Chromatography Today Help Desk

Important Considerations for Repeatable HPLC Assays of Chiral Compounds

The manufacturing process of many of the active pharmaceutical ingredients that are used in prescription drugs today may result in racemic mixtures of the compound being synthesised. In some cases, the efficacy and toxicity of the different mirror forms of the drug will be nominally the same and so the consequences of not having a single enantiomer pure drug are mild. However, there many cases where this is not true. Probably the most discussed drug that falls into this category is thalidomide and the birth defects associated with the application of this substance. The drug was prescribed in Canada, Europe, Australia, and parts of Asia in the late 1950's-1960's to pregnant women in their first trimester to treat nausea and vomiting caused by morning sickness. After the introduction of thalidomide as a pharmaceutical, there was a huge increase in birth defects, especially in Europe and Canada, caused by an enantiomeric impurity. It is estimated that as many as 10,000 children were born with major malformations due to their mothers taking this drug during pregnancy. The thalidomide molecule is a racemic glutamic acid analogue, consisting of S- and R+ enantiomers that interconvert under physiological conditions. The S- form potently inhibits release of tumour necrosis factor from peripheral mononuclear blood cells, whereas the R+ form seems to act as a sedative, probably mediated by sleep receptors in the forebrain, the different forms of the molecule are shown in Figure 1.

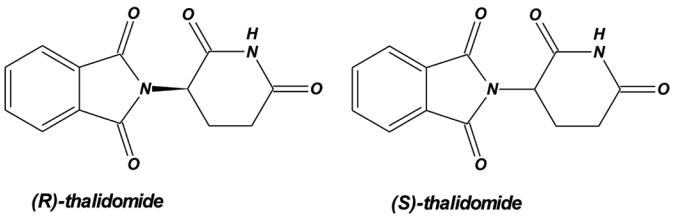

Figure 1. The two enantiomers of thalidomide

The two enantiomers of thalidomide

Quality procedures to ensure that the compound has only one form of an enantiomer are very dependent on retention times. If there is a shift in the retention time, there is a strong probability that the concentration of the wrong compound may be determined. For some separations, the separation factor is large enough to ensure that this is not a problem, however the nature of a chiral separation is that peaks elute close together.

This raises the question of what causes retention time shift? Unfortunately, there is no one solution, as there can be several factors. This article will look at causes of a shift in retention time. Although in this case it is applied to the analysis of enantiomers, it is very relevant to all analytes.

Check Valves

Single pump heads

One obvious cause of retention time shift is that the pump is not delivering the correct flow rate. This is easily assessed, by either connecting a flowmeter or by using a stop watch and a measuring cylinder / syringe (the latter is for low flow rates, with the needle attached to the outlet tubing from the LC system). If the pump has been identified as the cause of the retention time shift then resolving the issue needs to assess several criteria, which will depend on the type of pump that is being used. For isocratic and low pressure mixing pumps, there is a single pump head and the pump seals and check valves on this pump should be checked.

Single pump heads may include two pistons which will drive the flow under pressure through the LC system. There are several arrangements for pistons, either reciprocating so that one pump head is delivering flow whilst the other is refilling the solvent chamber or flow-through, where there is a primary and a secondary pump in line. The primary pump pumps liquid through the secondary pump which delivers flow whilst the primary pump refills the solvent chamber. This design differs from the dual reciprocating pump where each piston delivers flow independently to a mixing tee.

Dual pump heads

A high pressure binary pump will utilise two pumps that mix together at a tee. With this physical arrangement, inaccuracies in the flow rate can be attributed to either or both of the pump heads. One common issues associated with older style pump heads is that the pump head delivering the organic solvent does not contribute its share to the overall flow of the mobile phase. There are a range of materials that can be used for check valves (the most common being sapphire, ruby or ceramic) and selection of the correct check valve material may overcome this issue. As a temporary fix it is possible to clean the check valve in a water methanol mixture to allow the pump head to function appropriately. A better approach is to employ a ceramic check valve that will work better in high acetonitrile composition mobile phases. The use of mechanically assisted check valves will also eliminate the issue. The cause of this is explained in detail by an excellent article Dolan [1].

Compressibility Factor

Solvents compressed under pressure will have differing degrees of compressibility. This is something that will typically have to be programmed into the pump delivery system. Compressibility is the fractional change in volume per unit increase in pressure. Water has a compressibility factor of 46.4×10^{-6} Atm^{-1}, therefore, for each atmosphere increase in pressure, the volume of water would decrease 46.4 parts per million. The compressibility

that is quoted for most organic solvents in liquid chromatography is about 110 x 10^{-6} Atm^{-1}. If the compressibility of the liquid is not set correctly, then the pump will potentially deliver the wrong flow rates and cause an increase in the level of noise originating from the pump. Compressibility settings are unlikely to change during an assay but might create retention shifts when instrument systems are changed.

Pump seals and pistons

Pump seals and pistons are often thought to be a cause of incorrect flow delivery, however in reality it is rarely the seals or piston. If the seals are suspected, then the installation of new ones should be done. Great care should be taken, particularly if very high pressures are being employed, as the seals have to be bedded in properly. With the pistons, the use of buffers can result in small scratches which can cause leaks and make accurate solvent delivery impossible.

Solvents

Mobile phase selection can play an important role in the reproducibility of the retention time for an analyte. Particular attention should be paid to the equilibration time and also the sensitivity of the analytes to effects such as pH and injection solvent. The following sections will discuss the implications of incorrect selection of solvent, either in mobile phase or injection solvent.

Equilibration issues

Another consideration is the equilibration time that is given at the end of the chromatographic run. This has been discussed in a previous Chromatography Today helpdesk articles, and so it will not be discussed in great detail here. However, this tends to become significant when the method is being transferred between different systems or columns. Often, if there is an issue this will be highlighted by poor peak shapes, with the affected peaks typically fronting. Figure 2 shows the effect of not having the correct equilibration time with the first injection looking fine, but with shifts in the retention time being observed with subsequent injections.

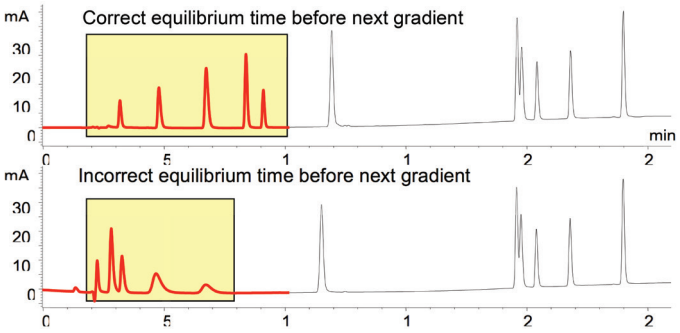

Figure 2. The effect of not equilibrating the column effectively before an injection.

pH

Stability of the retention time for any compound, whether it is chiral or achiral, can be very dependent on the variation of the log D as a function of the pH. In particular, it is important to keep the pH away from any pK^a points for that the compound, since this will result in inherently unstable retention times. Although this may not affect the elution order, this will have a detrimental effect on the accurate determination of the any compound. Figure 3 illustrates the effect that pH has on log k. It can be seen for neutral compounds that there is no appreciable effect.

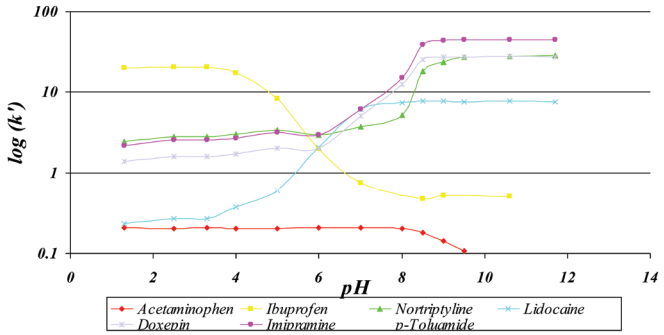

Figure 3. Effect of changing the pH on acidic, basic and neutral compounds, printed with kind permission M. Euerby.

Injection solvent

The final issue to consider is the injection solvent. The choice of the injection solvent may be affected by analyte solubility and also by any sample pre-treatment that may have occurred prior to the separation. It is important to understand that chromatography is fundamentally based on a partitioning effect between a mobile phase and a stationary phase, and that when the compound is injected the mobile phase is formed partially by the injection solvent. For very strong injection solvents, the compounds of interest will remain predominantly within the mobile phase and not interact with the stationary phase, with the retention times shifting towards the solvent front as a consequence. As with the incorrect use of equilibration times this may also result in poor peak shapes. As with all of the previous causes of retention time shift, it would be anticipated that this would affect both enantiomeric forms equally, however if only one enantiomer is being seen, which may reflect the synthetic pathway, it can result in a large uncertainty with the assay. Other injection considerations such as pH, buffer strength and matrix components can affect the potential partitioning between of the enantiomers and the stationary phase.

Conclusion

With chiral separations, where resolution is often marginal between enantiomers, there are a variety of potential pitfalls that separation scientists should be careful of. Being aware of the parameters that can cause a shift in the retention time will ensure the development of robust assays to ensure that good decisions are made based upon accurate results.

It should be noted that modern pumps have built-in diagnoses or there are sophisticated check-out and performance verification procedures provided by manufacturers, so many of the suggestions in this article may not be so pertinent to newer pumps. It should also be noted that these guidelines are relevant for all HPLC separations and not specifically for chiral separations, where mostly isocratic, premixed solvent are used.

References

1 J. Dolan, Jun 01, 2008 LCGC North America 26 (6) 532–538,