Holistic LC strategies, from UHPLC to HPLC and back

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UHPLC has recently become a powerful tool in pharmaceutical analysis, specifically for method development, routine and rapid analysis. However, there are often limited numbers of UHPLC instruments available within internal and external customers and they do not always have access to this technology. This article gives an overview of the development and application of a holistic LC strategy to facilitate the implementation of UHPLC within a global Pharmaceutical Development function. An approach is being adopted that will enable the translation (geometric scaling) of chromatographic methods from "core" generic UHPLC methods with <2 µm columns to HPLC with ≥3 µm columns. There are requirements for UHPLC to HPLC transfer and practical issues to consider, specifically: instrument dwell volume differences, ensuring consistent selectivity across different particle sizes, frictional heating effects and pressure-induced retention effects. Finally the article demonstrates examples of UHPLC to HPLC translations and the application in AstraZeneca R&D projects.

Introduction

The role of LC in pharmaceutical development

HPLC has long been established as one of the main analytical techniques used in pharmaceutical R&D for controlling the quality and consistency of the active drug substance, synthetic pre-cursors (intermediates and starting materials), and drug product (dosage form). For example, HPLC is used in supporting Process R&D by helping to understand the impact of changes in the synthetic route, processing conditions and the scale of manufacture. HPLC is frequently used in determining the purity of different batches of the active drug substance and so helps to ensure that materials used in clinical trials are of a similar quality to that which has been assessed in toxicological studies. HPLC is also used to determine whether any degradation of the active drug substance occurs within the drug product over time and is used to establish the shelf life.

Since the introduction of Ultra High Performance Liquid Chromatography (UHPLC) in 2004, AstraZeneca and several other Pharmaceutical companies have investigated UHPLC as an alternative to HPLC for the analysis of pharmaceutical development compounds. Wren *et al.* presented data showing that significant reductions in separation time can be achieved with UHPLC, without compromising the separation quality.[1] Furthermore, results from precision and comparative studies indicated that UHPLC was a suitable technique for routine pharmaceutical analysis. The initial attempt to devolve the technology within AstraZeneca development function in 2005 was unsuccessful owing to "child diseases" and the specific training requirements associated with the new technology. Additionally, the instrumentation was considered to be unreliable.

In 2007 following technological improvements and further experience of UHPLC technology a series of evaluations and implementation plans were undertaken in sequential stages. This included the evaluation of the latest UHPLC technology by an AstraZeneca "expert" (Stage 1). After selection of the most suitable instrument, a global evaluation of UHPLC was initiated in a small number of mid/late stage drug projects, led by local "super users" (Stage 2). The findings clearly demonstrated the expected advantages of the technique, with regards to improvements in the quality and productivity for routine LC analyses. The study also highlighted the improved speed facilitated better capability to analyse both labile samples and larger numbers of samples. Thereby introducing the possibility to lower long term instrument costs by reducing the numbers of LCs within AstraZeneca Pharmaceutical development (e.g. replace 2 to 3 HPLCs with 1 UHPLC).[2,3] A business case was drafted for the wider implementation of UHPLC using a larger number of users, projects and functions (Stage 3). However, before full implementation of UHPLC technology in R&D a key gap remained, in that some of our key internal and external customers (e.g. Chemistry Sciences and contract research organisations, respectively) didn't have access to UHPLC instrumentation and would still require HPLC methods in the medium term. In order to facilitate the devolvement of UHPLC it was apparent that HPLC methods offering comparable selectivity and resolution (at the expense of increases in analysis time) were required.

Holistic LC strategy utilising UHPLC and HPLC

To address the issue of limited UHPLC instrument availability and aid it's phased implementation into AstraZeneca R&D an holistic LC strategy was devised, incorporating both UHPLC and classical HPLC technology. An approach is being adopted globally that will facilitate the translation (geometric scaling) of chromatographic methods from UHPLC to HPLC. Specifically translating "core" generic UHPLC methods using 10cm columns with <2 µm or superficially porous phases to HPLC with 15 cm columns with an equivalent ~3 µm or superficially porous stationary phase and the same mobile phases (see Figure 1: generic "core" UHPLC[c] and

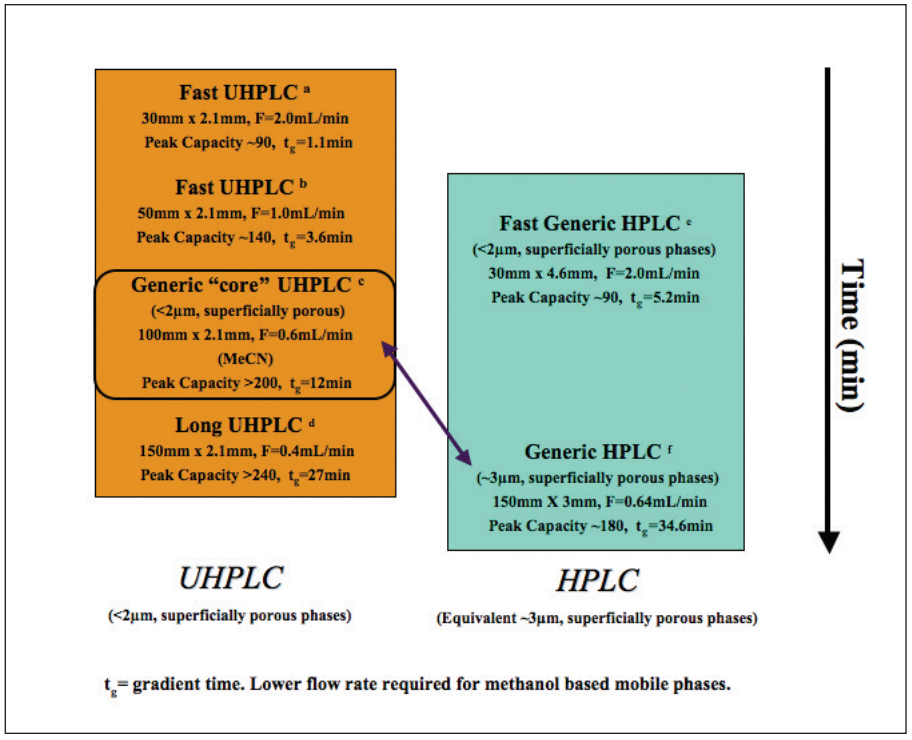


Figure 1. Schematic representation of using translatable UHPLC and HPLC providing a range of analysis times and peak capacities to suit different customer's needs.

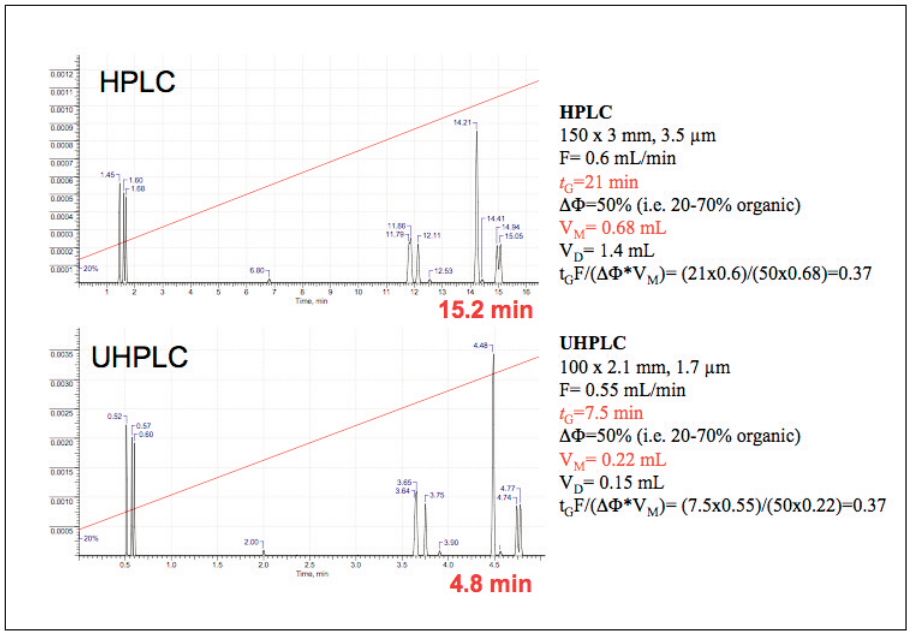


Figure 2. Chromatograms showing the geometric scaling of gradients and flow rate of a HPLC method (top) to UHPLC method (bottom) by keeping $t_G F/(\emptyset V_M)$ constant.

generic HPLC[f], respectively). In theory these HPLC methods offers comparable selectivity and resolution, but at the expense of an approximate 3 fold increase in analysis time. However, it does mean that UHPLC methods can be developed in the knowledge that if customers do not have UHPLC instrumentation then transfer back to HPLC is possible.

This strategy also offers a range of column dimensions with scaled gradient times and flow rates to provide a range of peak capacities and/or analysis times to meet a specific application's needs. For example, where only speed is important and peak capacity/resolution can be sacrificed (e.g. reaction monitoring methods), then faster UHPLC or HPLC methods are available which should exhibit comparable selectivity, but lower resolution compared to the "core" UHPLC method (Figure 1: Fast UHPLC[a], Fast UHPLC[b] and Fast HPLC[e]) . There is the possibility of further increasing peak capacity in UHPLC with longer columns and an increase in analysis time, but it is not possible to directly translate back to HPLC with comparable peak capacity (Figure 1: Long UHPLC[d]). Using the same principles it is possible to scale to semi-preparative LC methods using the same phase in 5 or 10 µm to isolate low quantities of impurities.

Method translations

The calculations needed for scaling methods whilst maintaining equivalent selectivity are well documented and are based on keeping the average retention factor constant (k_G) for each segment in the gradient (Equation 1).

$$k_G \propto t_G F/(\Delta\emptyset V_M) \qquad (1)$$

This requires the scaling of the gradient volume ($t_G F$) in proportion to the column dead volume (V_M), whilst maintaining the same initial and final mobile phase composition ($\Delta\emptyset$) in each gradient segment. This is achieved by scaling of the mobile phase flow rate (F) with the gradient time (t_G). Additionally injection volume should be scaled based on the difference in column dimensions in the two methods (See Figure 2 for an example).[4,5] In fact, several software programs for method translation are available from instrument vendors and Universities to simplify this process.[6]

Practical aspects of method translation

In practice there are sometimes complications when translating methods between UHPLC and HPLC instrumentation, specifically dwell volume effects, differences in gradient shape, frictional heating, pressure-induced retention effects and differences in selectivity across particle size, which are discussed in more detail below.

Dwell Volume Effects

Differences in dwell volume, column dead volume and flow rate can cause noticeable differences between the programmed gradient profile to that actually observed, which may affect retention in the entire chromatogram (i.e. a general shift for all peaks). It may also affect selectivity, particularly early eluting peaks and analytes that are not structurally similar, which could have very different retention characteristics (e.g. different slopes in log$k$ *vs.* % organic). Using the flow rates, column dimensions and dwell volumes it is possible to calculate which part of the chromatogram may be affected and the retention time and relative retention time shifts.

It should be noted that there is often a considerable difference in dwell volume between not only HPLC and UHPLC instrumentation, but also between different HPLC instruments. As such some methods often state the instrument dwell volume used during method development and routine

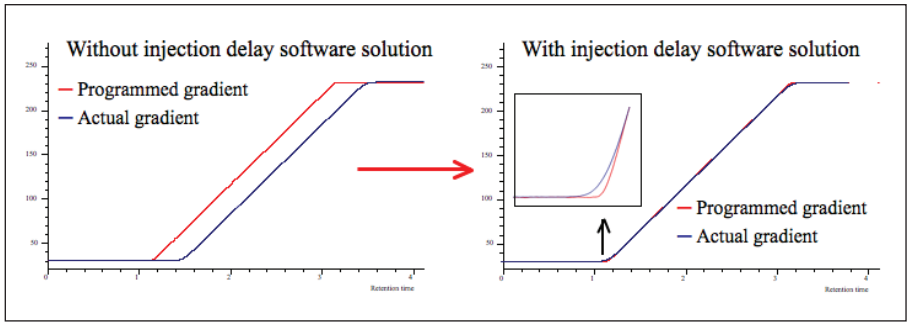


Figure 3. Programmed and actual gradient profiles on a UHPLC with 400 µL TFA mixer in combination with a prototype software solution for delayed injection to mimic the dwell volume of a UHPLC with 50 µL mixer.

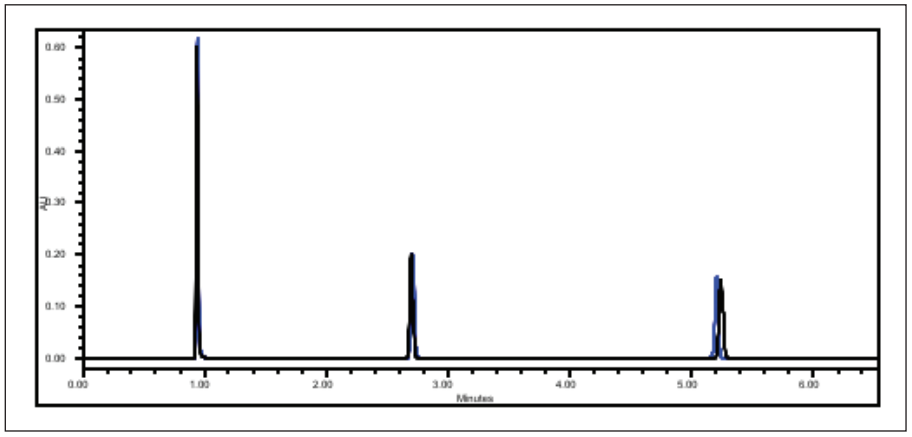


Figure 4. Chromatograms from a UHPLC with 400 µL TFA mixer in combination with a prototype software solution for delayed injection to mimic the dwell volume of a UHPLC with 50 µL mixer. Experiment uses a 32.2 sec injection delay with 400 µL mixer and 100 x 2.1 mm i.d. BEH C18 column, 0.6 mL/min, 40 °C, 40 to 100% acetonitrile in 10 min.

use. It is possible to compensate when transferring the new method onto instruments with a smaller dwell volume by introducing an isocratic step at the start of the gradient, to mimic the effect of the larger volume (i.e. transfer from HPLC to UHPLC). However, compensation when transferring from a low to a larger dwell volume LC system (i.e. transfer from UHPLC to HPLC) is not possible. The impact is larger for high dwell volume systems and low volume columns (i.e. short and/or narrow columns). In the case of transferring from UHPLC 100 mm x 2.1 mm to HPLC 150 mm x 4.6 mm only the initial 3% of the gradient is affected and selectivity changes in the gradient are not usually an issue in our experience. However, when transferring from UHPLC to Fast HPLC (30 mm x 4.6 mm) the effect is significant, where approximately the first 15% of the gradient is affected and selectivity changes in the early part of the chromatogram are more likely.

The impact of the dwell volume can be lowered by aiming for an average capacity factor (k_G) >3 and by using wider diameter columns. The generic methods (listed in Figure 1) have been designed in order to minimise these differences, by using larger column diameters on HPLC systems with larger dwell volumes. It is often possible to reduce dwell volumes of HPLC instruments, which is particularly beneficial for fast HPLC applications. This has been used successfully within a number of AstraZeneca laboratories. This requires removing mixers or using lower volume mixers, performing injector by-pass routines and utilising lower volume tubing, needle seats and UV flow cells to reduce the system dwell volumes and extra column band broadening.[7,8]

A recent prototype software solution has been developed by Waters, which enables delaying the injection to compensate for dwell volume effects. The software application delays the injection to mimic an instrument with a specific lower dwell volume or delays the injection until the gradient reaches the head of the column. Use of this injection delay can realign the actual gradient (Figure 3). Initial results in our laboratories show that larger dwell volume systems can mimic a lower dwell volume system with only minor changes in peak retention times (Figure 4). However, minor changes in the gradient on-set and off-set profile are observed owing to the larger mixing volume (Figure 3). This should only have a significant affect on "ballistic" gradients (e.g. 1 minute gradients with sharp changes in mobile phase composition) and not routine analytical methods as highlighted in Figure 1 (methods b-f). Generally this approach should enable dwell volume compensation and facilitate effective method transfer.

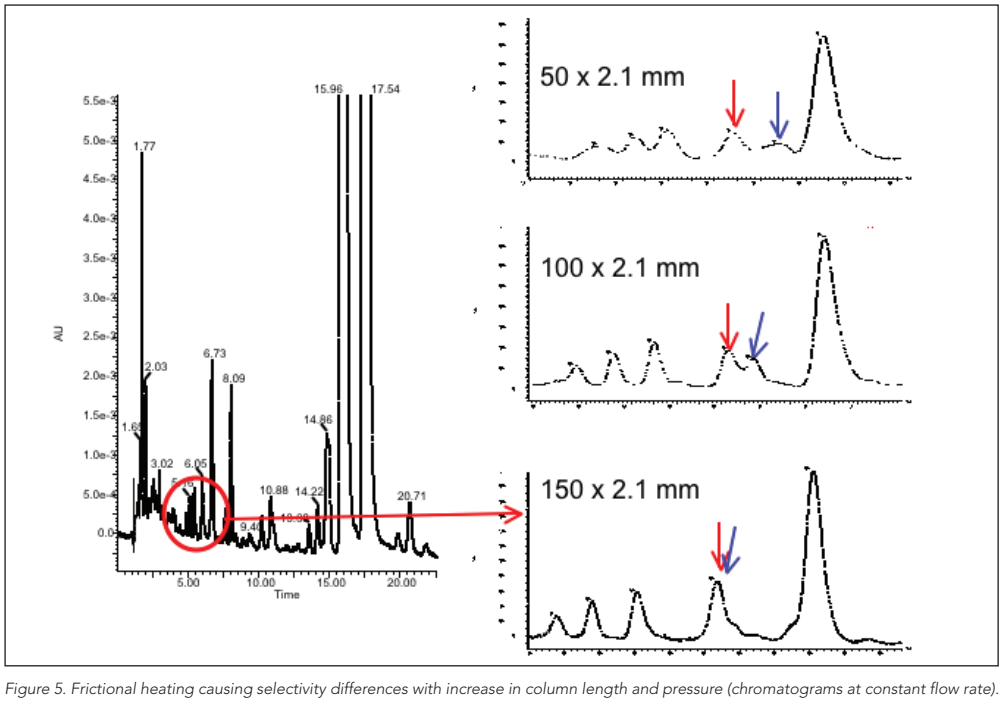


Figure 5. Frictional heating causing selectivity differences with increase in column length and pressure (chromatograms at constant flow rate).

Heat of friction and pressure-induced retention effects

The flow of mobile phase through particles in a packed LC column generates friction, and therefore produces heat resulting in a rise in temperature of the mobile phase. The higher pressures and linear velocities encountered in UHPLC compared to HPLC results in an increase in the heat of friction and also in the internal column temperature. This could cause

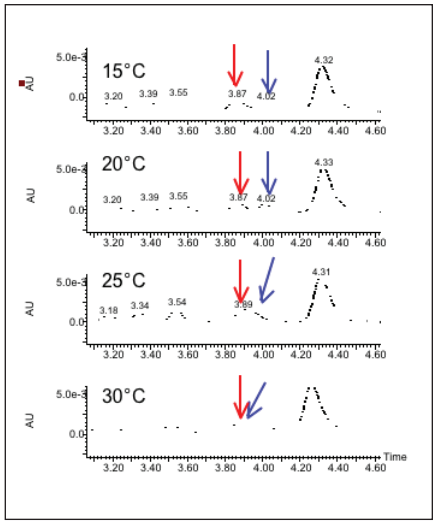


Figure 6. Compensation for frictional heating effects by decreasing temperature when using increased column length and pressure (examples using 100 mm x 2.1 mm i.d. column at the same flow rate).

selectivity differences and depending on the column characteristics and particularly on its diameter, could also reduce efficiency.[9,10] However, the smaller the column diameter, the better the heat dissipation and the less of a problem it creates. Accordingly, UHPLC instruments typically use ~2 mm or 1 mm columns instead of the 3/4.6 mm columns used in HPLC. Therefore UHPLC research using much higher pressures (1,000 to 7,000 bar) requires the use of capillary columns.

Accordingly, in practice it may be necessary to increase the temperature of a HPLC method if translating from UHPLC to obtain the same selectivity (or vice versa). It may even be required to increase the temperature when scaling from one column dimension and/or flow rate to another. During translation of a method on a 5 cm column to longer 10 and 15 cm columns to improve resolution of two minor impurities, it was observed that the resolution actually decreased when using the same temperature (30°C) flow rate with scaled gradient times (as seen in Figure 5). Heat of friction was suspected to be the cause and this theory was supported by the fact that decreasing the column temperature to 20 °C compensated for the frictional heating when using the longer 10 cm column (Figure 6).

Recent investigations into the effect of pressure on retention under UHPLC conditions compared with HPLC conditions have demonstrated large increases in retention with pressure. This is attributed to a reduction in the analyte's molar volume on transferring from the mobile to stationary phases owing to a loss of the analyte's hydration layer on entering the hydrophobic bonded layer on the stationary phase.[11,12]

Further research is on-going to study the effects of frictional heating and pressure-induced retention differences. Charged analytes are expected to be affected more than neutral species, but in practice these effects seem to be a rare problem, possibly owing to the fact that impurities are often structurally related and therefore have similar retention characteristics (e.g. similar slopes in logk vs. 1/T and logk vs. P plots). A further hypothesis by Fallas *et al.* is that any increase in retention by pressure-induced retention is partially cancelled out by decreases in retention from frictional heating (thermal) effects.[12]

Selectivity across different particle sizes

Currently there are an abundance of HPLC phases available, but significantly less UHPLC compatible phases, which limits the selection of stationary phases for translation between UHPLC and HPLC (and vice versa). For a method to be truly translatable then consistent selectivity between the larger 3-5 µm particle sizes used in HPLC instrument and the equivalent sub 2 µm materials for UHPLC is required. Superficially porous phases (~2.5 µm) have recently been shown to have comparable or even slightly better performance than sub 2 µm phases when used under optimised conditions with low dispersion LC instrumentation.[13] They have the benefit of being able to use the same particles in both HPLC and UHPLC (pressure limits often ≤600 bar) and therefore simplifies method transfer between UHPLC and HPLC, but they have limitations in pH stability and scaling to semi-prep.

Characterisation tests are used to define the separating ability of a phase to aid the decision of which column is suitable for a particular analysis and have been thoroughly investigated and reported in literature.[14-18] In particular there have been a number of investigations into the characterisation of reversed phase liquid chromatography columns based on the Tanaka protocol, which was originally introduced in 1989.17 Recently in our laboratories we have characterised a wide range of stationary phase types and specifically compared selectivity across particle sizes of the "same" phase. This work is useful not only to identify phases suitable for method transfer between UHPLC and HPLC, but also identify dissimilar and similar stationary phases. The characterisation tests are taken from the Euerby and Petersson adaptations of the Tanaka protocol.[18,19] The characteristics it assesses are the hydrophobicity and hydrophobic selectivity, shape selectivity, hydrogen bonding capacity, total ion exchange capacity and acidic ion

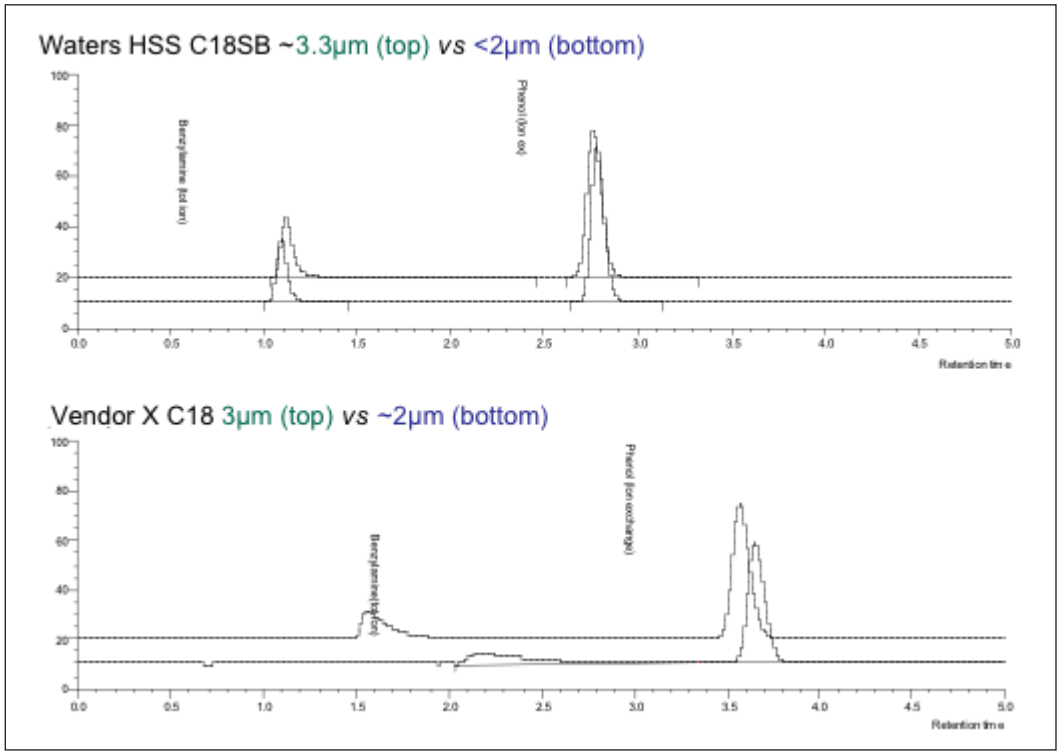


Figure 7. Comparison of total ion exchange characterisation using benzylamine and phenol at pH 7.6 with two different vendors stationary phases across 2 particle sizes. (examples using 50 mm x 2.1 mm i.d. column at the same flow rate).

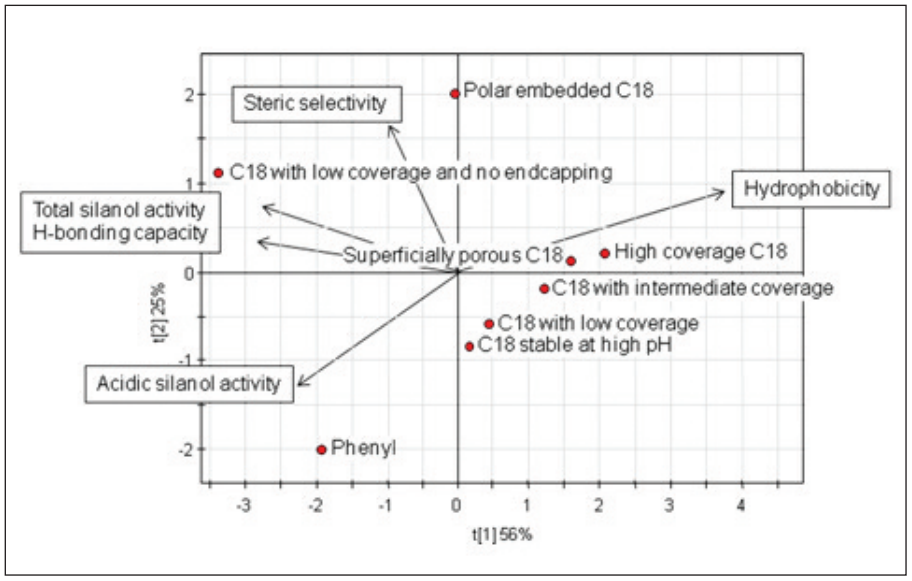


Figure 8. Principle components analysis (PCA) plot of a range of stationary phases tested, superimposed with a distribution of the characteristics.

exchange capacity and are supplemented by additional tests for polar embedded phases and aromatic phases (e.g. phenyl and perfluorinated phases). Whilst the vast majority of stationary phases we tested showed comparable selectivity across a range of particle sizes of the same phase, as in previous studies our results showed you cannot assume stationary phases with same trade name show comparable selectivity across all particle sizes.[20] Figure 7 shows examples of a "good" and also a "bad" column, where there are large differences in selectivity and also peak shapes across the particle sizes owing to differences in the total silanol content of the two different particles sizes.

Principal components analysis (PCA) is a chemometric tool that can has been used to determine similarities and differences between the characterised stationary phases, based on their separating abilities and is used to classify and group stationary phases. [21,22] From the PCA plots it's possible to identify similar and dissimilar columns and therefore used with AstraZeneca and other life science companies for identifying preferred columns for method screening/development or" replacement" columns (see Figure 8).

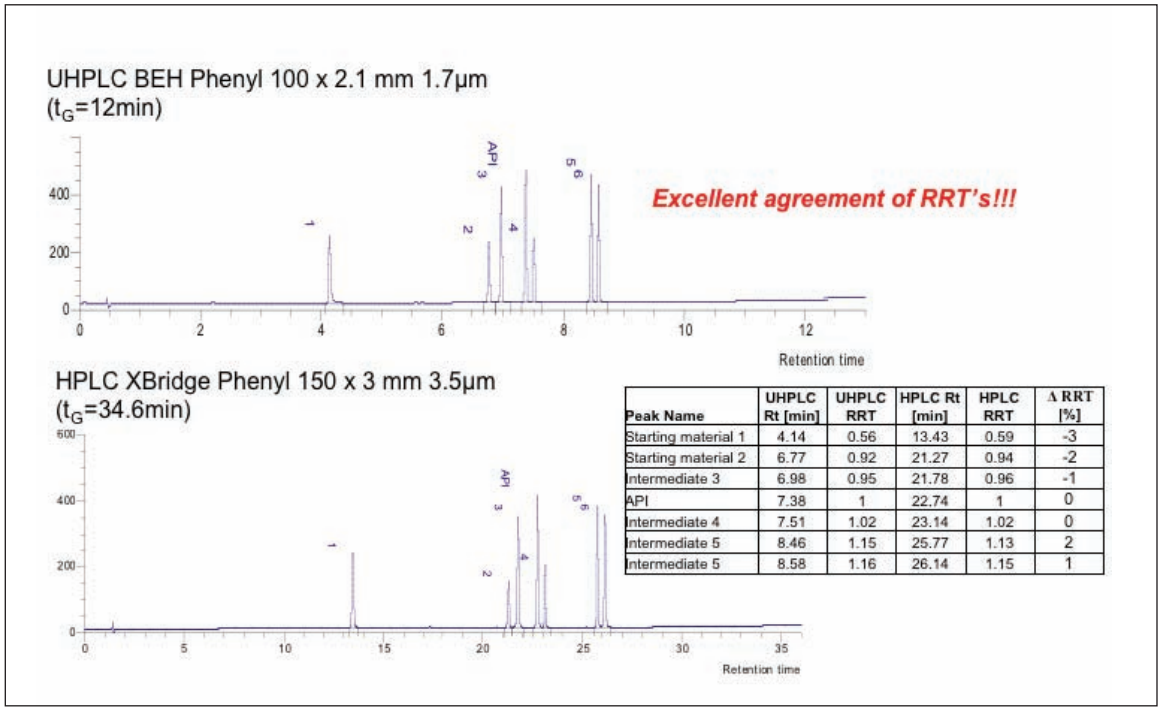
Application of method translations between UHPLC and HPLC method

In general when performing a UHPLC to HPLC translation (or vice versa) it is necessary to confirm the selectivity hasn't been affected. If this is the case consider an adjustment according to the guidance given above in the dwell volume and frictional heating sections. Furthermore, since different models of column thermostats (even from the same vendor) may have different properties it is recommended to include a system suitability test that allows compensation if there are indications that the temperature is a critical parameter.

In our experience the translation approach work is typically successfully and doesn't require any adjustments. Figure 9 shows an example where a generic UHPLC method was used to determine related impurities for all 5 synthetic stages of a new route. However as no UHPLC was available at the contract manufacturer, the UHPLC method was directly translated to an equivalent HPLC column and the gradient, flow and injection volume were geometrically scaled. This ensured that comparable relative retention times (selectivity) were maintained with no loss of resolution, although the analysis time was increased by a factor of 3. Furthermore, similar translations have been applied to obtain faster HPLC methods for reaction monitoring, at the expense of resolution (e.g. Figure 10).

Validation of translatable UHPLC and HPLC methods

As the translatable UHPLC and HPLC methods are designed to exhibit the same selectivity by using the same stationary phase chemistry, mobile phases and temperatures they might simply be considered versions of the same method, rather than separate methods. The only differences should be column dimensions and particle size (same stationary phase type) and geometrically scaled gradient profile, flow rates and injection volumes. These are based on standard and well established chromatography equations. Therefore, could this scenario be a simplified version of Quality by Design (QbD) in analytical



Peak Name	UHPLC Rt [min]	UHPLC RRT	HPLC Rt [min]	HPLC RRT	Δ RRT [%]
Starting material 1	4.14	0.56	13.43	0.59	-3
Starting material 2	6.77	0.92	21.27	0.94	-2
Intermediate 3	6.98	0.95	21.78	0.96	-1
API	7.38	1	22.74	1	0
Intermediate 4	7.51	1.02	23.14	1.02	0
Intermediate 5	8.46	1.15	25.77	1.13	2
Intermediate 5	8.58	1.16	26.14	1.15	1

Figure 9. Example of effective method translation from the generic "core" UHPLC method to the generic HPLC method.

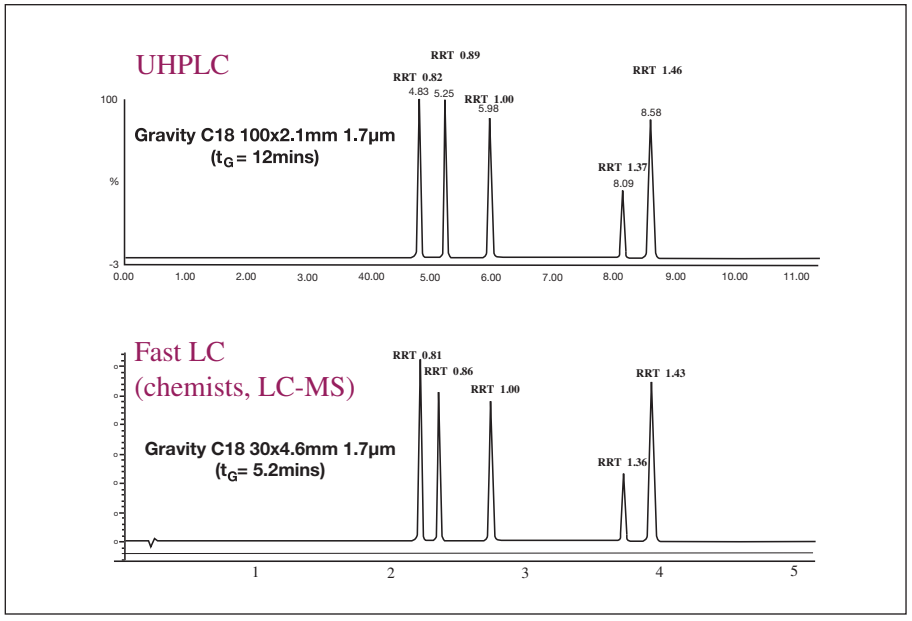


Figure 10. Example of effective method translation from the generic "core" UHPLC method to a generic fast HPLC method for reaction monitoring.

methods? In this case would it be necessary to validate both "methods" in full? If not, how much extra validation of the translated method is needed?

A common perception is that during method development and optimisation the use of chromatographic retention modelling software (e.g. DryLab or ACD LC simulator) and the factorial designs used for robustness testing should enable the identification of critical variables and critical ranges for these variables. It will necessary to confirm selectivity is the same in both UHPLC and HPLC and there are no effects arising from heat of friction, pressure-induced retention differences, dwell volume differences or from selectivity differences across the two particle sizes. Also need to demonstrate that sensitivity, linearity and recovery are acceptable as UHPLC's often employ more sensitive detectors and different injection principles or materials. These experiments on the translated method could be undertaken when validating the original method as the samples are readily available. Other than this a system suitability test SST that assesses the critical variables of the method and standard injector precision procedures should cover additional requirements during routine use (signal to noise, precision within range etc...). It's hoped this these questions will be answered soon as separation experts are starting to debate the current best practice in this area, but will require regulatory views also.

Conclusions

This article demonstrates the feasibility of translating methods between UHPLC and HPLC (or vice versa), by utilising geometrically scaled generic methods employing the same mobile phases and stationary phases in different particle sizes to obtain equivalent selectivity. Whilst there are some complications with this translation approach, specifically dwell volume effects, frictional heating, pressure induced retention differences and differences in selectivity across particle size. In practice the effects are rarely problematic during pharmaceutical analysis and are manageable by using the compensation procedures for dwell volume or frictional heating.

The benefits of adopting this approach have facilitated the smooth introduction of UHPLC into the AstraZeneca R&D. This has enabled the utilisation of UHPLC technology to improve LC analyses in terms of productivity (speed) and quality (resolution), but also having the option of proving equivalent HPLC methods "fit for purpose" methods to suit all customer's needs.

Acknowledgments

The authors would like to thanks Rachel Wilson (HiChrom Ltd, UK) and Mohammed Melludin (Loughborough University, UK) for performing the column characterisation studies and Doug McCabe, Eric Grumbach and Damian Morrison (Waters Corporation, Milford, MA, USA) for provision of prototype columns and help and advice. The authors offer their gratitude to Barry Upton (Waters Ltd, UK), Elizabeth Hodgdon and John Heden (Waters Corporation, USA) for providing a prototype software solution for delayed injection. We would also like to thank a Prof. Mel Euerby (HiChrom Ltd, UK), Lee Mallard, Simon Linke and Steven Tatterton (all AstraZeneca R&D) for the LC optimisation and method translation work.

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