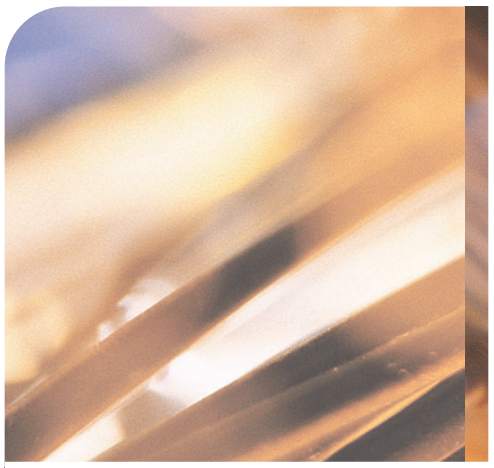

Chromatography Focus

EXPANDING POSSIBILITIES IN LIQUID CHROMATOGRAPHY - HIGH PERFORMANCE CCC

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"WITH THE ADVENT OF THE NEW GENERATION OF HPCCC INSTRUMENTS NEW ELUTION MODES IN LIQUID CHROMATOGRAPHY ARE AVAILABLE FOR SEPARATION SCIENTISTS TO USE"

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THE STATUS QUO

The vast majority of contemporary liquid chromatography techniques employ a liquid mobile phase passing over a solid stationary phase contained in a column. Your sample is then injected into the mobile phase and passes on to the column. The solid stationary phase will retain different components of the sample at differing rates, caused by interaction with the components of the sample, these components will then elute one after another by the time they reach the end of the column.

When using liquid-solid chromatography only one elution mode, typically Reverse Phase, is possible on any given column and this can be limiting.

- For example, although the separation maybe complete on the column (i.e. all components are resolved) you must continue to flow the mobile phase through the column to remove all the components from the solid phase column. This process increases run time, reducing productivity and can be costly due to solvent consumption.
- A solid stationary phase can only be stationary and to reap the benefits of a moving bed requires the use of multiple columns and complex valving arrangement.

Today pharmaceutical companies are developing compounds of increasing complexity and molecules must be purified to ever more stringent standards. Soild phase chromatography technology has served so well for so long, is now under severe pressure to perform and reference [1] clearly indicates the challenges that current techniques are facing.

To meet these challenges providers of liquid chromatography instruments are developing many new modes for operating solid phase columns– low viscosity mobile phases (SFC), ultra high-pressure liquid chromatography (UHLC) or multi-column chromatography (SMB).

However, all are just variations where a particular variable is altered and none is a fundamental or disruptive change. Hence, only a step change in performance is produced, but it is also introducing specialised equipment where ancillaries are specific rather than generic. The job is done, but at a cost.

Perhaps the time is right to consider a new approach rather than adapting an existing technique.

LIQUID STATIONARY PHASES

Perhaps the problem cannot be solved, if you use a solid stationary phase, but what if you could use a liquid stationary (or moving) phase instead. Since the early 1960s, there has been a liquid chromatography technique available that uses liquids for both mobile and stationary phases, namely Countercurrent Chromatography (CCC); however, it has largely been ignored.

The reason for this was that solid stationary phases could solve the separation challenges and the compounds were not exposing the limitations of the technique. Over the last few years, that situation has changed and now the use of liquid stationary phases is being considered.

The reasons are that most scientists appreciate the benefits of using two liquids for the mobile and stationary phases, the benefits being:

- Improved solubility capabilities
- Higher purification throughput
- Improved yields

The very problems that most scientists are trying to solve and manufacturers of solid phase columns are adapting their equipment to imitate even though it cannot emulate the performance.

So why has this liquid chromatography technique, Countercurrent Chromatography, not been more widely adopted in the last 40 years? This can be easily summarised by three features of the instruments that the technique is performed on:

Time of separation: Even with the introduction of "so called" high-speed CCC (HSCCC) machines in the early 1980s, which took many hours to perform a complete purification, whereas other liquid chromatography (LC) techniques performed similar tasks in minutes or tens of minutes.

Range of equipment available: Previously the only size of HSCCC machine available was at the semi-preparative scale (i.e., high hundreds of milligrams to low gram quantities). At the early stages of a drug development programme, this size of injection might be the total amount of material available, and would never be risked as a single injection.

Further, down the drug development process there is a requirement to process tens to hundreds of grams of material. Since there were no larger HSCCC machines available, purifications using this technique were impractical.

Reliability of equipment: Until the late 1990s, the reliability of HSCCC instruments was questionable. Given the value of the products requiring purification, it is hardly surprising they were not risked in these instruments.

However during the late 1990's, continuing through to 2004, there has been a revolution resulting in a new generation of instruments, High Performance Countercurrent chromatography [2] (HPCCC) that have completely addressed the above issues. These high g instruments allow separations to be performed at the milligram scale through to the kilo scale seamlessly.

HOW DOES THIS CHANGE THE STATUS QUO?

In CCC separations, the mobile phase is a liquid as before, but the stationary phase is also a liquid. In CCC, these liquids are immiscible and typically one will be an organic solvent and the other will be an aqueous solvent. In a CCC instrument, either the organic or the aqueous solvents could be mobile. Therefore, in CCC a normal phase (NP) separation is just one where the organic phase is mobile and the aqueous phase stationary and a reverse phase separation (RP) the converse is true. It is also possible that both organic and aqueous phases can be moving at the same time, in either counter or co-current directions. This enables a completely new range of elution modes that cannot be considered when using solid stationary phases.

With these new elution modes and current range of HPCCC instruments the possibility exists for fundamental change, using existing ancillary equipment associated with solid phase liquid chromatography.

ELUTION MODES POSSIBLE USING HPCCC

The following gives you an overview of the different elution modes that are possible using HPCCC instruments:

Standard elution mode: This is the same as used in solid phase liquid chromatography. The stationary phase is retained while the mobile phase flows in one direction only. Either phase can be the mobile phase and therefore either normal (organic phase mobile) or reversed (aqueous phase mobile) phase separations are possible.

Elution extrusion: This strategy takes advantage of the fact that the analyte may be fully separated inside the column before being eluted. Because a liquid stationary phase is used, it is possible to recover the separated compound without completing a full elution cycle. In elution extrusion, the separation begins in the same way as standard elution mode. However, once the separation is complete the stationary phase is extruded which elutes the last component(s) of the separation, keeping not only the separation efficiency but reducing run time and solvent consumption.

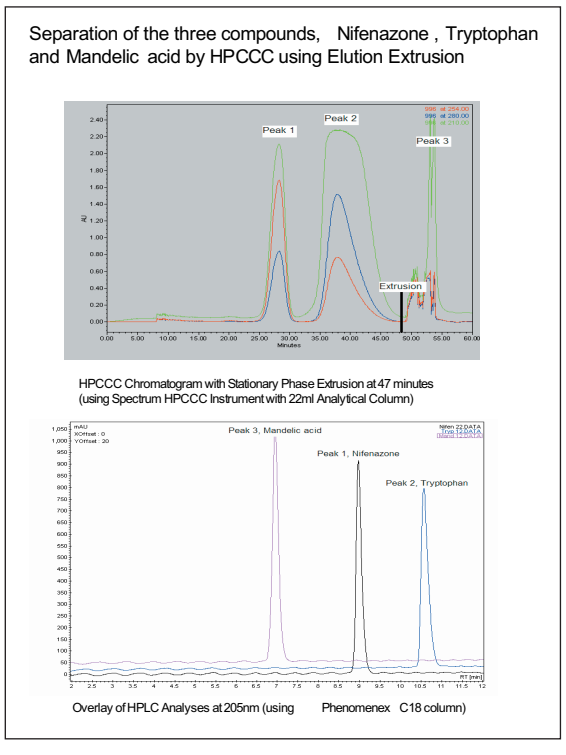

Figure 1. Separation of the three compounds, Nifenazone, Tryptophan and Mandelic acid by HPCCC using Elution Extrusion.

This figure demonstrates the use of elution extrusion mode for compounds which are retained in the stationary phase of a HPCCC column. The top panel is a chromatogram from the elution of compounds from a HPCCC column showing the first two compounds separating in the HPCCC centrifuge and the final compound of the mixture which is eluted with the stationary phase.

Dual-mode elution: When operating a dual-mode elution strategy, the aqueous phase is initially pumped as the mobile phase (i.e. normal phase operation) and after a set period of time, the organic phase is pumped as the mobile phase (i.e., reverse phase operation). This switching procedure is repeated until the desired resolution for purification is achieved.

The advantage of this method is that compounds that have a strong affinity for the original stationary phase can also be separated more quickly maintaining resolution but reducing run time. In the case of compounds which have overlapping elution peaks, this method can prise apart these peaks avoiding the need to screen multiple gradients and different columns.

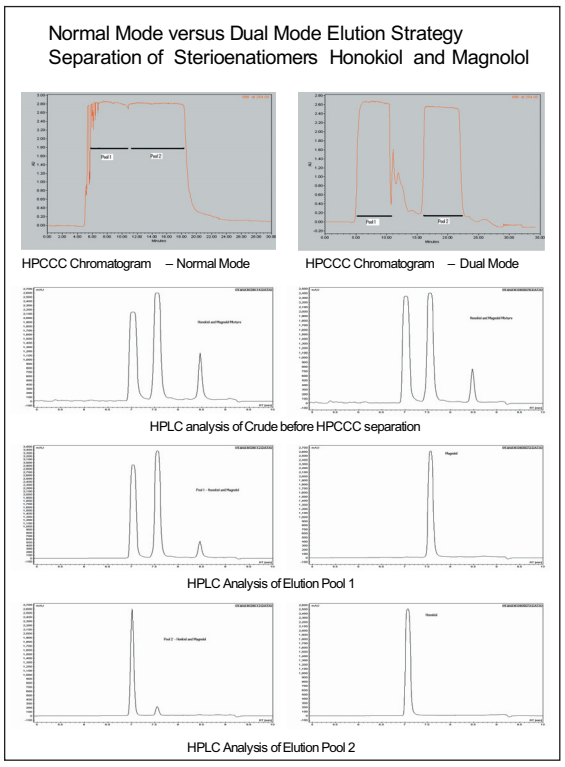

Figure 2. Normal Mode versus Dual Mode Separation of Stereo-enantiomers Honokiol and Magnolol

This figure demonstrates the benefits of using a Dual Mode elution for HPCCC separations, where first one phase then the other is pumped after each other in opposite directions.

This elution mode is useful for the separation of compounds which would normally co-elute or have overlapping elution peaks. The top panels show the chromatograms for the standard elution mode (Normal Phase only) and Dual Mode (Normal Phase then Reverse Phase then Normal Phase etc). From these HPCCC chromatograms and the analyses of the elution peaks by HPLC, below, you can see the benefits of this technique.

pH zone refining: This elution strategy utilises the phenomena that charged entities (ions) prefer the aqueous phases and uncharged molecules prefer organic phases so uses basic organic phases and acidic aqueous phases (or vice versa). The analytes dissolved in the stationary phase are eluted by the mobile phase according to their pKa values and solubility.

This strategy enables the separation of compounds at higher loading capacities, which are structurally identical but have different pKa's.

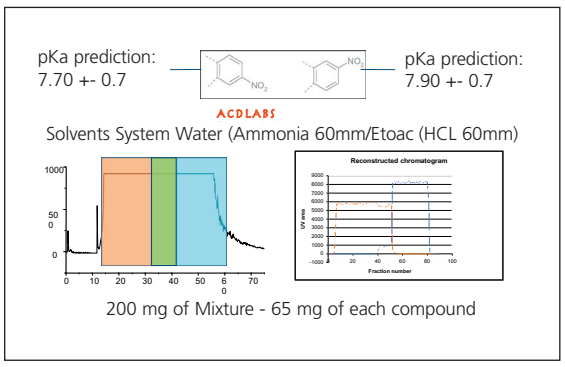

Figure 3. pH Gradient Elution: Regioisomers separation Urology, Alpha 2a antagonists

However, more developments are currently being researched. Of particular interest is the use of the co-current elution, which if combined with the ability to place a chiral selector in either of the phases would lead to a new option for enantiomer separation. Rather than SMB (Simulated Moving Bed), which is carried out using multiple solid phase columns, we will have TMB (True Moving Bed) and that potentially could reduce the cost of chiral purifications at all scales dramatically. Inevitably, this leads to the possibility of continuous chromatography, where the stationary phase is continuously being refreshed and smaller units can be used 24x7, reducing capital expenditure and improving productivity.

Co-current elution: Here the mobile and stationary phases are pumped simultaneously. Depending on the retained volume of each phase in the column, the residence time of each will be different, eluting the sample in a defined band whose volume or width is determined by the respective flows. This allows the stationary phase to be continuously refreshed, allowing continuous processing.

The challenges facing separation scientists today are very different from only ten years ago, let alone 30 or 40 years ago and therefore new solutions are required to work along side other liquid chromatography techniques, in order for scientists to get their job done. With the advent of the new generation of HPCCC instruments new elution modes in liquid chromatography are available for separation scientists to use. These new options can reduce overall processing time, dramatically reduce solvent consumption and provide a purification solution that traditional technologies cannot. Rather than just trying to adapt, this brings new thinking to bear.

REFERENCES

1. Lopez et al., Strategies for the purification of Synthetic Products in the Pharmaceutical Industry., LC-GC December 2005

2. Keay et al., Advances in High Performance Countercurrent Chromatography., The Column June 2007

ACKNOWLEDGEMENT

S.T.A. Dubant, of Pfizer, for producing the chromatograms in Figure 3.