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A BRIEF REVIEW ON ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Ultra Performance Liquid Chromatography (UPLC) is a relatively new technique. It is an analytical method commonly used in analytical chemistry and in the pharmaceutical industry. Its operation is based on column chromatography that is often used to investigate mixtures. This technique gives new possibilities in liquid chromatography. The use of which decreases the length of column, saves time and reduces solvent consumption. It shows a great enhancement in speed, resolution and sensitivity of analyses by using particle size less than 2μm. The quality analyses of various pharmaceutical formulations are transferred from HPLC to UPLC system. The separation on UPLC is performed under very high pressure (up to 100MPa). Separation efficiency remains maintained or is even improved by UPLC. This review introduces the theory of UPLC.

KEYWORDS: Chromatography, UPLC, Resolution, HPLC.

INTRODUCTION

UPLC systems were first introduced in 2004. UPLC refers to Ultra Performance Liquid Chromatography. It improves in three areas: chromatographic resolution, speed and sensitivity analysis. In this system uses fine particles (less than 2.5 μm). So reduces length of column, saves time and reduces solvent consumption.[1] Ultra Performance Liquid Chromatography (UPLC) could be considered to be a new direction of liquid

chromatography. UPLC, as its first producer Waters proclaims, means "speed, resolution and sensitivity" As it is very well known from Van Deemter equations, the efficiency of chromatographic process is proportional to particle size decrease.[2] According his model describing band broadening, which describes relationship between *height equivalent of theoretical plate* (HETP) and *linear velocity*, one of the terms (path dependent term), is dependent on a diameter of particle packed into the analytical column. UPLC comes from HPLC.UPLC is used in many laboratories all over the world. One of the main advantage of this technique is growth and development is due to the advancement of materials used for packaging is used in stimulating the separation. An underlying principle of HPLC dictates that as column packing particle size decreases, efficiency and thus resolution also increases. As particle size decreases to less than 2.5µm, there is a significant gain in efficiency and it's doesn't diminish at increased linear velocities or flow rates according to the common Van Deemter equation. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time) can be extended to new limits which is known as Ultra Performance. The separation on UPLC is performed under very high pressure (up to 100MPa).[3] The immaculate separation method UPLC has many advantages like robustness; ease of use, changeable sensitivity selectivity but the main limitation is lack of efficiency in comparison to gas chromatography or capillary electrophoresis. The present review focuses on the basic principle, instrumentation of UPLC and its advantages over HPLC, and pharmaceutical applications of UPLC technique.

Chromatography

Chromatography is used to separate mixtures of substances into their components. In Greek 'Chromo' meaning "color" and 'Graphic' meaning "writing". All forms of chromatography work on the same principle. They all have a stationary phase (a solid or a liquid supported on a solid) and a mobile phase (a liquid or a gas). It involves mass transferring between stationary and mobile phase.

- **Mobile Phase-**It is the phase that moves in definite direction. The selection of mobile phase based on the polarity of stationary phase, nature of sample and chromatographic method. e.g. Acetone.

- **Stationary phase-**It is non- moving phase in chromatographic separation. It is the substance fixed in place for the chromatography procedure. e.g. silica.

UPLC analyses were performed on Waters Acquity Ultra Performance Liquid Chromatographic system (Waters, Prague, Czech Republic) with PDA detector, cooling auto sampler and column oven enabling temperature control of analytical column. Data were collected and processed by chromatographic software Empower. With this UPLC system special analytical column was connected. X-Terra sorbent of second generation packed into Acquity UPLCBEH C_{18} (2.1mm×50 mm, 1.7m) was used as a stationary phase. UPLC analyses utilized flow-rates in a range0.50–0.60 ml min^{-1}. All analyses were performed at 25°C (laboratory temperature). Two micro liters were used as injection volume using partial loop mode for sample injection.

Liquid chromatography is an analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent.[4]

Principle of UPLC

The UPLC is based on the principal of use of stationary phase consisting of particles less than 2 μm.[5] The underlying principles of this evolution are governed by the Van Deemter equation, which is an empirical formula that describes the relationship between, linear (flow rate) and plate height (HETP or column efficiency).[6,7] The equation is as follows:

H = A + B/V + CV

Where,

A, B and C are constant

H= HETP

A = Eddy diffusion

B = Longitudinal diffusion

C = Equilibrium mass transfer

V = flow rate

- **Eddy diffusion-** The mobile phase moves through the column which is packed with stationary phase. Solute molecules will take different paths through the stationary phase at random. This will cause band broadening.

- **Longitudinal diffusion-**The concentration of analytes is less at the edges of the band than at the center. Analyte diffuses out from the center to the edges. This causes band broadening.

The eddy diffusion A is smallest when the packed column particles are small and uniform. The B term representing longitudinal diffusion or the natural diffusion tendency of molecules diminishes at high flow rates and so this term is divided by B. The C term equilibrium mass transfer is due to kinetic resistance to equilibrium in the separation process and this kinetic resistance is the time lag involved in moving from the gas phase to the packing stationary phase and back again. The greater the flow of gas, more a molecule on the packing tends to lag behind molecules in the mobile phase. Thus, this term is proportional to B.[8]

Chromatographic resolution is described by[9]

Rs=√N/4(α-1/) (k/k+1)

Where,

Rs = Resolution

N = Separation efficiency (therapeutic plate)

α = Selectivity factor

k = Retention factor (Capacity factor)

Separation efficiency (N)

N=L/H=L/h dp

Where,

L = Column length

H = Height of therapeutic plate

h = Reduced plate height

dp= Particle diameter

Therefore

Rs α N α 1/dp

Chemistry of small particles

As the particle size decreases to less than 2.5µm, not only there is significant gain in efficiency, but the efficiency doesn't diminish at increased flow rates. By using smaller particles, speed and peak capacity (number of peaks resolved per unit time in gradient separation) can be extended to new limits, termed Ultra Performance Liquid Chromatography.

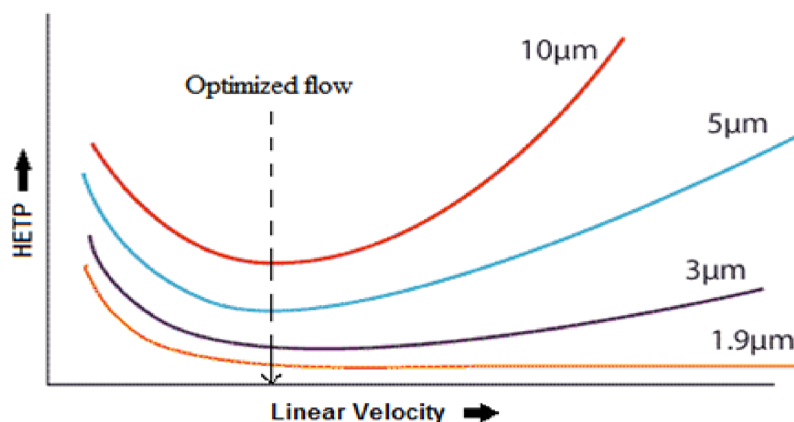

Figure 1: Van Deemter plots for various particle sizes.[10]

Figure 1 indicates that the decrease in particle size results in an increase in efficiency of column and on the other hand increase in linear velocity (flow rate) increase the efficiency for the column for particle size less than 1.9 µm and after the optimized flow it remains same, while for column with particle size greater than 1.9µm, efficiency again decrease after certain optimized flow. A commercially available non-porous, high iffiest small particle has poor loading capacity and retention due to low surface area. To maintain retention and capacity must use novel porous particles that can withstand high pressures. In 2000, hybrid of silica and polymeric column was introduced which consist of classical sol-gel synthesis that incorporates carbon in the form of methyl groups, these columns are mechanically strong. They are highly efficient and can be operate at wide range of pH.[11]

Instrumentation

The schematic diagram of UPLC and various parts of the instrument are shown in Figure 2, Figure 3 and Figure 4 respectively.

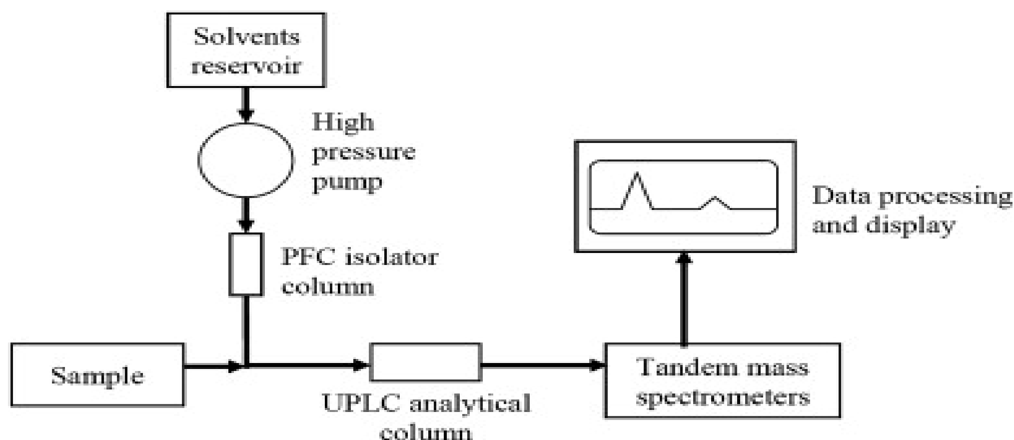

Figure 2: Schematic diagram of UPLC.[12]

Figure 3: **The Acquity UPLC System.**[13]

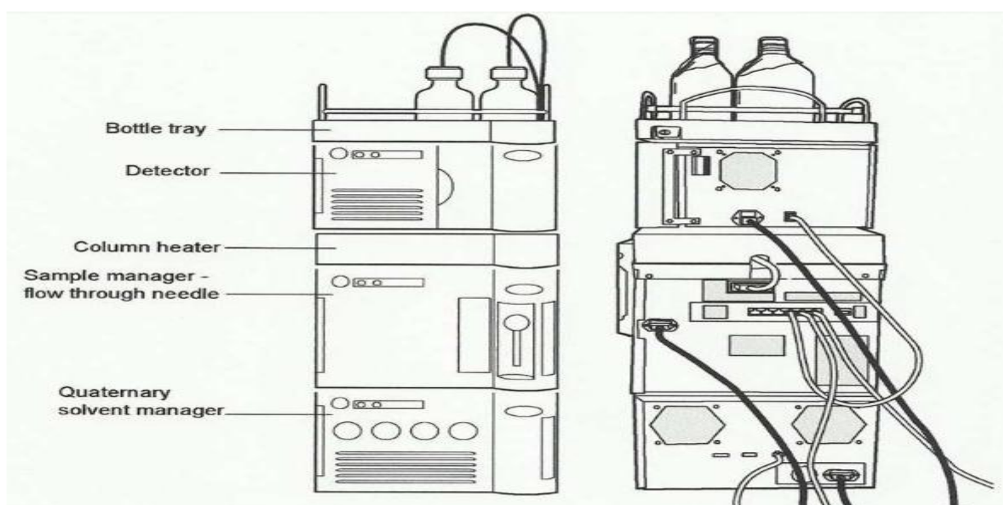

Figure 4: UPLC System.

A design with advanced technology in the pump, auto sampler, detector, data system and service diagnostics was required to full fill the purpose. The basic instrumentation of UPLC is discussed below:

1) Pumping devices

An ideal pump for UPLC has a capacity of delivering solvent at higher pressure around 15000 psi for the optimum flow rate with maximum efficiency across 15 cm long column packed with 1.7 μm particles.

The two basic classifications are[14]:

a) Constant pressure pump

b) Constant flow pump

Constant pressure pump: The constant pressure is used for column packing.

Constant flow pump: This type is mostly used in all common UPLC applications.

Standard UPLC pump requirements

- ✓ Sample injection volume is as less as 3 – 5 micro liters
- ✓ Pump operates at 10000 psi pressure
- ✓ Particle size in stationary phase packing material is less than 2 micro meter

A solvent reservoir is in the device. This solvent is called eluent or mobile phase. The pump pumps the mobile phase at a constant speed and pushes to the column. UPLC uses two serial pumps with pressure limit of 1000 bar and have inbuilt solvent selector valves, which have the capability to choose the accurate solvent ratio from up to four solvents**.** When the one piston the eluent pushes the column, the other piston sucks the eluent from the reservoir. If this principle is not used, stable pressure and flow velocity cannot be achieved.[15]

2) Sample injection

In UPLC, 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 extreme pressure fluctuations, the injection process must be relatively pulse-free and 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 in turn requires a high sample capacity. Low volumes injections with minimal carryover are also required to increase sensitivity. There are also direct injection approaches for biological samples.

The injector injects the test sample into the mobile phase between the pump and column. Often this is done automatically by means of a car sampler because it gives better results and better reproduction. There are different types of injectors available with different amounts of injection ports. The zespoort injector is most commonly used in single column analyzes.[15]

3) UPLC columns

In UPLC columns particle size of packing material is approximately 1.7 µm. which increases speed of separation of components. Separation of the components of a sample requires a bonded phase that provides retention and selectivity. The UPLC columns are made up of small particles having size less than 2 µm. The role played by small particle size in UPLC technique has been mentioned below. The particles are bonded in matrix as the bonded stationary phase is required for providing both retention and selectivity. Various types of columns manufactured by ACQUITY are available in the market, which can be used by UPLC technique.[16]

Acquity UPLC BEH C_{18}- These are straight alkyl chain, bonded and columns are considered to be the universal columns with leading mobile phase pH (1-12). These columns provide the widest pH range.

Acquity UPLC BEH C_8- Due to its shorter alkyl chain length, these columns exhibit low hydrophobicity than C_{18} column, resulting in lower retention and faster elution of analyte peaks.

Acquity UPLC BEH Phenyl columns- Columns utilize a trifunctional C_6 alkyl tethering between the phenyl ring and the silyl functionally. This employs trifunctionally-bonded phenyl hexyl ligand which provides reproducibility, chemical stability and peak shape for all analyte types.

Acquity UPLC BEH Amide columns- The combination of trifunctionally bonded amide phase with BEH small particles provides exceptional column life time. The use of a wide range of phase pH i.e. from pH 2 to 11.

Acquity UPLC Shield RP_{18}- Unique and patented bonding chemistry provides complementary selectivity to a C_{18} column and enhances the peak shape for basic compounds and yielding compatibility with 100% aqueous mobile phases.

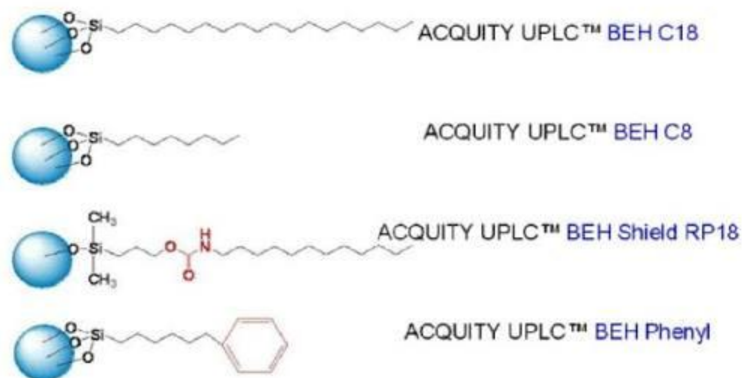

Figure 5: Acquity UPLC BEH Column Chemistries.[17]

Based on technology columns are differentiated as follows:[18]

BEH technology: BEH C_{18}, BEH C_8, BEH shielded RP_{18}, BEH Phenyl, and BEH amide columns.

Peptide separation technology: BEH 130, BEH 300.

Protein separation technology: BEH123, BEH200, BEH450 SEC columns.

Glycan separation technology: BEH Glycan column.

Oligo neuclotides separation technology: OST C_{18} columns.

Charged surface hybrid technology: CSH C_{18}, CSH Phenyl-Hexyl and CSH Flouro-Phenyl columns.

HSS technology: HSS T3, HSS C_{18}, HSS C_{18} SB, HSS PFP, HSS Cyno columns.

Factors affecting column efficiency- According to chromatographic theory, column efficiency (N) is inversely proportional to particle size (dp). Thus, smaller particles provide higher resolution. The highly efficient 1.7µm BEH particles allow chromatographers to maximize the efficiency (N) of their separation when used in the Acquity UPLC system. The factors affecting column efficiency are:

a. Column length
b. Particle size
c. Linear velocity
d. Retention factor
e. Temperature of the column
f. Solvents

4) Column Heater

The column heater heats the column compartment to any temperature from 5^0C to 65^0C.

5) Detectors[18]

The detector is the instrument used for qualitative and quantitative detection of analytes after separation. The system can be configured with a TUV, ELS, PDA and FLR detectors or a combination of them.

TUV (Tunable Ultraviolet) detector: It is a two channel, absorbance detector. Controlled by Empower or Mass Lynx software for both LC/MS and LC applications.

PDA (Photo Diode Array) detector: It is an optical detector absorbs UV-Visible light that operate between 190-500nm.

ELS (Evaporative Light Scattering) detector: The detector Controlled by Empower or Mass Lynx software, which incorporates a flow-type nebulizer that is optimized for UPLC system performance.

FLR (Fluorescent) detector: It is a multi-channel, multi wavelength detector, which has an excitation wavelength that ranges from 200 to 890 nm, an emission wavelength that ranges from 210-900 nm, offers 3D scanning capability for easier method development.

Coupling of an UPLC to a mass spectrometer is shortened to LC/MS. The data goes from the detector to a computer that creates a chromatogram. A chromatogram is a representation of the separation that has occurred in the analysis. A chromatogram shows a baseline representing the mobile phase as it flows through the detector. If an analyte is 'seen' by a detector, a response will arise. This results in a peak in the chromatogram. The height of the peak depends on the concentration of the analytes that passes through the detector at that time. The liquid can be collected after the detector to further test.[15]

Comparison between HPLC and UPLC

The characteristics of HPLC and UPLC and advantages of UPLC over HPLC are summarized in Table 1.

Table 1: Comparison between HPLC and UPLC.

Characteristic	HPLC	UPLC
Particle size	<4µm	1.7µm
Maximum backpressure	35-40 MPa	103.5 MPa
Analytical column	Alltima C_{18}	Acquity UPLC BEH C_{18}
Column dimensions	150×3.2mm	150×2.1 mm
Injection volume	20 L	3-5 L
Pressure limit	Up to 4000 psi	15000 psi
Total run time	10 min	1.5 min
Sensitivity	Less	Higher
column coagulation	Does not takes place	Takes place
Analysis time	More	Less

Applications

This technique has been successfully applied to pharmaceutical analysis of numerous drugs, such as Aspirin, Metoprolol (MT), Ramipril (RM), Diclofenac, Fluconazole, Hespiridine, Secnidazole, etc. with the improvement in the factors like retention time and mobile phase consumption. Narasimham and Barhate developed and validated UPLC method for the simultaneous determination of β-blockers and diuretic drugs in pharmaceutical formulations with the objective of reducing analysis time and maintaining good efficiency.

- **Identification of metabolites**

The identification of major metabolites is done by performing *in vitro* discovery studies. The weak spots of metabolites of drug candidate molecules are recognized and hence protected by altering the compound structure.

Biotransformation of new chemical entities (NCE) is necessary for drug discovery. When a compound reaches the development stage, metabolite identification becomes a regulated process. It is of the utmost importance for lab to successfully detect and identify all circulating metabolites of a candidate drug. UPLC/MS/MS addresses the complex analytical requirements of biomarker discovery by offering unmatched sensitivity, resolution, dynamic range and mass accuracy.[19]

- **Detection of impurities**

UPLC technique offers exact required data and is operational at alternate low and high collision energies. The fast change of collision energy produces both precursor and product ions of all analytes present in the sample, which allows rapid identification and profiling of impurities.

- **Dissolution testing[12]**

The dissolution profile of a drug signifies reliability and batch to batch uniformity of API (Active Pharmaceutical Ingredients) in the formulations. By using UPLC, dissolution testing can be fully automated as it can perform functions like data acquisition, analysis of samples aliquots, management of test results and distribution.

- **Forced Degradation Studies[12]**

The FDA and ICH require stability testing data to understand how the quality of an API (active pharmaceutical ingredient) or a drug product changes with time under the influence of environmental factors such as heat, light, pressure and moisture or humidity. UPLC combined with specific Photodiode array detector and MS detection will give confidence for identifying degradation products and thus shortening the time required to develop stability-indicating methods.

- **Drug discovery**

UPLC improves the drug discovery process by means of high throughput screening, combinational chemistry, high throughput in vitro screening to determine physiochemical and drug’s pharmacokinetics.

- **High throughput quantitative analysis**

UPLC coupled with time of flight mass spectroscopy give the metabolic stability assay. UPLC coupled with time of flight mass spectroscopy give the metabolic stability assay.

- **Analysis of dosage form**

It provides high speed, accuracy and reproducible results for isocratic and gradient analysis of drugs and their related substance. Thus method development time decreases.

- **Analysis of amino acids[20]**

UPLC used from accurate, reliable and reproducible analysis of amino acids in the areas of protein characterizations, cell culture monitoring and the nutritional analysis of foods.

- **Analysis of natural products and traditional herbal medicines[5]**

UPLC provides high-quality separations and detection capabilities to identify active compounds in highly complex samples that result from natural products and traditional herbal medicines. Metabolomics-based analysis, using UPLC, exact mass MS, and Marker Lynx

Software data processing for multivariate statistical analysis, can help quickly and accurately characterize these medicines and also their effect on human metabolism.

- **Determination of pesticides[21]**

UPLC couples with triple Quadra-pole tandem mass spectroscopy will help in identification of trace level of pesticides from water.

Thus Ultra Pressure Liquid Chromatography set a new standard in the science of chromatography. Working range with 15000 to 16000 psi pressure and column packed with less than 2 micrometer in size helped in various fields.

- **ADME (Absorption, Distribution, Metabolism, Excretion) Screening**

Pharmacokinetics studies include studies of ADME (Absorption, Distribution, Metabolism and Excretion). ADME studies measure physical and biochemical properties – absorption, distribution, metabolism, elimination, and toxicity of drugs where such compounds exhibit activity against the target disease.[21]

The high resolution of UPLC enables accurate detection and integration of peaks in complex matrices and extra sensitivity allows peak detection for samples generated by lower concentration incubations and sample pooling.

- **Drug Abuse[22]**

UPLC-MS/MS method can be used to develop and evaluate a fast, robust and specific screening platform for the determination and quantification of a variety of commonly used drugs of abuse (opioids, benzodiazepines *etc.*) in urine.

Advantages[23]

The advantages of UPLC are as follows:

- Decreases run time and increases sensitivity.
- Provides the selectivity, sensitivity and dynamic range of LC analysis.
- Multi residue methods are applied
- Uses of fine particle (2µm) for packing of stationary phase make analysis fast.
- Consumption of solvent is less
- Assures end-product quality, including final release testing.
- In chromatogram resolved peaks are obtained.

- Delivers real time analysis in step with manufacturing processes.
- Separation on UPLC is performed under very high pressures up to 100 MPa.
- Columns are withstanding high back pressure system.

Disadvantages[20]

- Due to increased pressure requires more maintenance and reduces the life of the columns of this type.
- In addition, the phases of less than 2µm are generally non-generable thus have limited use.
- 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 straight forward.

CONCLUSION

UPLC is a new revolution in chromatography. Due to the smaller particle size of UPLC columns leads to highly selective, efficient and chemically stable columns with high speed of analysis which results in shorter retention times with reproducible results and highly robust not even more than 2mins unlike 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 main advantage is a reduction of analysis time which meant reduced solvent consumption but UPLC have limited use. It was found that the sensitivity of UPLC was much higher than that of conventional HPLC. UPLC is more convenient for complex analytical determination of pharmaceutical preparations.

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