then equilibrated with water before the analyte is loaded onto the column.

Conversely, analyte may remain on the cartridge after elution. Depending on the analyte, this may be for one of several reasons, the most simple of which in reverse phase chromatography is that the eluent is not polar enough. However, if the analyte is retained on the column via hydrophobic interactions, increasing the concentration of organic solvent in the eluent may help, as may switching to a stronger organic solvent, such as acetonitrile instead of methanol. If the analyte may be interacting with residual silanols in the packing material of the column via hydrogen bonding, then the addition of a protic solvent such as methanol to an eluent of acetonitrile or tetrahydrofuran may resolve the issue.

If the sample is too concentrated there may also be issues with the solids crashing out in the system, causing a blockage; this may also occur when components of the mixture are insoluble in the mobile phase being used. In addition, peaks may overlap if the ultraviolet signal is saturated and may not be clearly defined by the baseline in between them, making quantitation difficult.

Column equilibration

Equilibrating a column is a reversible process; it is necessary to equilibrate a column when the constituents of the mobile phase are changed. The time needed to equilibrate a column depends on the concentration of the new mobile phase and the retention factor of the existing components on the column (i.e. how easy it is to remove them). The volume of mobile phase needed to equilibrate the column, in combination with the flow rate, will therefore determine how long the equilibration will take. In broad terms, the higher the concentration of the mobile phase component and the lower the retention factor of the component to be replaced, the quicker the equilibration. In general, flushing the column with the new mobile phase for several minutes should be sufficient to equilibrate it, but more accurate methodology can be used if required to determine the exact volume needed*.

This simple situation assumes that the column surface does not already contain any methanol or other solvents; however, if the column has previously been equilibrated with a different solvent, for example acetonitrile, this will need to be replaced for total equilibration with the new solvent. The quickest way to do this is to wash the column with a mobile phase containing a high concentration of methanol before adjusting to the (lower) desired concentration, for example 1% methanol.

Some solvents have a very large retention factor, for example when using (polar) methanol in normal phase silica chromatography. If it is necessary to change the solvent system here to a non-polar organic phase, it is much easier to remove the methanol if the column is first washed with a mobile phase of intermediate elution strength, such as ethyl acetate. This will allow easier (and quicker) final equilibration with the desired non-polar mobile phase, such as hexane. If water has been used on the column previously, it is advised to use a similar strategy, gradually decreasing the strength of the mobile phase, rather than trying to equilibrate directly with hexane, which would most likely take several days. A typical sequence is methanol, then ethyl acetate, then dichloromethane, and finally hexane.

Carryover and ghost peaks

Ghost peaks can confuse a spectrum, making an otherwise clean sample appear contaminated. The primary causes of these artefacts are impurities in the mobile phase and sample carryover. When using an isocratic gradient, sample carryover from previous injections may occur if the injector washing step is not adequate, leaving sample on the needle or in the syringe, or if the sample itself is too concentrated. This will lead to a peak of gradually decreasing intensity in subsequent injections (*Figure 2*). It is best to dissolve samples for isocratic chromatography in the mobile phase if possible, rather than a mixture of solvents. This will eliminate the possibility of samples precipitating in the injector or elsewhere in the system. If a sample precipitates on meeting the mobile phase, it may completely block the system, or at the very least, cause the same problem of ghost peaks of decreasing intensity in subsequent injections.

In gradient chromatography, the same issues of carryover and precipitation also apply; there are two main additional causes of ghost peaks. If the water being used in reverse phase chromatography is not of sufficient purity, there may be a build-up of impurities on the column, which will adsorb at the starting solvent composition and then be eluted as the strength of the mobile phase increases during the gradient, often leading to a messy chromatogram with many small baseline peaks. Bacterial growth on the filters used at the end of the mobile phase tubing may also cause the same phenomenon so these should be checked regularly – small air bubbles on the filters are often an indication of bacterial growth.

When analysing proteins by reverse phase chromatography, sample carryover may be an issue, but in this case the cause is likely to be incomplete elution from the column, rather than contamination from the injector. Ghost peaks of decreasing intensity will be observed in subsequent injections, at the same retention time that was seen in the original sample.

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*Typical values for these key factors are given here: concentration of mobile phase in column (x) 1–20 μm/m^2; packing surface area inside column (y) 300 m^2/g; mass of packing material in column (z) 0.5 g. The concentration of adsorbed components is equal to the concentration of mobile phase multiplied by the packing surface area multiplied by the mass of packing material (x.y.z). So, using these figures as a guide, the average concentration of adsorbed components is 150–3000 μm/ml (0.15–3 mmol/ml). Using methanol as an example (molecular weight 32), and using a figure for the stationary phase concentration of 3 mmol/ml, it is possible to calculate that 96 mg of methanol per ml of column volume is needed to completely saturate the surface. If a mobile phase containing at least 10% methanol is used, then there will be enough methanol in 1 ml of mobile phase to completely saturate the column. To ensure complete equilibration, it is advised to double the equilibration volume, so in this case 2 ml of mobile phase will be sufficient. If the concentration of methanol in the mobile phase is lower, it will require a higher volume to equilibrate the column.

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In most cases, flushing the lines and washing the injector and injection port should eliminate ghost peaks; in some cases back-flushing the column (physically removing the column and reattaching it 'upside-down' before flushing mobile phase through it) may be necessary to remove stubborn impurities.

System pressure

Consistent system back-pressure is crucial for spectrum reproducibility. High back-pressure can be caused by several things including incorrect composition of the mobile phase, temperature fluctuations, incorrect column choice or a blockage in the system.

Different solvents have different inherent viscosities so if the make-up of the mobile phase is incorrect, as a result of a mix-up of lines or a solvent bottle running dry, the back-pressure may increase. Failure of an automatic solvent blending system may also cause fluctuations in the back-pressure. If a column heater is in use, in order to work at elevated temperature, a malfunction here may also cause variation in the back-pressure. Solvent viscosity changes by a factor of 25% per 10°C so even a small change in temperature could cause a large difference in back-pressure. Column back-pressure also changes as a function of the particle size of the column packing, so a column with 5 µm packing will lead to a higher system pressure than the 10 µm version of the same column.

The most common cause of high back-pressure is a blockage in the system. Ideally, the guard column will be the culprit, given that its sole purpose is to prevent solid impurities from reaching the (far more expensive) column. A blockage in the guard column is easily identified by removing the guard column and reattaching the tubing directly to the analytical column. If a blank injection with the standard gradient no longer exhibits high pressure then the guard column is likely to be partially or completely blocked. It is possible to try to clean the guard column by sonication in an appropriate solvent mixture (or 100% polar solvent) but in many cases it is best to discard the blocked guard column and use a new one, as it will have performed its function and any future blockage may not be effectively prevented from reaching the analytical column. If the blockage still persists after removal of the guard column then a process of elimination will be necessary, checking each piece of tubing and other system components in turn to discover where the blockage is located. It is useful to have a record of the usual system pressure for comparison when a blockage is suspected, or indeed to diagnose high back-pressure at any time.

Conclusions

High performance liquid chromatography is a very powerful technique for separating and analysing components of a mixture, whether that is compounds in an analytical chemistry lab or mixtures of proteins being used for clinical research. High performance liquid chromatography can also be coupled to a mass spectrometer to facilitate analysis by mass of the components of a mixture in addition to ultraviolet; this can be a very powerful addition for example when analysing whole proteins for residue replacements or fluorescent tagging, when the ultraviolet peak may not shift noticeably but the molecular weight of the molecule in question will change.

There are many pitfalls associated with using high performance liquid chromatography in the lab, but by careful analysis of the problem and often using a process of elimination, the cause and therefore strategies for future prevention can be identified. **BJHM**

Conflict of interest: none.

The author would like to thank Muddasar Farooq for helpful discussions and early input into this article and Uwe Neue for invaluable clarity in the Waters HPLC troubleshooting guide (Neue, 2002), from where several concepts in this article are reflected.

The diagram and text for Figure 1a *are reproduced and edited with permission from Waters (2013).*

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Further reading

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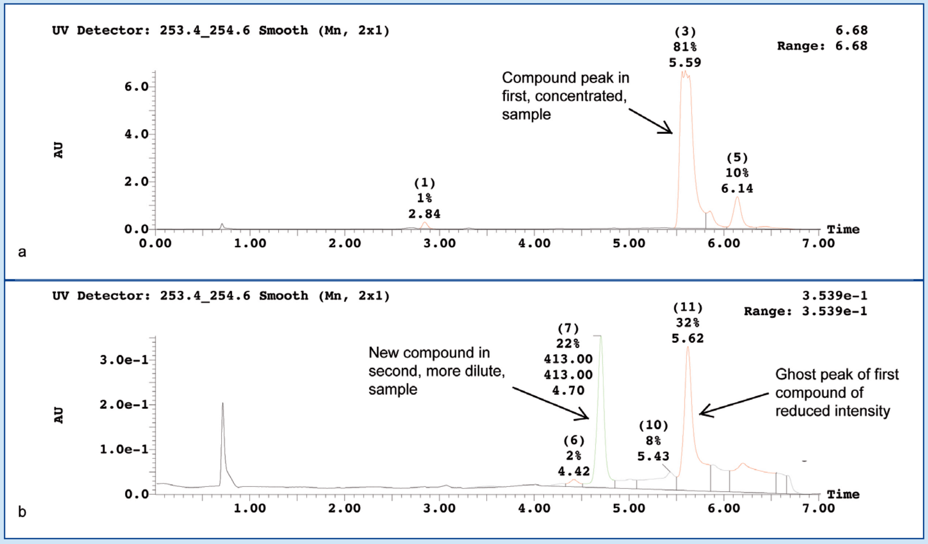
Figure 2. Example of carryover. a. The sample in the top chromatogram was too concentrated, leading to (b) contamination of the column and the appearance of a ghost peak of the same retention time in the next sample, which was actually a clean compound.



Compound peak in first, concentrated, sample

New compound in second, more dilute, sample

Ghost peak of first compound of reduced intensity

KEY POINTS

- High performance liquid chromatography is a type of chromatography that is used to separate, identify and quantify mixtures of compounds.
- This typically involves using a stationary phase, a mobile phase of varying polarity and an ultraviolet detector.
- Common pitfalls associated with high performance liquid chromatography include low recovery, sample carryover and messy spectra. These can be avoided by appropriate sample or column preparation, correct choice of solvent and maintaining a consistent system pressure.

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