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Novel Achievement of HPLC: UPLC

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Abstract: Most of the pharmaceutical manufacturer tries to reduce the cost and shorten the time of new drug development. This leads to better achievement called as Ultra performance liquid chromatography i.e. UPLC which involves HPLC with very high pressure and column having very small particle size. The factor responsible for the development of the technique was evolution of packing materials used to effect the separation. The principles behind this evolution are governed by the van Deemter equation that describes the relationship between linear velocity and plate height. UPLC is a new category of analytical separation science that retains the practicality and principles of HPLC, while increasing the overall interlaced attributes of speed, sensitivity and resolution. UPLC present the possibility to extend and expand the utility of this widely used separation science. This review focus on Principle, Instrumental design, Method development and Optimization guideline and Application of it.
Keywords: Deemter equation, HPLC, UPLC.

INTRODUCTION

High performance liquid chromatography (HPLC) is a well known technique that has been used in laboratories worldwide from more than last 30 years. The factor responsible for the development of the technique was evolution of packing materials used to effect the separation. The principles behind this evolution are governed by the van Deemter equation that describes the relationship between linear velocity and plate height.

According to the van Deemter equation, decrease in particle size increases the efficiency of separations while on other hand efficiency diminishes on increased flow rates or linear velocities. But at a particle size less than 2.5 mm, not only is there a significant gain in efficiency, but the efficiency does not diminish at increased flow rates or linear velocities. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time in gradient separations) extended to new limits, termed ultra performance liquid chromatography (UPLC) [1]. UPLC / UHPLC stands for "Ultra High Performance Liquid Chromatography"or "Ultra High Pressure Liquid Chromatography".

Pumps in conventional HPLC systems reach a pressure of max. 400 bars. Pumps in
UHPLC systems can reach pressures of 1000 bar and more. This allows the use of
smaller particles (< 2.0 mm) and still produce acceptable flow rates (up to 5 ml/min)[2].

The use of smaller particles allows to

- Obtain better resolution (separation efficiency) or
- Perform faster chromatography or a combination of both or
- Increase sensitivity, due to sharper (narrower) and higher peaks

This technology takes full advantage of chromatographic principles to run separations using columns packed with smaller particles and/or higher flow rates for increased speed, with superior resolution and sensitivity [1]

CHEMISTRY OF SMALL PARTICLES

Smaller particles provide not only increased efficiency, but also the ability to work at increased linear velocity without a loss of efficiency, providing both resolution and speed. Efficiency is the primary separation parameter behind UPLC since it relies on the same selectivity and retentivity as HPLC. In the fundamental resolution (Rs) equation: resolution is proportional to the square root of *N*.

$$Rs = \frac{\sqrt{N}}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k}{k+1}\right)$$

System Efficiency — Selectivity — Retentivity

But since *N* is inversely proportional to particle size (*dp*): as the particle size is lowered by a factor of three, from, for example, 5 µm (HPLCscale) to 1.7 µm (UPLC-scale), *N* is increased by three and resolution by the square root of three or 1.7. *N* is also inversely proportional to the square of the peak width:

$$N \propto \frac{1}{w^2}$$

This illustrates that the narrower the peaks are, the easier they are to separate from each other. Also, peak height is inversely proportional to the peak width:

$$H \propto \frac{1}{w}$$

So as the particle size decreases to increase *N* and subsequently *Rs*, an increase in sensitivity is obtained, since narrower peaks are taller peaks. Narrower peaks also mean more peak capacity per unit time in gradient separations, desirable for many applications,
e.g., peptide maps. Still another equation comes into play when migrating toward smaller particles:

$$F_{opt} \propto \frac{1}{dp^2}$$

This relationship also is revealed from the van Deemter plot. As particle size decreases, the optimum flow F opt to reach maximum *N* increases. But since back pressure is proportional to flow rate, smaller particle sizes require much higher operating pressures, and a system properly designed to capitalize on the efficiency gains; A system that can both reliably deliver the requisite pressures and that can maintain the separation efficiency of the small particles with tightly managed volumes. Efficiency is proportional to column length and inversely proportional to the particle size.

$$N \propto \frac{L}{dp}$$

Therefore, the column can be shortened by the same factor as the particle size without loss of resolution. Using a flow rate three times higher due to the smaller particles and shortening the column by one third (again due to the smaller particles), the separation is completed in 1/9 the time while maintaining resolution. Although high efficiency, nonporous 1.5-µm particles are commercially available, they suffer from poor loading capacity and retention due to low surface area. Silica-based particles have good mechanical strength but can suffer from a number of disadvantages, which include a limited pH range and tailing of basic analytes. Polymeric columns can overcome pH limitations, but they have their own issues including low efficiencies, limited loading capacities and poor mechanical strength[3].
Packing a 1.7µm particle in reproducible and rugged columns was also a challenge that needed to be overcome, however. A smoother interior surface for the column hardware, and re-designing the end frits to retain the small particles and resist clogging were necessary. Packed bed uniformity is also critical, especially if shorter columns are to maintain resolution while accomplishing the goal of faster separations[4].

PRINCIPLE [4]

The principle of UPLC is the same as HPLC (H, for high) namely adsorption chromatography using adsorbent of very fine size, to increase the surface area and thus adsorption. Naturally, the column length has to be less and much higher pressure is needed to maintain percolation of the developing solvent. As it becomes ultra, its effectiveness for preparative work decreases. The efficiency of HPLC increased as particle sizes of the column packings decreased. it was found that HETP decreases to a minimum value and then increases with increasing flow rate. However, with the 1.7 µm particles used in UPLC, HETP is

lowered compared to the larger particles and does not increase at higher flow rates. This allow faster separations to be carried out on shorter columns and/or with higher flow rates, leading to column increased resolution between specific peak pairs and increased peak capacity, defined as the number of peaks that can be separated with specified resolution in a given time interval.

Efficiency is three times greater with 1.7 µm particles compared to 5 µm particles and two times greater compared to 3.5 µm particles. Resolution is 70% higher than with 5 µm particles and 40% higher than with 3.5 µm particles. High speed is obtained because column length with 1.7 µm particles can be reduced by a factor of 3 compared to 5 µm particles for the same efficiency, and flow rate can be three times higher. This means separations can be nine times faster with equal resolution. Sensitivity increases because less band spreading occurs during migration through a column with smaller particles (peak width is less and peak height greater).

UPLC INSTRUMENT DESIGN [5]

An ultrahigh performance liquid chromatography system must be specially designed to withstand higher system pressures during chromatographic analysis. The system adjustments involve a high pressure fluidic binary pump, which is able to work up to 100 MPa (15000 psi) or more as well as an autosampler unit. Sample injection should be characterized by fast injection cycles, low injection volumes, and negligible carryover. Important characteristics of a suitable detector are long path-length and low volume detection cell so as to enable the highest possible sensitivity. Other requirements are a high sampling rate, minimal dispersion, and a high acquisition rate (at least 20–40 points/s). System volumes should be minimized in order to maintain speed, resolution, and sensitivity of analysis.

The Acquity system from Waters is the only UPLC system that is commercially available. To operate with smaller particles and higher pressure than conventional HPLC and obtain the improved performance described above, the following design elements were incorporated.

Microbore Column

The microbore column is typically 5 to 10 cm long x 2.1 mm diameter and is packed uniformly with porous 1.7 Nun particles utilizing a bridged ethylsiloxane/silica hybrid (BEH) structure with a narrow particle-size distribution, produced by condensing 1,2-bis(triethoxysilyl)ethane and tetraethoxysilane . Trifunctional C18 bonding chemistry and a proprietary end-capping procedure are used to optimize peak shape. The interior surface of the column hardware must be smooth to facilitate packing of smaller particles, and end frits must retain small particles without clogging. Acquity UPLC columns have an integral heater for insulation from ambient temperature fluctuations and eCord microchip technology that records manufacturing information (certificate of analysis) and can be updated with real time column history information during use (e.g., pressure, number of injections, and number of injections).

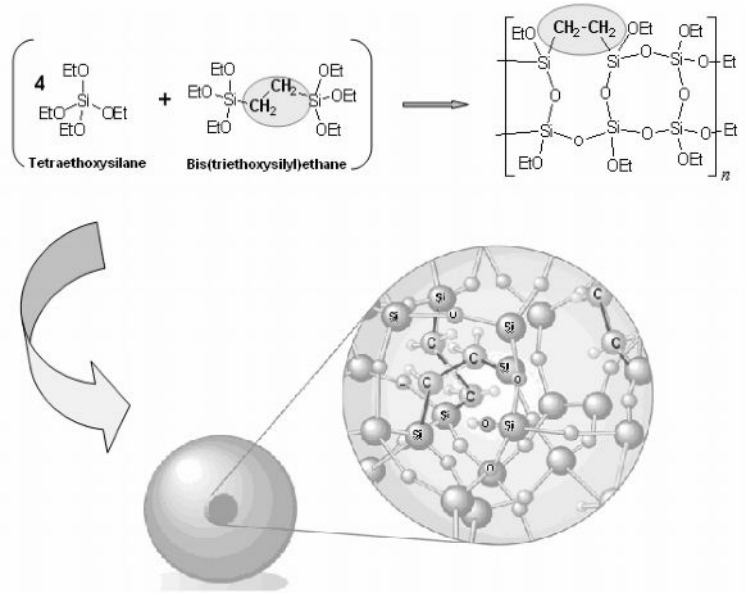

Figure 1. Structure of second generation X-Terra particles.

Solvent Delivery System

The solvent delivery system must achieve smooth, constant, reproducible high pressure pumping. UPLC systems routinely operate at 8000-15 000 psi or 535-1000 bar; the calculated pressure drop is about 15 000 psi across a 15 cm long column packed with 1.7 µm particles. The delivery system must also compensate for solvent compressibility for a wide range of pressures and a variety of solvents used in isocratic and linear and nonlinear gradient elution. The Acquity UPLC Binary Solvent Manager has two solvent delivery modules operating in parallel for high pressure blending of two solvents in <140 µL internal system volume, and dissolved gasses are removed by vacuum from up to four eluents plus two wash solvents.

The Detector

The detector must have a high sampling rate for sensitive detection and reliable quantification of the narrow peaks produced (<1 s half-height peak width), and have minimal dispersion (volume) so the separation achieved on the column is not lost. The detector also requires new electronics and firmware to support Ethernet communications that accommodate the high data rates. The potential sensitivity increase of UPLC is estimated at 2-3 times that obtained by HPLC, depending on the detection method. Acquity photodiode array (PDA) and tunable Vis-UV (TUV) detectors utilize fiber optic flow cell designs that incorporate Teflon AF, an internally reflective surface to improve light transmission efficiency by eliminating internal absorptions. The pathlengths are 10 nun, total internal volumes 500 nL, and acquisition rates 20 (PDA) and 40 (TUV) points/s. Mass spectrometry (MS) detection has also been used with UPLC.

The Injection Device

The injection device should be pulse free and have the following characteristics: a small swept volume to reduce possible band spreading, fast injection cycle, high sample capacity, automated operation over long periods, low injection volumes with minimal carryover, and temperature control. Sample volumes of 2-5 µL are typical in UPLC.

The Acquity Sample Manager has the following features: use of two SBS (Society for Biomolecular Screening)-compliant microliter plates or vial holders in any combination, temperature control from 4- 40 ^{0}C, needle-in-needle sample probe for injection of 1 µL out of 4 µL for sample-limited applications, and pressure-assisted injection of 0.1-50 µL volumes. The optional Sample Organizer extends sample capacity to 22 standard microliter plates, 14 intermediate plates and vial holders, or 8 deep-well plates or vial holders, with random access of any combination for transport into the injection compartment for processing.

Overall system dead volume (tubing and fittings) must be minimized as much as possible and leak-free valves and connections are needed for successful UPLC. Empower or MassLynx software provides instrument control, monitoring, and diagnostic capabilities.

METHOD DEVELOPMENT [6]

Initial transfer of the HPLC assay to UPLC was accomplished by simply applying a scaling factor to the mobile phase flow rate and the sample injection volume. This scaling factor derived from the ratio of the column cross sectional areas in order to retain the mobile phase linear velocity. Chromatograms from UPLC method contain very narrow peaks, and the excessive resolution indicated opportunity for method improvement. The mobile phase flow rate is increased until limited by column backpressure. However, subsequent column lifetime studies indicated that reducing total run time by increasing organic solvent content is more economical. A dramatic decrease in solvent consumption is also obtained. Chromatograms in Figure 2 compare the original HPLC method to those of the initial scaling and the final UPLC conditions.

METHOD OPTIMIZATION GUIDELINES AND OBSERVATIONS [6]

During the course of optimizing the UPLC method, considerations to expedite future
method transfers were developed, and the following recommendations were made:

- Increase elution solvent strength to reduce run times taking advantage of the high resolution potential of UPLC columns.
- Increase mobile phase flow rate secondarily to solvent strength in order to promote longer column lifetimes. While high mobile phase linear velocities with good resolution are possible, as with any column, routine operation at 80% maximum rated pressure led to shortened lifetimes. UPLC operation around 8000 psi or less provided comparable or lower column cost per assay than HPLC. Maintaining low flows as much as possible also reduces solvent and waste disposal costs, although these are already an order of magnitude less than HPLC.
- Reduce column re-equilibration times by taking advantage of the low system dwell volume. Programmed changes in the mobile phase take time to reach the column. The small UPLC dwell volume (measured as 110 µL, 15% of that of the HPLC) allowed in part the abbreviation of the original assay. Column re-equilibration accomplished during next sample loading in the UPLC, further increasing throughput.

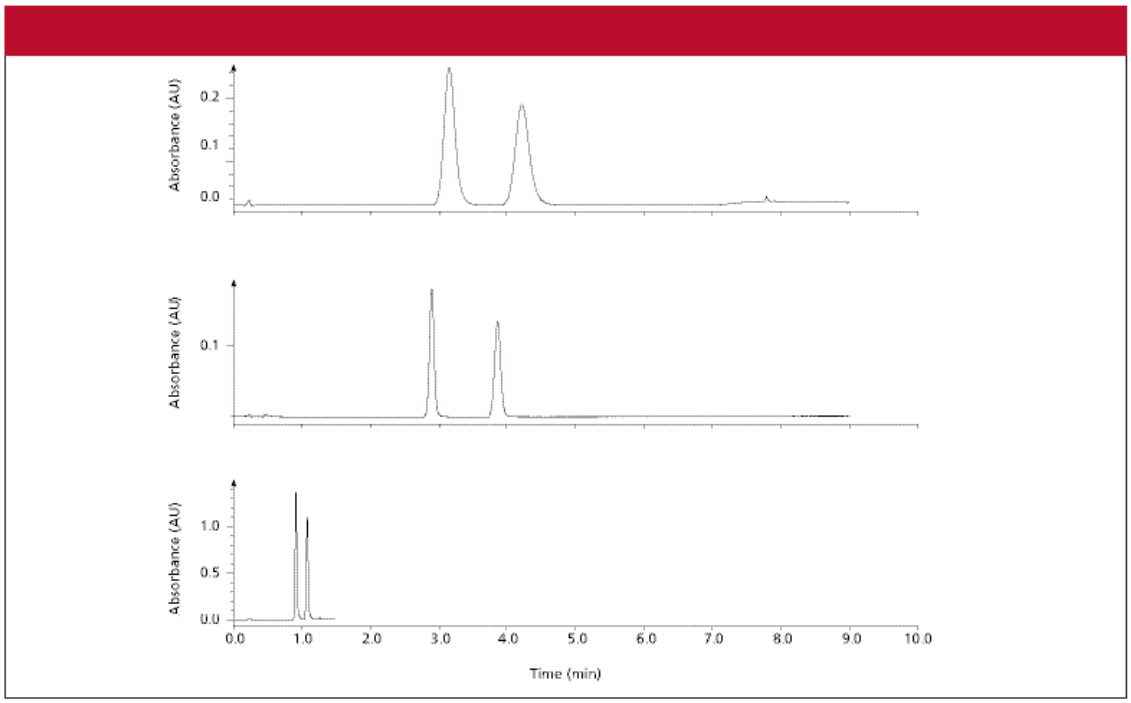

Figure 2: Chromatograms (from top to bottom): original HPLC, initial scaling to UPLC showing peak shape improvement and possibility for further method optimization, and final UPLC method. Order of peak elution: internal standard (IS) then Cpd A.

- Reduce injection volumes appropriately for the column diameter to achieve good peak shapes. Peak splitting occur when too large of a strong sample solvent bolus overwhelms the packing at the column head. While this assay method tolerated 5 µL injections, volumes of 1–3µL are more typical starting points. Smaller injection volumes may be compensated by enhanced peak height from use of the high resolution columns and by the low carryover from the UPLC injector (measured as 10% of the HPLC carryover for this analyte) to achieve an equivalent or even lower LOQ). An alternative to smaller injection volumes might be to lower sample solvent strength to accomplish sample focusing on the head of the column.
- Utilize partial loop-fill injections in preference to full loop-fill. Partial loop-fill precision was good even at volumes up to 80% of the loop total volume . Typical laboratory practice is to limit sample volume injections to roughly 50% of the total loop volume. The UPLC injection system, which utilizes air-gap sandwiching of the sample, allows better utilization of the sample loop and higher injection precision, reducing the need for use of the full loop-fill mode. From a practical point of view, full loop fill requires substantially greater sample movement considering overfill functions. This likely increases subsequent needle washing, this may impact sample throughput and increase wear of the washing hardware. Larger sample volume transfers also increases exposure to sample particulates, lowering long-term instrument reliability.
- If full loop-fill mode is utilized, perhaps for very high precision requirements ensure adequate loop overfilling. A significant laminar flow velocity differential in the loading sample between its wall interface and center is created in the very narrow bore tubing of the UPLC injector. Overfilling the sample loop by at least four loop volumes was found necessary to fully displace wash solvent from the 5 µL injector loop. For this instrument, the manufacturer has determined and set as the default the optimum overfill volume with typical sample solvents for each sample loop size. Operators can specify other overfill volumes for unusual sample compositions.
- Choose the proper composition and volume of weak sample wash to obtain good peak shape. A portion of the weak sample wash solvent will be co-injected with partial-loop filled samples. The weak solvent wash should therefore mimic the initial conditions mobile phase in solvent strength. Utilizing the weak wash solvent as sample diluents in the sample loop may enhance sample focusing onto the column. The volume of the weak wash must be sufficient to purge the former strong wash solvent from the loop.

ADVANTAGES [2, 7]

- ✓ Better resolution (separation efficiency)
- ✓ Faster chromatography
- ✓ Better sensitivity (sharper and higher peaks)
- ✓ Less solvents
- ✓ Withstand high back pressure system

DISADVANTAGES [2]

- ✓ Higher price of instruments, spare parts and columns
- ✓ Also detector and data collection system (CDS) may not cope with sharper peaks (data acquisition rate).
- ✓ So far only binary pump systems (not ternary or quaternary). This may make method transfer not straightforward.
- ✓ Number of stationary phases still limited (improves quickly)

POTENTIAL AREAS OF USE [2]

- ✓ Analysis of complex mixtures (e.g. impurity profiles, formulation inerts)
- ✓ At-line analysis in manufacturing (analysis at the vessel)
- ✓ Analysis of large amounts of samples
- ✓ For LC/MS to get better spectra (improved signal to noise)

APPLICATIONS [3, 4]

- ➢ With UPLC increased resolution in shorter run times can generate more information faster without sacrifices. Higher sample throughput with more information per sample may decrease the time to market, an important driving force in today's pharmaceutical industry. The corresponding HPLC separation takes in excess of 12 min; UPLC accomplishes the same separation in under 30 s.
- ➢ UPLC can also be used to significantly improve the success of the drug discovery process. Drug discovery is heavily dependant upon the early prediction of metabolic fate and interactions of drug candidate molecules.
- ➢ Sensitivity, selectivity, and analysis time (sample throughput) are also some of the challenges analysts face when analyzing environmental samples such as soil and water. Explosives residues in soil or environmental waters are of both forensic and environmental interest. These types of assays prove challenging because of the selectivity needed to resolve positional isomers.
- ➢ In addition, for complex samples like natural product extracts, added resolution can provide more information in the form of additional peaks. HPLC versus UPLC separation comparison of a ginger root extract sample where both speed and resolution are improved.
- ➢ UPLC coupled with MS technology provided parent and fragment mass information of lipids in one chromatographic run, thus, providing an attractive alternative to current LC methods for targeted lipid analysis as well as lipidomic studies.
- ➢ Applications areas of UPLC specified in Waters literature include high throughput library screening, metabolite identification and bioanalysis, peptide mapping, stability indicating analyses, and quantitative analysis.

CONCLUSION

At a time when many scientists have reached separation barriers pushing the limits of conventional HPLC, UPLC present the possibility to extend and expand the utility of this widely used separation science.UPLC begins to fulfill the promise of increased speed, resolution and sensitivity predicted for liquid chromatography. This achievement enables method development to be more efficient, allowing products to be brought to market faster. Hence use of such UPLC systems will become the option of choice for the development of fast LC methods in pharmaceutical development in the near future.

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