REVIEW

UPLC: A MODERN ANALYTICAL TECHNIQUE

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ABSTRACT: UPLC is a modern analytical technique which gives a new direction for liquid chromatography. UPLC refers to ultra performance liquid chromatography, which enhance mainly in three areas: "speed, resolution and sensitivity. In twenty first centenary pharmaceutical industries are focusing for new analytical methods which are economy and shorten time for development of drugs. The separation and quantification of UPLC is done under very high pressure (up to 15000 psi). This review includes theories & principles of Chromatography along with the Comparison between HPLC and UPLC and also most recent applications of UPLC are also included.

KEYWORDS: Ultra performance liquid chromatography; High separation efficiency; Cost effective; High pressure.

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INTRODUCTION

High-performance liquid chromatography (HPLC) is an important liquid chromatography (LC) technique that is used for the segregation of different components in mixtures[3]. It is also used for the identification and quantification of compounds in the process of drug development. To achieve the dramatic increase in resolution, speed and sensitivity in LC, a significant advancement in the instrumentation and column technology were made. To achieve the above targets, Waters in 2004, launched and trademarked Ultra Performance Liquid Chromatography (UPLC) which is based upon small, porous particles (sub 2micron particles). Van Deemter equation is the principle behind this evolution which correlates the connection between linear velocity and plate height[2]. The small particles require a high pressure to work with UPLC *i.e.*, 6000 psi which is typically the upper limit of conventional HPLCs. It was observed that when the particle size is decreased below 2.5 µm, there is a remarkable increase in the effectiveness and this effectiveness increases the linear speed or rate of flow. This method reduces the mobile phase volume consumption by at least 80% compared to HPLC with a shorter runtime of about 1.5 min. The smaller sized particles increase the pressure up to 1000 bars or more which can alone increase the retention factor of the separation[5]. Lower injection volume is required for UPLC which results in higher efficiency and increase in resolution. The higher column temperature reduces the mobile phase viscosity resulting in the high diffusion coefficient and flow rate without significant loss in efficiency and increase in column back pressure. The cost and make the technology is environment friendly[6].

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[3].

$$Rs = \underbrace{\frac{\sqrt{N}}{4}}_{\text{System Efficiency}} \underbrace{\left(\frac{\alpha - 1}{\alpha}\right)}_{\text{Selectivity}} \underbrace{\left(\frac{k}{k+1}\right)}_{\text{Resentivity}}$$

But N is inversely proportional to particle size (dp): as the particle size is lowered by a factor of three. For example, 5 µm (HPLC scale) 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[3].

$$F_{max} \propto \frac{1}{dpc}$$

This relationship also is revealed from the van Deemter plot. As particle size decreases, the optimum flow F to reach maximum N increases. But since back pressure is proportional to flow rate, smaller particle sizes require much higher operating pressures. 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 using a flow rate three times higher due to the smaller particles, the separation is completed in 1/9 the time while maintaining resolution. Although high efficiency, nonporous 1.5-µ 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. Packed bed uniformity is also critical, especially if shorter columns are to maintain resolution while accomplishing the goal of faster separations[7].

PRINCIPLE:

The principle of UPLC is based on the Van Deemter relationship which explains the correlation between flow rate and plate height. The Van Deemter equation shows that the flow range with the smaller particles is much greater in comparison with larger particles for good results.

$$H = A + \frac{B}{v} + Cv$$

Where H represents height equivalent to the theoretical plate (HETP), A, B & C are the constants and v is the flow rate (linear velocity) of the mobile phase. The aim is to minimize HETP to improve column efficiency[8]. The term A does not depend on velocity and indicates eddy mixing. It is smaller if the columns are filled with small and uniform sized particles. The term B denotes the tendency of natural diffusion of the particles. At high flow rates, this effect is smaller, so this term is divided by v. The term C represents the kinetic resistance to equilibrium during the process of separation. The kinetic resistance is the time lag that involved in moving from the mobile phase to the stationary phase and back again. The higher the flow rate of the mobile phase, the more molecules on the packing material inclines to lag behind molecules in the mobile phase. Thus, this term is inversely proportional to linear velocity. Consequently, it is likely to enhance the throughput, and without affecting the chromatographic performance, the separation can be speeded up[9]. The use of UPLC has necessitated the improvement of existing instrumentation facility for LC, which takes the benefit of the separation performance (by decreasing dead volumes) and consistent pressures (about 500 to 1000 bars, compared with 170 to 350

bars in HPLC). Efficiency is proportionate to the length of the column and inversely proportional to the radius of the particles. Consequently, the column length can be reduced by the similar factor as the particle radius without affecting the resolution[3].

ADVANTAGES OF UPLC

1. It is more selective and sensitive with high resolution performance and faster resolving power. 2. It also reduces process cycle time and assures end-product quality with reduced cost of operation and decreased run time[1].

3. It increases sensitivity and provides quick analysis through the use of a novel column material of very small particle size[3].

4. It decreases the consumption of solvent and increases sample throughput and also provides real-time analysis in step with manufacturing processes[4].

DISADVANTAGES OF UPLC

1. Due to increased pressure requires more maintenance and reduces the life of the columns of this type[8].

2. In addition, the phases of less than 2 µm are generally non-regenerable and thus have limited use[10].

3. Higher price of instruments, spare parts and columns[6].

4. Also detector and data collection system (CDS) may not cope with sharper peaks (data acquisition rate).

5. So far only binary pump systems (not ternary or quaternary). This may make method transfer not straightforward[9].

TABLE 1: COMPARISION BETWEEN HPLC AND UPLC:

Sl.no	Characteristics	HPLC	UPLC
1.	Particle size	3 to 5µm	Less than 2µm
2.	Maximum back up pressure	35-40Mpa	103.5Mpa
3.	Analytical column	AlltimeC18	ACQUITY UPLC BEH C18
4.	Column dimensions	150×3.2mm	150×2.1mm
5.	Column temperature	30^0C	65^0C
6.	Injection volume	5ml	2ml

INSTRUMENTATION

The instrumentation of UPLC includes- sample injection, UPLC columns and detectors.

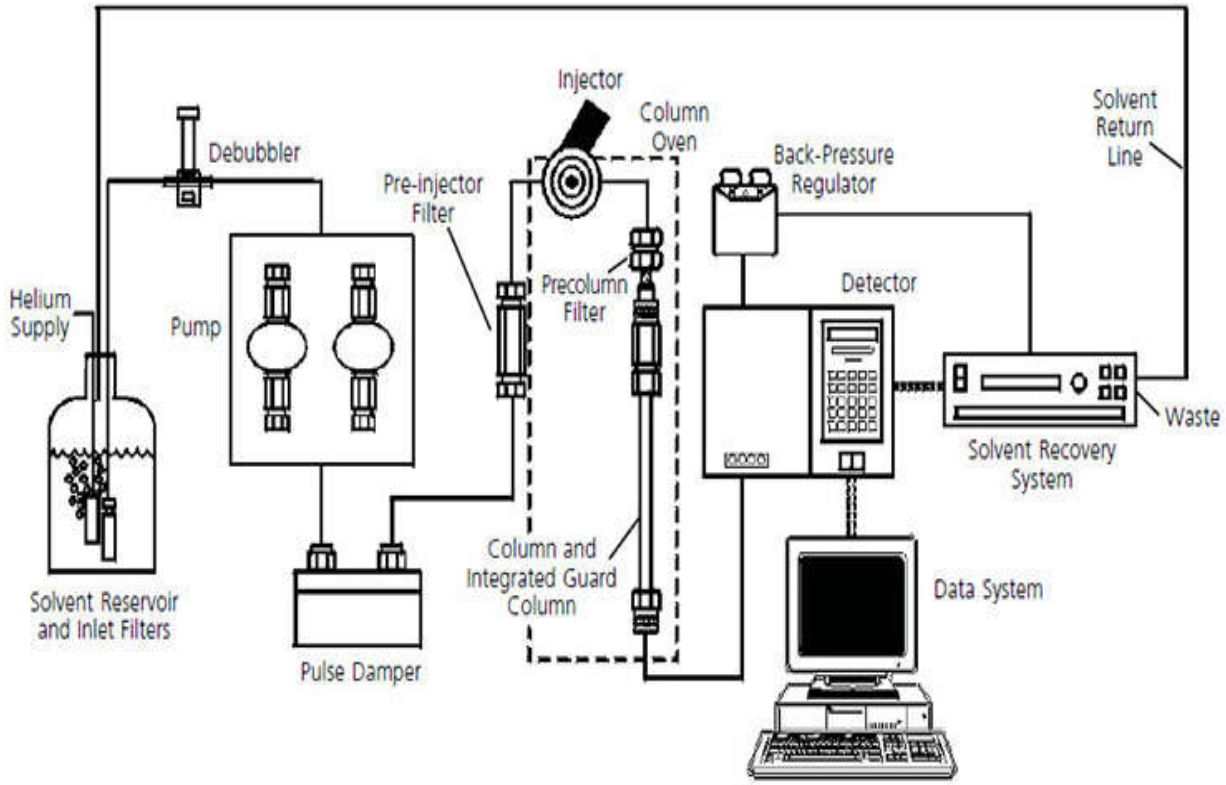

Figure 1: Block diagram of UPLC System

Sample Injection

The use of the injector is to add precisely measured, a small volume of solution containing the sample in the mobile phase. The injection must be done reproducibly and accurately. Conventional injection valves may be manual or programmed and to guard the column from extreme pressure instabilities, the injection process must be comparatively pulse-free. To reduce the potential band spreading, the swept volume of the device is desired to be minimal. A quick injection cycle time is required to fully avail the speed afforded by UPLC. To increase the sensitivity, low volume injections with minimal carryover are required. The volume of the sample in UPLC is usually 2-5 µl. Nowadays, direct injection approaches are utilized for the biological samples.

UPLC Column

Resolution is increased in a 1.7µm particle packed column because efficiency is better. Separation of the components of a sample requires a bonded phase that provides both retention and selectivity. Four bonded phases are available for UPLC separations:

(i) ACQUITY UPLC TM BEH C8 (straight chain alkyl columns)

(ii) ACQUITY UPLCTM BEH C18 (straight chain alkyl columns)

(iii) ACQUITY UPLC BEH Shield RP18 (embedded polar group column) and

(iv) ACQUITY UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C6 alkyl)

Each column chemistry provides a different combination of hydrophobicity, silanol activity, hydrolytic stability and chemical interaction with analytes.

ACQUITY UPLC BEH C18 and C8 columns are considered the universal columns of choice for most UPLC separations by providing the widest pH range. They incorporate tri functional ligand bonding chemistries which produce superior low pH stability. This low pH stability is combined with the high pH stability of the 1.7µm BEH particle to deliver the widest usable pH operating range.

ACQUITY UPLC BEH Shield RP18 columns are designed to provide selectivity that complements the ACQUITYUPLC BEH C18 and C8 phases.

ACQUITY UPLC BEH Phenyl columns utilize tri functional C6 alkyl tethered between the phenyl ring and the silyl functionality. This ligand, combined with the same proprietary end capping processes as the ACQUITY UPLC BEH C18 and C8 columns, provides long column lifetimes and excellent peak shape. This unique combination of ligand and end capping on the 1.7µm BEH particle creates a new dimension in selectivity allowing a quick match to the existing HPLC column. An internal dimension (ID) of 2.1 mm column is used. For maximum resolution, choose a 100 mm length and for faster analysis, and higher sample throughput, choose 50 mm column. Half-height peak widths of less than one second are obtained with 1.7µm particles, which gives significant challenges for the detector. In order to integrate an analyte peak accurately and reproducibly, the detector sampling rate must be of high enough to capture enough data points across the peak. The detector cell must have minimal dispersion (volume) to preserve separation efficiency. Conceptually, the sensitivity increase for UPLC detection should be 2-3 times higher than HPLC separations, depending on the detection technique. MS detection is significantly enhanced by UPLC; increased peak concentrations with reduced chromatographic dispersion at lower flow rates promote increased source ionization efficiencies.

The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient. There are built-in solvent select valves to choose from up to four solvents. There is a 15,000-psi pressure limit (about 1000 bar) to take full advantage of the sub-2µm particles. The sample manager also incorporates several technology advancements. Using pressure assisted sample introduction, low dispersion is maintained through the injection process, and a series of pressures transducers facilitate self-monitoring and diagnostics. It uses needle-in-needle sampling for improved ruggedness and needle calibration sensor increases accuracy. Injection cycle time is 25 seconds without a wash and 60 sec with a dual wash used to further decrease carry over. A variety of micro titer plate formats (deep well, mid height, or vials) can also be accommodated in a thermostatically controlled environment. Using the optional sample organizer, the sample manager can inject from up to 22 micro titer plates. The sample manager also controls the column heater. Column temperatures up to 65°C can be attained. To minimize sample dispersion, a "pivot

out" design allows the column outlet to be placed in closer proximity.

Different types of columns being used in UPLC are packed with particles which are produced through different technologies. These are as follows:

1. Charged Surface Hybrid [CSH] particle technology,
2. Ethylene Bridged Hybrid [BEH] particle technology,
3. High Strength Silica [HSS] particle technology and
4. Peptide Separation Technology (PST).

CSH Particle Technology

CSH Technology is the newest methodology in the development of hybrid materials which utilizes low-level surface charged particles for the enhancement of the selectivity and sharpness of the peaks. Hybrid based packing material approach provides sharp peaks especially for basic compounds under low pH with higher efficiency and chemical stability. CSH C_{18}, CSH Phenyl hexyl, and CSH Fluoro phenyl are the different types of CSH particles being widely used.

These columns have the advantage of exceptional peak shape, increased loading capacity (CSH C_{18}); complementary selectivity to straight chain alkyl phases (CSH-phenyl-hexyl); selectivity for positional isomers, halogenated and polar compounds (CSH-fluoro phenyl). The other advantages include- higher stability at a wide range of pH, improved batch to batch reproducibility and fast column equilibration after any change in the pH of the mobile phase.

Applications of CSH technology based columns include the analysis of basic compounds even in their ionized form. While analyzing the basic compounds under low pH and reversed phase conditions, poor peak shape and retention often result. Whereas, CSH Phenyl hexyl columns, provide exceptional peak shape for basic drugs under acidic mobile phase conditions.

BEH Particle Technology

For more than a decade, hybrid particle technology [HPT] has delivered incomparable versatility and performance, enabling chromatographers to push the limits of LC separations. The XTerra particle was the first commercially available option to improve the issues (poor peak shape for basic compounds and column longevity due to chemical instability) without the drawbacks of unpredictable selectivity produced by alternative materials such as zirconia, organic polymers, and graphitic carbon. With the commercialization of 2.5 µm XTerra particles, the concept of fast HPLC with small particles was born, improving the productivity of chromatographic laboratories globally.

Straight chain alkyl columns (BEH C_{18} and C_8), embedded polar group column (BEH Shield RP18) and UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C_6 alkyl) are the different types of BEH particle technology based columns which are being widely used.

These columns provide higher retention, selectivity (C_{18}); exceptional efficiency, peak symmetry, chemical stability (C_8); enhanced peak shape and higher compatibility with 100% aqueous mobile phase (RP18); chemical stability, reproducibility and peak shape (phenyl). Applications include the rapid

assay of cytochrome p450 isoenzymes which are responsible for more than 90% of drug metabolism on the market today. Cytochrome p450 isoenzymes are used to determine the level of drug inhibition, induction or drug-drug interaction that takes place. The ultra-low dispersion and system dwell volume of UPLC technology enables the rapid analysis of these enzymes in less than 30 seconds.

HSS Particle Technology

HSS particle technology is the advanced technology, born from an innovative synthetic process in which the mechanical stability is improved while the pore volumes remain similar to that of HPLC silica-based materials. This results in an advanced technique which provides higher retention in comparison to the hybrid particles. HSS T3, HSS C_{18}, HSS C_{18} SB, HSS PFP and HSS CN are the different types of HSS particles being widely used.

These columns provide balanced retention of polar and hydrophobic molecule (T3); exceptional peak shapes, increased retention (C_{18}); greater retention of basic compounds (CS $_{18}$ SB); ideally suited for planar aromatic, positional, halogenated compounds (PFP); ultra stable retention and compatible with both reversed-phase and normal-phase techniques (CN).

Applications of this technology include the analysis of tetracycline antibiotics which are commonly prescribed for the treatment of bacterial infections. The ACQUITY UPLC HSS C8 column enables a single UV-based method for the simultaneous separation of oxytetracycline including its degraded and related products (which are produced due to drug fermentation) as well as additional veterinary antibiotics

PST Columns

PST utilizes the C_{18} BEH Technology particles whose particles sizes range from 1.7 µm to 10 µm and the column dimension ranges from 75 µm to 30 mm internal diameter (i.d) and column length from 50 mm to 250 mm. They are used in all kind of research and development that involves analysis and isolation of peptides. The PST columns provide sharp symmetrical peaks.

Solvent Delivery System

The solvent delivery system must perform reproducible high pressure pumping with a smooth and constant flow of solvents. UPLC systems routinely operate at 8000-15000 psi. The delivery system must also remunerate for a variety of solvents used in an isocratic, linear & nonlinear gradient elution and solvent compressibility for a wide range of pressures. The Acquity UPLC binary solvent manager has two solvent delivery modules operating in parallel for high pressure merging of two solvents in <140 µL internal system volume. The dissolved gases are removed by vacuum up to four eluents plus two wash solvents.

The Detector

The detector employed for the UPLC should be able to give a high sampling rate with narrow obtainable peaks (<1 s half-height peak width) and the dispersion of the peaks should be minimum so that the wastage of the separated solute is less on the column. The UPLC technique provides the sensitivity of separation two to three times more than the

previous analytical method HPLC, which is also due to the method employed for the detection. The

detectors employed in the UPLC are Acquity photodiode array (PDA) and Tunable Vis-UV (TUV) in which Teflon AF is used which provides an internally reflective surface and enhances the light transmission efficiency by eliminating the internal absorptions. These have path lengths 10 nun, acquisition rates 20 (PDA) and 40 (TUV) points, and total internal volume 500 nL. Mass spectrometric detection has also been used with UPLC.

Applications of UPLC

Analysis of natural products and traditional herbal medicine

These technique is popularly use for the separation of natural products and traditional herbal medicine. It has a highly advanced detection and separation capabilities to identify active compounds that are presents in the samples of natural products and herbal medicines.

Study of metabonomics /metabolomic

Metabonomics studies are carried out in labs to accelerate the development of new medicines. It provides a quick and robust method for detecting the changes, improves understanding of potential toxicity, and allows observing the capacity. The correct application of metabolomics and metabolomic information helps in the discovery, development, and manufacturing processes in the biotechnology and chemical industry companies.

Identification of metabolite

Biotransformation of new chemical entities (NCE) is necessary for drug discovery. When a compound reaches the development stage, its identification becomes a regulated process. UPLC addresses the complex analytical requirements of new discovery by providing unmatched resolution, sensitivity, and mass accuracy**.**

ADME (Absorption, Distribution, Metabolism, Excretion) Screening:

Pharmacokinetics studies include studies of ADME. It studies important physical and biochemical properties like absorption, distribution, metabolism, elimination, etc. where such compounds show its activity against the target disease.

Manufacturing / QA / QC

Identification of purity, quality, safety and efficacy are the most important factors that need to be considered while manufacturing a drug product. For the successful production of quality pharmaceutical products, the raw materials need to meet the purity specification. These can be achieved with the help of UPLC technique.

Impurity profiling

These techniques easily detect the impurities present if it is presents in very trace levels too. UPLC combines with same mass.LC/MS, which by running with different low and high collision energies, has been successfully used for the detection of drug and endogenous metabolites.

Improved Resolving Power in Peptide Maps

Peptide mapping is an essential technique for the characterization of proteins. Due to exceptionally reduced instrument and column dispersion, the analyzes of tryptic digest of phosphorylase by UPLC technology provides significantly improved resolution, peak capacity, and sensitivity compared to HPLC, allowing the detailed characterization of the protein.

Multi-Residue Analysis of Pharmaceuticals in Waste Water

The water used in the pharmaceutical companies is found to have the traces of various cholesterol-lowering statin agents, anti-ulcer agents, antibiotics, beta-blockers, analgesics, anti-inflammatory agents, lipid regulating agents, psychiatric drugs, and histamine H2 receptor antagonists. UPLC coupled with Q-TOF-MS is used to confirm and screen these drugs in the samples of waste water treatment plant.

Method Development / Validation

Method development and validation is a complex process and consumes a lot of time. For the development of a robust and reliable method, many combinations of different parameters *e.g.* mobile phase, temperature, pH, column and gradient chemistry *etc*. UPLC is an important method used in the laboratory which reduces the cost and increases the efficiency of analysis required for developing and validating the method. With UPLC, the speed of the separation increases and efficiency improves, which results in the fast development of methodologies. High stability of the UPLC columns provides the possibility of selection of column temperature and pH from a wide range.

Forced Degradation Studies (FDS)

Forced degradation/ stress testing is performed under accelerated environment. The experimental conditions cause the candidate compound to degrade under extreme conditions like acid and base hydrolysis, peroxide oxidation, photo-oxidation and thermal stability to identify the resultant degradation products. Use of UPLC with photodiode array and MS analysis supports the identification of degradation products and also reduces the time needed to evolve stability indicating methods.

CONCLUSION:

UPLC decrease the analysis time, which in turn reduces consumption of solvent that plays a vital role in analytical method development. It also facilitates the analysis of complex mixtures in relatively short time and the peaks obtained with the help of this method are clearer than that of HPLC. UPLC increases productivity in both chemistry and instrumentation by providing more information per unit of work as it gives increased resolution, speed, and sensitivity for liquid chromatography. The UPLC column even can withstand high back up pressure.

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