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A NEW BOON IN CHROMATOGRAPHY UPLC –A REVIEW

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Abstract:

Miniaturization embracing instrumentation, column particle size and column dimensions is one of major current trends in separation techniques. High performance liquid chromatography (HPLC) has utilized most widely in analytical laboratories. Though HPLC is dynamic and versatile technique, there is critical necessity for reliable, faster, more accurate chromatography technology. UPLC technique in chemistry and instrumentation accepted widely due to its ability to provide more information per unit of work as UPLC fulfills the criteria like increased speed, resolution and sensitivity predicted for liquid chromatography UPLC is preferable for obtaining chromatographic separation where packing material have particle size less than 2.5µm. Today's pharmaceutical companies give more emphasis on to cut cost and lessen time for development of new drug with improvement in quality of the products. A typical assay was transferred and optimized for UPLC system to achieve both higher sample analysis throughput and better assay sensitivity. Analysis of operation cost and sample throughput found UPLC cost advantageous over HPLC. UPLC is a rising chromatographic separation technique whose packing materials have smaller particle size lesser than 2.5µm which improves the speed, resolution and sensitivity of analysis. When many scientists experienced separation barriers with conventional HPLC, UPLC extended and expanded the utility of chromatography. The main advantage is a reduction of analysis time which also reduces solvent consumption. The analysis time, solvent consumption and analysis cost are very important factor in many analytical laboratories. The time spent for optimizing new methods can also be greatly reduced.

Key words: *UPLC, chromatography, HPLC, Instrumentation.*

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INTRODUCTION

High performance liquid chromatography (HPLC) has utilized must widely in analytical laboratories, over the past 30 years. Though HPLC is dynamic and versatile technique, it is time consuming and wastes producing too. There is critical necessity for reliable, faster, more accurate chromatography technology.[1] One of uplift for growth & continuous use of HPLC has been the evolution of the packing material which is used to separation. The underlying principles of this evolution are dictated by van Deempter equation, which is an empirical formula that explains the relationship between linear velocity and plate height. As particle size is one of variable, a van Deempter equation can be used to explain chromatographic performance. As per van Deempter equation, as particle size decreases to less than 2.5µm, not only gain in efficiency but also efficiencies does not change with increased flow rates or linear velocities speed, peak capacity (number of peaks resolved per Unit time of chromatography) can be extended to new limits which are termed as Ultra performance liquid chromatography. With UPLC, it is possible to consider chromatographic principles to run separations using shorter columns and higher flow rates for increased speed, with superior resolution and sensitivity [1, 2].

Ultra performance liquid chromatography a new intelligent device management technology presents the possibility to extend, expand the utility of conventional HPLC. It utilize low particle size column , fast sampling rate, UV detector and system design that is able to withstand very high back pressure over the standard HPLC. These improvements incorporate a very high efficiency and fast analysis.

Characteristics of Small Particles:-

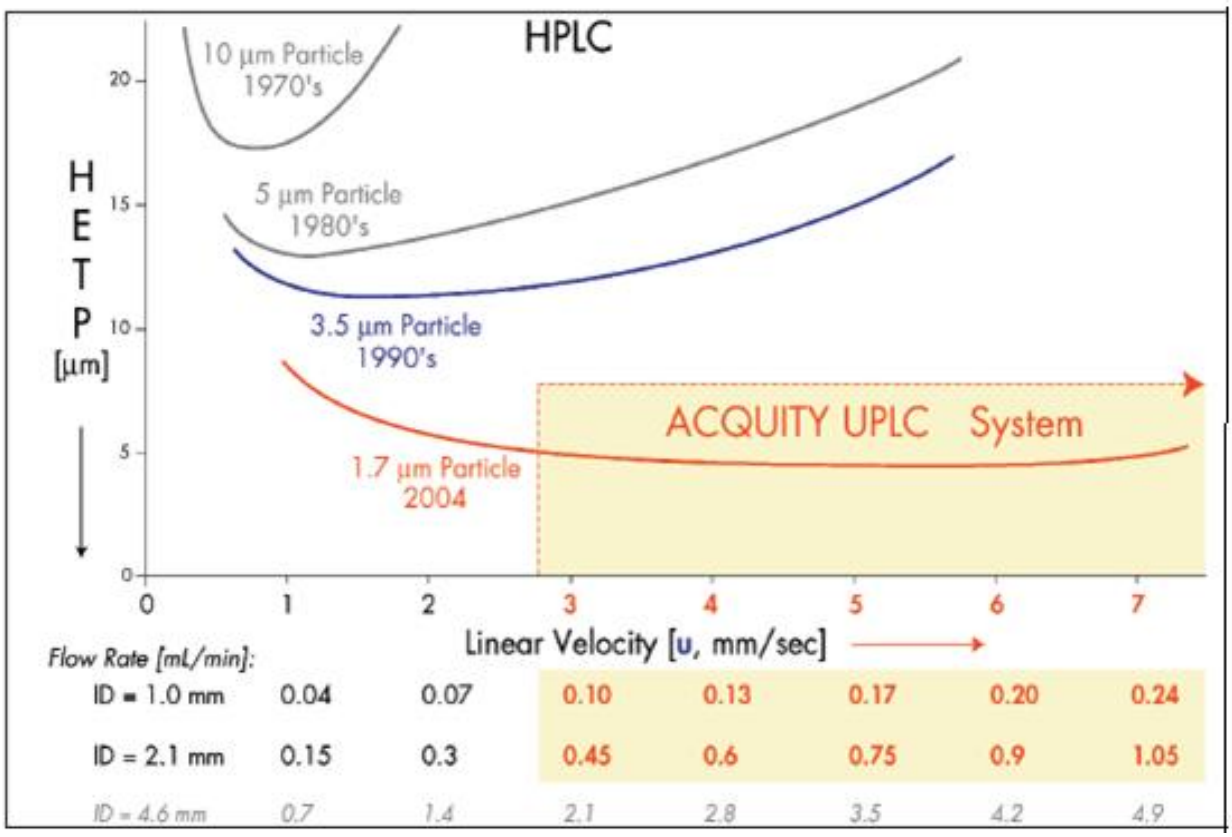


Fig 1: Van Demeter Plot Illustrating the Evolution of Particle Sizes over the Last Three Decades.

Figure 1 explains smaller particle provides increased efficiency and ability to work at increased linear velocity without loss of efficiency with resolution and speed. Efficiency of UPLC depends on same sensitivity and retentively (α-1) as HPLC.

Fundamental resolution equation is given as-

$$Rs = \frac{\sqrt{N}}{4}(\alpha\text{-}1)$$

Above equation indicates that resolution is proportional to square root of N. But N is inversely proportional to particle size. (dp)

$$N = \alpha \frac{1}{dp}$$

N is also inversaly proportional to square of peak width.

N ά 1

□ 2

This explains that the narrower the peaks easier they are to separate from each other .

Yet another equation comes into existence when migrating towards smaller particles.

Fopt □ 1

□

dP

This relationship derived from the Van Deempter plot. As particle size decreases, the optimum flow Fopt to reach maximum, N increases. As back pressure is proportional to flow rate, smaller particle size require much higher operating pressures & system properly designed to increase efficiency. UPLC system can deliver requisite pressures & that can maintain separation efficiency of smaller particles with managed tightly volumes. Higher resolution & efficiency can be extended when analytical speed is primary objective. Efficiency is proportional to column length inversely proportional to the particle size.

Thus, column can be shortened by the same factor as particle size without loss of resolution. The development and packing of sub 2 µm particle in reproducible & rugged high efficiency column is significant challenge, although high efficiency nonporous 1.5µm particles are commercially available, they cause poor loading capacity and retention due to low surface area. To maintain retention & capacity similar to HPLC, UPLC uses a novel porous particle that can withstand the high pressures. Silica based particles have better mechanical strength, but suffer from number of disadvantages which include limited PH range but polymeric columns can overcome PH limitations. This polymeric column have low efficiencies, limited loading capacities and poor mechanical strength.[2,3]

In 2000 first generation hybrid, X Terra Tm particle technology introduced by waters, that took advantage of silica & polymeric column. X Terra columns are mechanically strong, with high efficiency and operate over an using Sol gel Synthesis that incorporate carbon in the form of methyl group. In order to provide enhanced mechanical stability UPLC, Second generation bridged hybrid (BEH) technology was developed called as ACQUITY UPLC BEH technology. ACQUITY UPLC BEH, 1.7µm particles derived their enhanced mechanical stability by bridging the methyl group in silica, matrix, that can stand up both high pressures and high PH, packing a 1.7µm particle and ruggal column was also challenge that should be overcome hardware & resistance for clogging were necessary. All ACQUITY UPLC BEH columns include e cord tm microchip technology that includes manufacturing information for each column, such as quality control tests and certificate of analysis. The e cord database should be updated with real time method information such as number of injections pressure information [3].

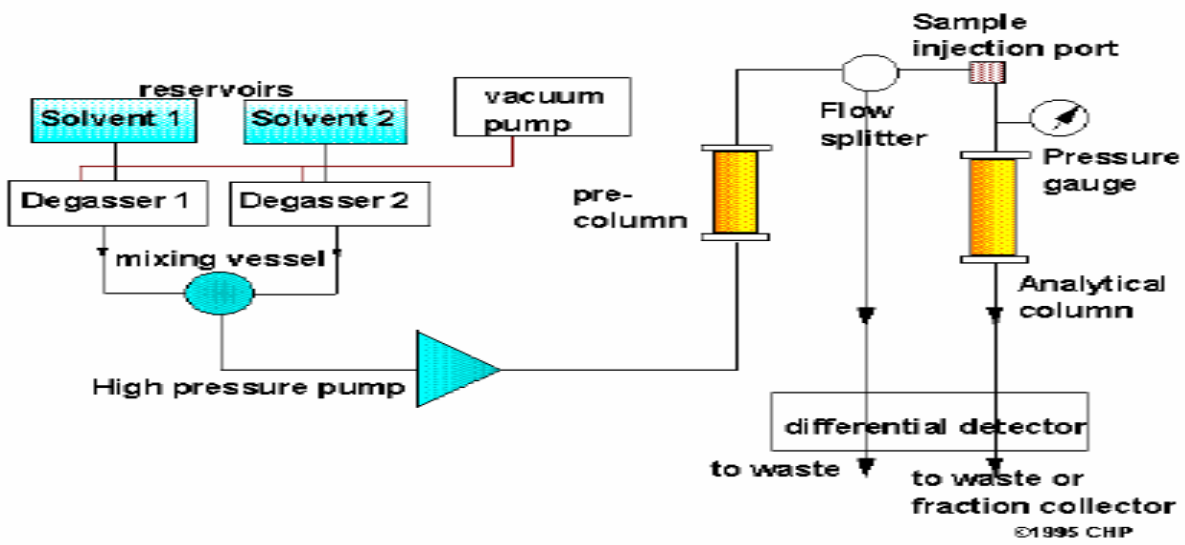
Advantages [5-9]

1) It provides selectivity, sensitivity with decrease in run time.
2) UPLC have fast resolving power ,quantifies compound quickly
3) Solvent consumption and operational cost is less.
4) It maintains resolution performance.
5) Analysis is faster as uplc based upon use of very fine particle size.
6) Decreases run time and increases sensitivity
7) Provides the selectivity, sensitivity, and dynamic range of LC analysis
8) Maintaining resolution performance.
9) Expands scope of Multiresidue Methods
10) Operation cost is reduced
11) Less solvent consumption
12) Reduces process cycle times, so that more product can be produced with Existing resources.
13) Delivers real-time analysis in step with manufacturing processes
14) Assures end-product quality, including final release testing

Disadvantages

1) Due to increased pressure requires more maintenance and reduces the life of the columns of this type.
2) Performance OF UPLC similar or even higher have been demonstrated by using stationary phases of size around 2 µm without the adverse effects of high pressure.
3) The phases of less than 2 µm are generally non-regenerable and thus have limited use

INSTRUMENTATION



Components in a mixture are separated on a column packed with silica-bed particles (referred to stationary phase) by pumping a solvent (referred to mobile phase) through the column. Depending on the unique affinity of each component (referred to the analyte) between the mobile phase and the stationary phase, each analyte migrates along the column at different speeds and emerges from the column at different times, thus establishing a separation of the mixture. Analyte with higher affinity for the mobile phase migrate faster down the column, where those with higher affinity for the stationary phase migrate slower. This migration time (referred to retention time) is unique for each analyte and can be used in its identification. With the appropriate use of a detection method after the column, each analyte can also be quantified for analysis. Smaller column particle size can improve chromatographic resolution, but increased solvent delivery pressure is needed. Further reduction of column particle size can allow for higher solvent flow rates, reducing analysis time without sacrificing resolution. UPLC decreases the time and costs associated with analytical separations, but to consistently maintain these technology advantages; you need column hardware that ensures the speed and precision of your processes [10-15].

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 was derived from the ratio of the column cross sectional areas in order to retain the mobile phase linear velocity. Chromatograms from this UPLC method had very narrow peaks, and the excessive resolution indicated opportunity for method improvement. The mobile phase flow rate was increased until limited by column backpressure. However, subsequent column lifetime studies indicated that reducing total run time by increasing organic solvent content was more economical. A dramatic decrease in solvent consumption was also obtained. Chromatograms in Figure 1 compare the original HPLC method to those of the initial scaling and the final UPLC conditions. Parameters of the HPLC and final UPLC methods are listed in Table I. Method Optimization Guidelines and Observations 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 (see Table 1).

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.

Table 1: Parameter of HPLC and UPLC

Parameters	HPLC Assay	UPLC Aaaay
Columns	AlltimaC18,50x4.6mm	ACQUITY UPLC BEHC18,50x2.1MM
Particle size	4 µm	1.7µm
Flow Rate	3.0ml/min	0.6ml/min
Maximum backpressure	35-40MPa	103.5MPa
Needle wash	Methanol	Strong Needle Wash 200ul methanol
Column Temperature	35°C	65°C
Injection volume	20ul	5ul

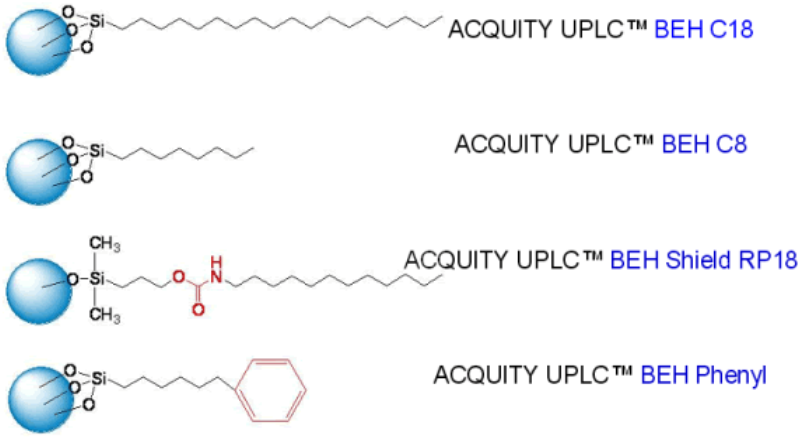


Fig.1 ACQUITY UPLC BEH Column chemistries

As with any column, routine operation at 80% maximum rated pressure led to shortened lifetimes. In experience, 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 _L, 15% of that of the HPLC) allowed in part the abbreviation of the original assay. Column re-equilibration was accomplished during next sample loading in the UPLC, further increasing throughput.

- Reduce injection volumes appropriately for the column diameter to achieve good peak shapes. Peak splitting can occur when too large of a strong sample solvent bolus overwhelms the packing at the column head. While this assay method tolerated 5 _L injections, volumes of 1–3 _L are more typical starting points in our experience. Note that 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 (meas 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 (Figure 3). 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 through put and increase wear of the washing hardware. Larger sample volume transfers also increases exposure to sample particulates, lowering long-term instrument reliability.

Columns [16-19]

Waters HPLC column portfolio extends from state-of-the-art silica phases in the SunFire line to state-of-the-art Hybrid Particle Technology in the XTerra line, with many significant chromatographic product milestones achieved along the way. column family includes:

1) XTerra Columns—

with patented hybrid particle technology and the optimal combination of attributes needed for drug discovery, drug development, and isolation and purification.

Atlantis Columns—the industry's solution to polar compound retention, designed to retain and separate difficult-to-analyze polar compounds, fully LC/MS compatible and available in two chemistries: dC18 and HILIC Silica

2) SunFire Columns—the new performance standard in silica based HPLC technology. A new state-of-the-art reversed-phase C18 and C8 bonded silica, SunFire provides the best peak shape, low pH stability, efficiency and batch-to-batch reproducibility. In addition, the greater mass loading capability combined with the unique patent-pending Optimum Bed Density (OBD) design make SunFire Prep Columns unmatched in performance, scalability and column lifetime.

Design Requirements for UPLC System:-

A completely new design system with advanced technology in the solvent, auto sampler, detector, data system and service diagnostic are required, as HPLC technology does not have capability to take full advantage of Sub 2µm particles. The ACQUITY UPLC system designed for low system and dwell volume to minimize dispersion.

Small particle achievements high peak capacity separation requires a greater pressure range then that achievable by HPLC instrumentation. The calculated pressure drop at the optimum flow rate for maximum efficiency across a 15cm long column packed with 1.7µm particle is about 15,000Psi. Therefore a pump capable of delivering solvent smoothly & reproducibly at these pressures & compensates for solvent compressibility & operates in both gradient and an isocratic separation mode is required. Sample introduction is critical conventional injection valves either automated or manual are not designed and hardened to work at extreme pressure. To protect the column from experiencing extreme pressure fluctuations. The injection process must be relatively pulse free. The swept volume of the device also needs to be minimal to reduce potential band spreading.

A fast injection cycle time is needed to fully capitalize on the speed afforded by UPLC, which requires a high sample capacity. Low volume injection with minimal carry over is also required to realize increased sensitivity benefits.

With 1.7µm particles, half height peak width of less than one second can be obtained. In order to accurately and reproducibly integrate an analyte peak detector sampling rate must be high enough to capture enough data across the peak. The detector cell must have minimal dispersion to preserve separation efficiency. Consequently sensitivity increase for UPLC detection should be 2-3 times higher than HPLC separations, depending on detection techniques. UPLC significantly increase MS detection, increased peak concentration with reduced chromatographic dispersion at lower flow rates promotes increased source ionization efficiencies for sensitivity improvement.

The ACQUITY UPLC system consists of a binary solvent manager, sample manager, detector and optical sample organizer. The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient mixed under high pressure. There are built in solvent select values to choose from up to four solvents. There is a 15,000 psi pressure limit (about 1000 bar) to take advantage of sub 2µm particles several advanced technology incorporates by the sample manager. Low dispersion is maintained through the injection process using pressure assist sample introduction and series of pressure transducer facilitate self monitoring and diagnosis. It uses needle sampling for improved ruggedness. Injection cycle time is 25 s without a wash and 60 s with a dual wash used to further decrease carry over. The sample managed can inject

from samples from upto 22 micro titer plates. The sample manager controls the column heater up to 65^0c. To minimize excess tubing and sample dispersion, a 'piviot-out' design provides versatility to allow the column outlet to be placed in closer proximity to the source inlet of an ms detector.

The tunable UV visible detector includes new electronics and firmware necessary for UPLC detection conventional absorbance based optical detector are concentration sensitive detectors and for UPLC use the flow cell volume would have to be reduced in standard UV visible detectors to maintain concentration and signal. According to Beer's law smaller volume conventional flow cells would reduce path length upon which the signal strength depends and worse a reduction in cross-section means the light path is reduced and transmission drops, increasing noise. UPLC sensitivity would be compromised if conventional HPLC flow cell used. The ACQUITY UPLC system detector cell consists of a light guided flow cell equivalent to an optical fiber. A 10mm flow cell path with volume of only 500 ml maintained by transferring light efficiently down the flow cell in an internal reflectance mode. Tubing & connections in the system are efficiently routed to maintain low dispersion and to take advantage of leak that interact with the software to alert the user to potential problems.

APPLICATION [20-25]

1. UPLC Chromatograph are used to making compromises and one of the most common scenarios involves sacrificing resolution for speed. Complex samples like natural products extracts, added resolution can provide more intimation in the form of additional peaks Metabolite identification & bio-analysis will benefit from speed resolution and sensitivity. Peptide mapping accomplished in shorter time for characterization purposes.

2. UPLC system can also be used to improve the success of the drug discovery process. Drug discovery highly dependent upon the early prediction of metabolic fate and interaction of drug candidate molecules. To control poor candidates from progressing through the discovery process factors as metabolic stability, toxic metabolite production p450 inhibition and induction are monitored.

3. Analyst faces some of the challenges while analyzing environmental samples such as soil and water. These types of assays prove challenging because of selectivity needed to resolve positional isomers. Typical HPLC analysis require viscous, buffered mobile phases operated at high temperatures and analysis time exceeding 30 min, while UPLC system requires seven minutes with a much simpler more robust mobile phase than that commonly used in HPLC assays.

CONCLUSION

Acuity UPLC using 1.7µm particles provide significally more resolution while reducing run times and improves sensitivity for the analysis of many compound types. At a time with conventional HPLC when many scientists have reached separation barrier, Ultra Performance Liquid Chromatography presents the possibility to extend and expand the utility of chromatography. UPLC technique in chemistry and instrumentation accepted widely due to its ability to provide more information per unit of work as UPLC fulfills the criteria like increased speed, resolution and sensitivity predicted for liquid chromatography. For both techniques Tailing factor and resolution, peak area repeatability and Peak retention time were similar, but in UPLC higher backpressure than HPLC is observed .It can be reduced by increasing the column pressure. Thus overall UPLC technique accepted widely due to its ability to fulfill the criteria like increased speed, resolution and sensitivity.

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