CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

http://doi.org/10.5281/zenodo.1401201

Available online at: http://www.iajps.com *Review Article*

HPLC – HIGH PERFORMANCE LIQUID CHROMATOGRAPHY & UPLC – ULTRA PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM – A REVIEW ON MODERN LIQUID CHROMATOGRAPHY

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Abstract:
Chromatography is a technique which is used for the separation of constituents in a mixture. This technique involves 2 phases stationary and mobile phases. separation of this constituents is based on the difference between partition coefficients of the two phases. High Performance Liquid Chromatography is analytical technique for separation, dentification and quantification of constituents in mixture. HPLC is an advanced technique of column liquid chromatography.
UPLC – Ultra performance liquid chromatography, modern technique in liquid chromatography.
It Improves three areas – chromatographic resolution, speed and sensitivity analysis. It uses fine particles and saves time also reduces solvent consumption. Separation on UPLC is performed under very high pressure (upto 100 mpa) using sub – 2 mm particles and mobile phase at high linear velocities. It has no negative influence on analytical column or other chromatographic system.
This new category of analytical separation retains the principle of HPLC and create new steps for improvement in chromatographic performance.
In 21st century pharmaceutical companies were focusing on new ways to shorten time for development of drugs. This review mainly focuses on comparison between HPLC and UPLC technique its principle, instrumentation and applications.
Keywords: *UPLC, HPLC, Resolution, Sensitivity, High pressure.*

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Please cite this article in press Siddhant S. More et al., ***HPLC – High Performance Liquid Chromatography & UPLC – Ultra Performance Liquid Chromatographic System – A Review on Modern Liquid Chromatography.,*** *Indo Am. J. P. Sci, 2018; 05(08).*

1. INTRODUCTION:

HPLC is modern liquid chromatography, solvent generally flows through the column with gravity, but in HPLC technique, solvent will be forced under high pressures upto 400 atm so that sample can be separated into different constituents with the help of difference in relative affinities. In HPLC, pumps are used to pass pressurized liquid solvent including the sample mixture which enters into a column filled with solid adsorbent material. The interaction of sample component will be varied and this causes difference in flow rates of each component and leads to separation of components of column. Chromatography is a mass exchange process including adsorption. HPLC depends on pumps to pass a pressurized fluid, pressurized fluid are commonly a blend of solvents (e.g. water, acetonitrile and/or methanol) which is known as 'mobile phase'. Its organization and temperature plays an important part in the partition procedure by affecting the connections occurring between sample segments and adsorbent. HPLC is recognized from traditional ("low weight") liquid chromatography because operational pressures are fundamentally higher (50 bar to 350 bar), while normal liquid chromatography regularly depends on the power of gravity to pass the portable stage through the segment. Because of the small sample amount isolated in scientific HPLC, column section measurements are 2.1 mm to 4.6 mm distance across, and 30 mm to 250 mm length. Additionally, HPLC segments are of smaller sorbent particles (2 µm to 50 µm in normal molecule size). This gives HPLC high resolving power (the capacity to recognize components) while isolating mixtures, which makes it a prominent chromatographic method.

Conventional separation method HPLC has many advantages like robustness, ease of use, good selectivity, and adjustable sensitivity but main disadvantage is lack of efficiency due to low diffusion coefficient in liquid phase and slow diffusion of analytes in stationary phase.

UPLC is a new invention in liquid chromatography. Ultra-performance liquid chromatography (UPLC) is a well known technique that has been used in laboratories from last 10 years.

Ultra Pressure Liquid chromatography, it improves in three areas:

- ✓ **Resolution,**
- ✓ **Speed,**
- ✓ **Sensitivity.**

UPLC instrument operates at high pressure than that of HPLC. System uses fine particles (< than 2.5 µm) and / M.p at high linear velocities decreases the length of column, this also reduces solvent consumption and saves time. Water in 2004, launched and trade marketed UPLC which is based upon small porous particles. Principle of this evolution are governed by the van deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (column efficiency), according to van deemter equation, as particle size decreases to less than 2.5 µm, not only there is a significant gain in efficiency, but the efficiency does not diminish at increased flow rate or linear velocities.

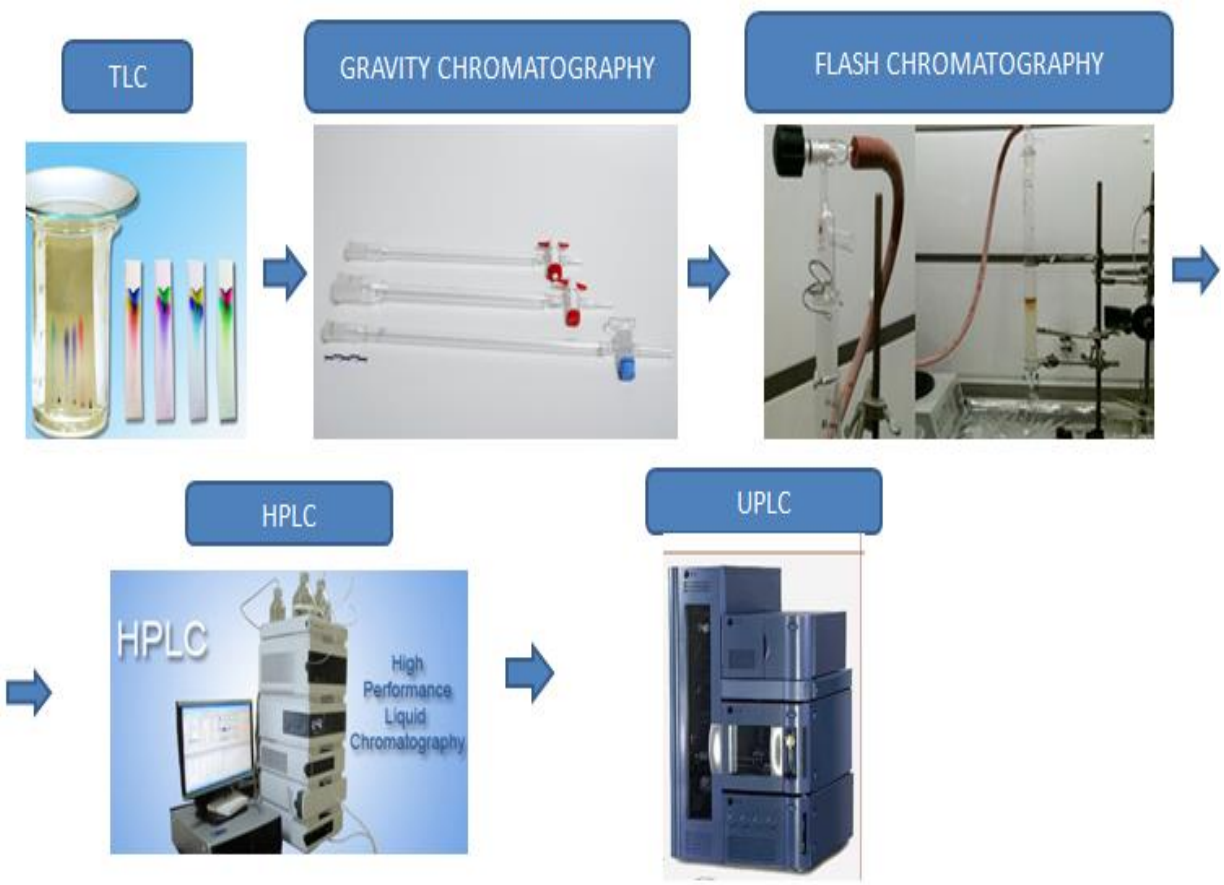


Fig.1. Evolution in chromatography

Fig.2. High performance liquid chromatography

Fig.3. Ultra performance liquid chromatography

1.1 What is UPLC

UPLC - Ultra performance liquid chromatography, it was introduced by waters corporations when they introduced their acquity LC systems.

It Uses sub – 2 µm particles, which were operated at higher flow rate by doubling the overall operating pressure than a conventional system. This resulted in significantly shorter analysis times.

1.2 Use of UPLC system

In this Elevated – temperature chromatography, it allows high flow rates by lowering the viscosity of mobile phase, which reduces the column back pressure. Monolithic columns contain a porous support (polymerized) structure which provides lower flow resistance than any conventional particle-packed columns.

2. Principle

In HPLC chromatography, column plays a significant role in the separation of different compounds because it contains stationary phase (3 to 10 µm). The stationary phase is of polar and non-polar particles according to the type of column, Polar and non-polar columns are used according to the nature of the sample to be analysed.

When a mixture of compound enters a column, compounds are separated on the basis of their polarity. If stationary phase is non-polar then it attracts the non-polar compounds and a polar compound get elutes first then a non-polar compound and if the stationary phase is polar then a non-polar compound elutes first.

Principle of UPLC is based on using stationary phase which has particles less than 2 µm (while HPLC columns are typically filled with particle size of 3 to 10 µm). It is governed by van deemter equation, which describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency)

$$H=A+B/v+Cv$$

Where A, B and C are constants and V is linear velocity, the carrier gas flow rate

A = Eddy mixing

B = axial diffusion

C = solute's mass transfer

The A term is independent of velocity and represents "eddy" mixing. It is smallest when the packed column particles are small and uniform. The B term represents axial diffusion or the natural diffusion tendency of molecules. This effect is diminished at higher flow rates and so this term is divided by v. The C term is due to kinetic resistance to equilibrium in the separation process. The 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, the more a molecule on the packing tends to lag behind molecules in the mobile phase. Thus this term is proportional to v.

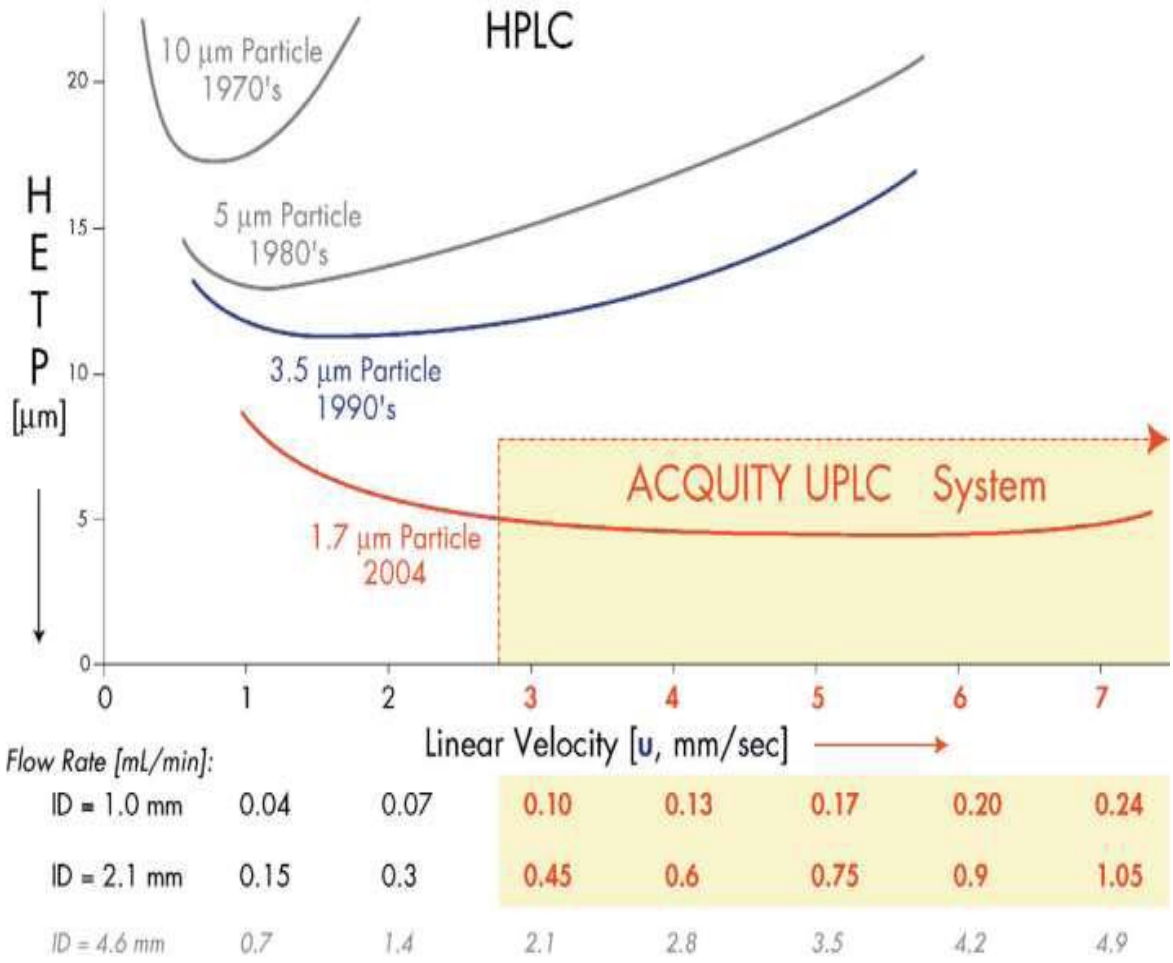


Fig.4. Relationship between linear velocity and HETP

Chromatographic resolution is described by

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

Where,
Rs = Resolution
N = Separation efficiency (theoretical plate)
α= Selectivity factor
k = Retention factor (Capacity factor)

separation efficiency (N)

$$N = \frac{L}{H} = \frac{L}{hd_p}$$

Where,
L = Column length
H = Height of theoretical plate
h = Reduced plate height
dp = Particle diameter
Therefore,

$$Rs \propto N \propto \frac{1}{d_p}$$

As particle size decreases by factor of three, there is three times increase in efficiency and also in resolution. Similarly, efficiency (N) is directly proportional to the length of the column (L)

3. Advantages of UPLC

- Decreased in run time and increased sensitivity.
- More selectivity, sensitivity, and dynamic range of LC analysis.
- Higher resolution performance.
- Expands scope of Multiresidue Methods.
- UPLC's fast resolving power quickly quantifies related and unrelated compounds.
- Faster analysis through the use of very fine particle size .
- Operation cost is minimize.
- Decreases consumption of solvent.
- Quicker analysis.

4. Disadvantages of UPLC

- High back pressure compared to HPLC which reduces the life of the column
- So far performance similar or even higher has been demonstrated by using stationary phases of size around 2 µm without the adverse effects of high pressure.
- Particle size of less than 2 µm are generally non - regenerable.

4.1 Draw Back

- Cost mixing
- Solvent pumping
- Lack of variety in commericial columns at 1.7 µm

5. Small particle chemistry

Van deemter equation is incomplete without smaller particles than those used in HPLC. Although high efficiency, non porous 1.5 mm particles are commercially available, they suffer from poor loading capacity and retention because of its low surface area. To maintain that retention and capacity similar to that of HPLC, efficiency plays a significant role In UPLC as it depends on same selectivity and retentivity as hplc. UPLC must use porous particles that can withstand high pressure. Silica based particles have good mechanical strength but can suffer from many disadvantages, which include a limited PH range and tailing of basic analytes. Polymeric column can overcome this PH limitations. In order to provide enhanced mechanical stability required for UPLC, a second generation bridged ethane hybrid (BEH) technology was developed.

According to Van deemter equation efficiency increases with smaller size particles but this can lead to a rapid increase in back pressure, while most of the HPLC system can operate only upto 400 bars, so for this reason short columns filled with particles of about 2 µm are used with these system, to accelerate the analysis without loss of efficiency.

6. Comparison between HPLC and UPLC

Principles of both (HPLC and UPLC) are same, only difference is in design of column material particle size which is less than 2 µm. This makes a big difference in performance and to maximize advantages of column, creating a powerful, robust and reliable solution

Sr.no	Characteristics	HPLC	UPLC
1	Particle size	3 to 10 µm	Less than 2 µm
2	Maximum back pressure	35 to 40 Mpa	103.5 MPa
3	Analytical column	Alltima C18	Acquity UPLC BEH C18
4	Column dimensions	150 ×3.2 mm	150 X 2.1 mm
5	Column temperature	30°C	65°C
6	Sample throughput	Less	More
7	Sample preparation	Simple	Tedious
8	Column coagulation	Does not takes place	Takes place
9	Analysis time	More	Less
10	Sensitivity	Less	Higher
11	Plate count	More than 2000	More than 7500

7. Instrumentation

7.1 The HPLC instrumentation

It involves pump, injector, column, detector, integrator and display system. In column actual separation occurs. The parts include:

7.1.1 Solvent Reservoir: In HPLC the mobile phase or solvent is a mixture of polar and non-polar liquid components. Depending on the composition of sample, the polar and non-polar solvents will be varied.

7.1.2 Pump: pump forces a liquid (i:e mobile phase) through the column at specific flow rate and then passes to detector. 6000 – 9000 psi is the operating pressure of the pump. This operating pressure depends on column dimensions, particle size of column, flow rate and mobile phase composition.

7.1.3 Sample Injector: The injector can be a solitary infusion or a computerized infusion framework. Injector introduce a liquid sample into the flow stream of mobile phase. An injector for HPLC should give infusion of the fluid specimen within 5 to 20 µL of volume with high reproducibility and under high pressure.

7.1.4 Columns: Columns are made of clean stainless steel,(gives high pressure capabilities) are somewhere around 50 mm and 300 mm long and have an inward distance across of somewhere around 2 and 5 mm. They are loaded with a stationary phase with a molecule size of 3 µm to 10 µm. Preferably the temperature of the mobile phase and the column should be kept consistent during investigation.

7.1.5 Detector: The HPLC detectors, situated toward the end of the column detect the individual analytes as they elute from the chromatographic column. Regularly utilized detectors are UV-spectroscopy, fluorescence, mass spectrometric and electrochemical identifiers.

7.1.6 Data Collection Devices or Integrator: Signals from the detector might be gathered on graph recorders or electronic integrators that fluctuate in many-sided quality and in their capacity to process, store and reprocess chromatographic information. It also determines the time of elution of the analytes. The PC coordinates the reaction of the indicator to every part and places it into a chromatograph that is anything but difficult to interpret.

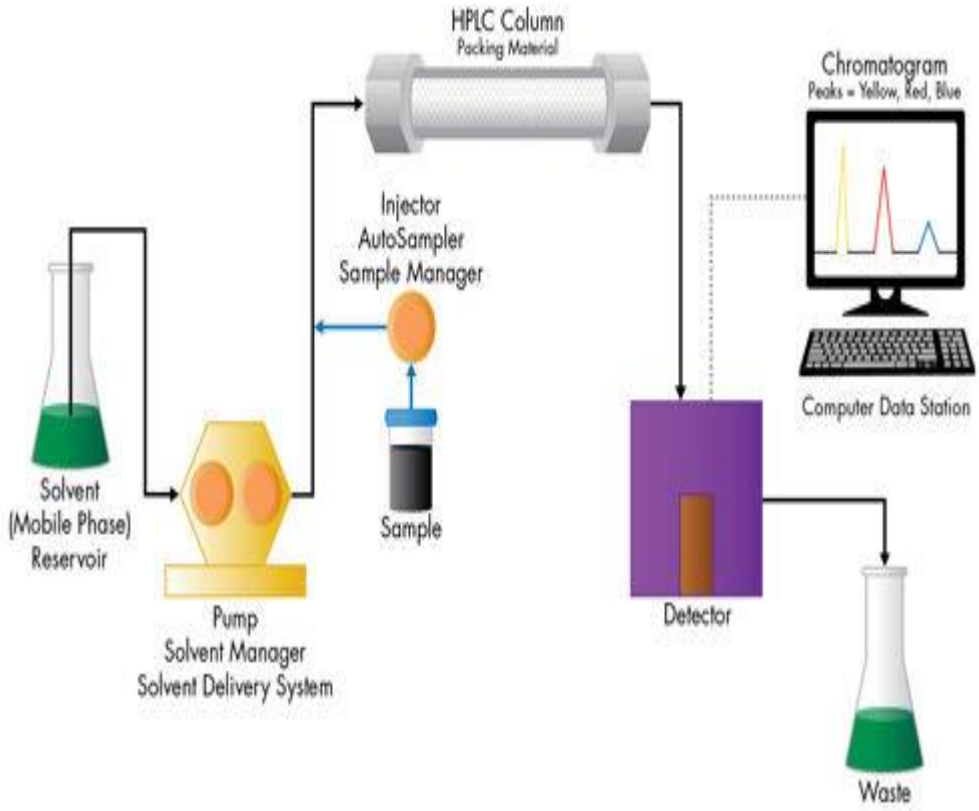


Fig.5. Schematic diagram of instrumentation of UPLC

7.2 UPLC instrumentation

UPLC system is so designed to match the performance needs of innovative column chemistries with robust hardware, easy to use software and specialized support services.

7.2.1 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, therefore it must be done reproducibly and accurately. To protect the column from extreme pressure instabilities, the injection process must be pulse-free and the swept volume of the device should be at minimal to reduce potential band spreading. A fast injection cycle time is needed to fully capitalize on the speed afforded by UPLC, which in turns requires a high sample capacity. Low volume injections with minimal carryover are also required so as to increase sensitivity. Nowdays, there are also direct injection approaches for biological samples

7.2.2 UPLC column

The UPLC columns are Made up of small particles having size less than 2 µm. The particles are bonded in matrix, as the bonded stationary phase is required for providing both retention and selectivity.

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 stationary phase columns manufactured by ACQUITY are available in the market, which can be used by UPLC technique.

(i) ACQUITY UPLCTM BEH C8 ,
(ii) ACQUITY UPLCTM BEH C18,
(iii) ACQUITY UPLC BEH Shield RP18 and
(iv) ACQUITY UPLC BEH Phenyl (phenyl group tethered to the silyl functionality with a C6 alkyl),
(**BEH-B**ridged **E**thane **H**ybrid)

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

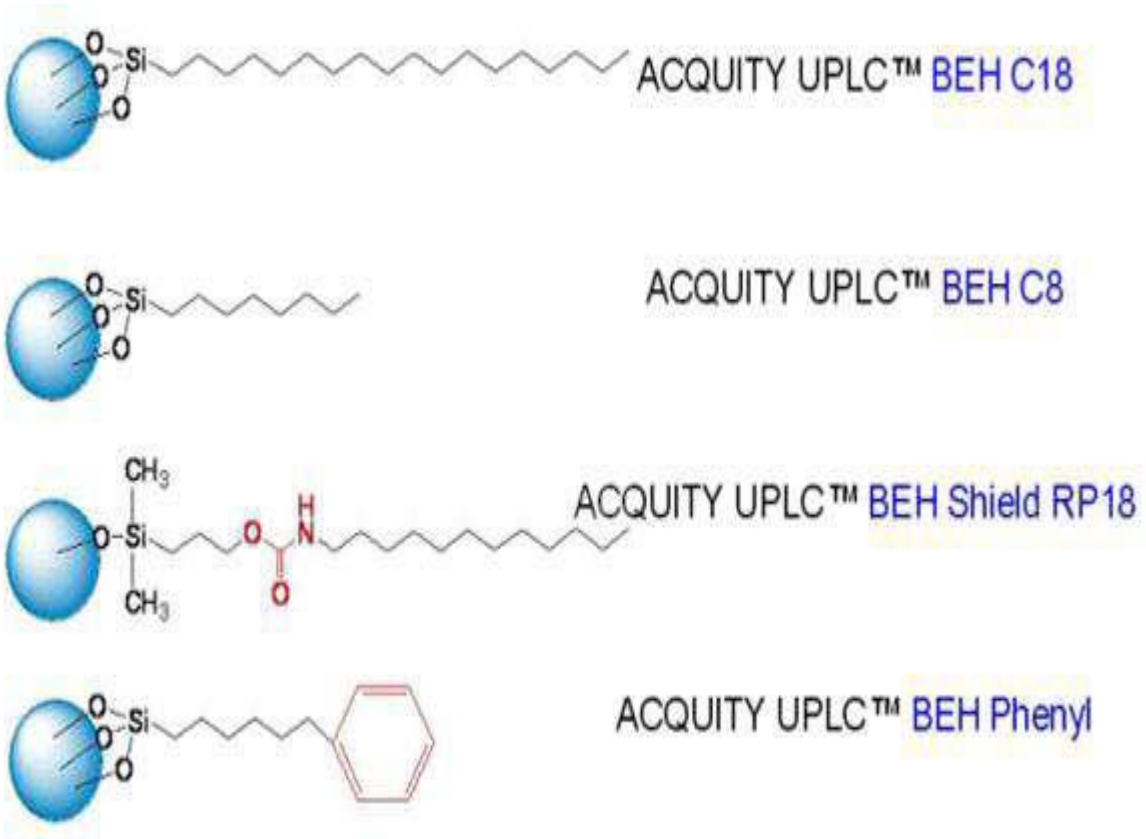


Fig.6. Columns in UPLC

ACQUITY UPLC BEH C18 and C8 columns are universal columns of choice for most of 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. ACQUITYUPLC BEH Shield RP18 columns are designed to provide selectivity that complements the ACQUITYUPLC BEH C18 and C8 phases. ACQUITY UPLC BEH Phenyl columns utilize a tri functional C6 alkyl tether 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 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. The ACQUITY UPLC System consists of a binary solvent manager, sample manager including the column heater, detector, and optional sample organizer.

7.2.3 Binary Solvent Manager

In binary solvent manager there are two individual serial flow pumps that delivers a parallel binary gradient. It is a high pressure pump that moves solvent through the system. It provides pulse free solvent flow and delivers solvent at flow rates of 1 ml/min at 103421 Kpa [1034 bar, 1500 psi] and up to 2 ml/min at reduced pressures to 62053 Kpa [621 bar, 9000 psF] . The solvent manager can pump two solvents immediately.

7.2.4 Sample Manager

The Acuity sample manager injects the sample, it draws from Micro titer plates or vials into the chromatographic flow stream. The Sample manager can perform an injection within 15 seconds. The sample manager also have control over column heater. column can attained temperature up to 65 °C.

7.2.5 Column Heater

It is attached to the top of the sample manager and serves as instrument's top cover.

7.2.6 Pumps

The UPLC pump is one of the most important components in a liquid chromatography system which provides continuous constant flow of the eluent through the UPLC injector, column, and detector.

The two basic classifications are

- Constant pressure pump
- Constant flow pump

7.2.7 Detectors

Detectors used are mostly UV-VIS detectors. Detection of analytes is based on absorbance that is concentration sensitivity detectors. In UPLC the flow cell volume would have to be reduced to maintain concentration and signal. Based on Beer's Law, smaller volume conventional flow cells would also reduce the path length upon which the signal strength depends. A reduction in cross-section means the light path is reduced, and transmission drops with increasing noise. Therefore, if a conventional HPLC flow cell were used, UPLC sensitivity would be compromised. The detectors are use in UPLC analysis is UV/Visible detector .The ACQUITY Tunable UV/Visible detector cell consists of a light guided flow cell equivalent to an optical fiber. Light is efficiently transferred down the flow cell in an internal reflectance mode that still maintains a 10mm flow cell path length with a volume of only 500mL. Tubing and connections in the system are efficiently routed to maintain low dispersion and to take advantage of leak detectors that interact with the software to alert the user to potential problems. Half-height peak widths of less than one second, obtained with 1.7 µm particles, which gives challenges to the detector. To integrate an analyte peak accurately and reproducibly, the detector sampling rate must be high enough to capture enough data points across the peak.

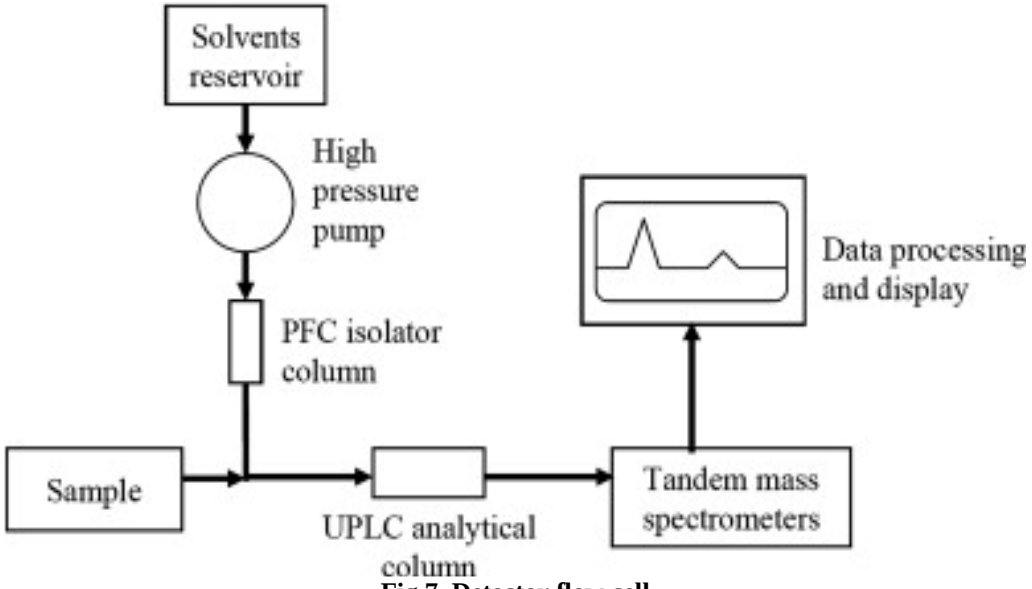


Fig.7. Detector flow cell

Types of Detectors:

Detectors can be classified as:

- Optical detectors
- Tunable ultra violet detectors
- Evaporative light scattering detectors
- Fluorescence detector

7.2.7.1 Optical Detectors:

Optical Detectors are used in UPLC analytical techniques, which has low dispersion characteristics, simple operation, and high data acquisition rates as well as cost effective maintenance and support.

The system can be configured with a TUV, PDA or ELS optical detector or any combination of the three.

7.2.7.2 Tunable Ultra Violet Detector

The TUV optical detector is a two channel ultra violet / visible absorbance detector which is design for use in acquity UPLC system. The detector has two flow cell options.

- The analytical cell flow, with a volume of 500 nano liters and a path length of 10 nm and
- The high sensitivity flow cell with a volume of 2.4 micro litres and 25 mm path length.

Both utilizes the waters patented light guiding flow all technology. The TUV detector operates at wave length ranging from 190 to 700 nm.

Features

- Maximum signal-to-noise response enabled by light-guiding flow cell technology, which eliminates internal absorption, for minimal bandspreading and for maintaining concentration
- High sensitivity for low-level detection for simultaneous quantitation of major and minor components

7.2.7.3 Acquity Uplc Els Dectectors

The ACQUITY UPLC ELS Detector helps to analyze more molecules (including sugars, triglycerides, phospholipids, antibiotics, and natural products) in a single run.

Anywhere large numbers of compounds are screened rapidly – this detector offers a convenient design, easy maintenance, and long lamp lifetimes.

The detector incorporates a flow type nebulizer that is optimized for acquity UPLC system performance

Features

- Provides reproducible, reliable results with precise control over nebulization and desolvation processes for the measurement of temperature-sensitive molecules
- Ensures the benefits of UPLC performance across the entire flow rate range with high data capture rates
- One nebulizer for the entire flow rate range provides simplified set-up and the benefit of maximized performance.

7.2.7.4 Acquity Uplc Flr Detector

The ACQUITY UPLC® Fluorescence (FLR) Detector delivers sensitivity and selectivity to fluorescence-based applications.

Features

- Advanced optical design to maximize light throughput, and reduce light scatter, allowing for better signal-to-noise performance
- Intuitive system console provides simple

navigation to manage instrument parameters for easy system control

- Intuitive software interface and diagnostic tools instill confidence that the detector is performing optimally.

7.2.8 Optional Sample Organizer

The optional sample organizer stores micro miter or vial plates and transfers them to and from the sample manages, automating their processing and increasing through put

8. Case study

8.1 Chromatograms of Simvastatin

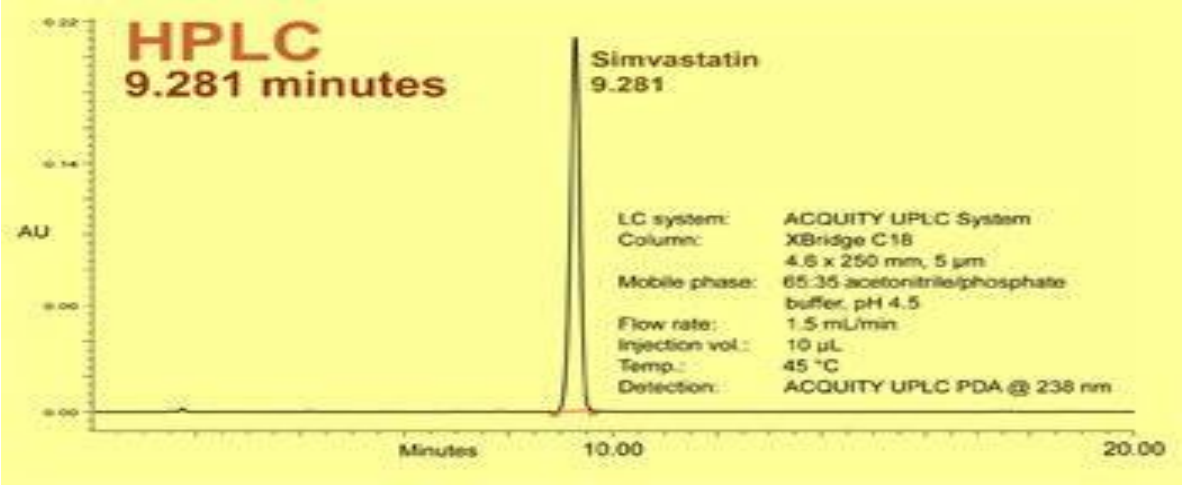


Fig.8. Chromatogram of simvastatin by hplc

Lcsystem -
Column-Xbridge.4.6×250mm
M.ph-65:35acetonitrilephosphatebuffer.Flowrate-1.5ml/min
Inj.vol-10microltr

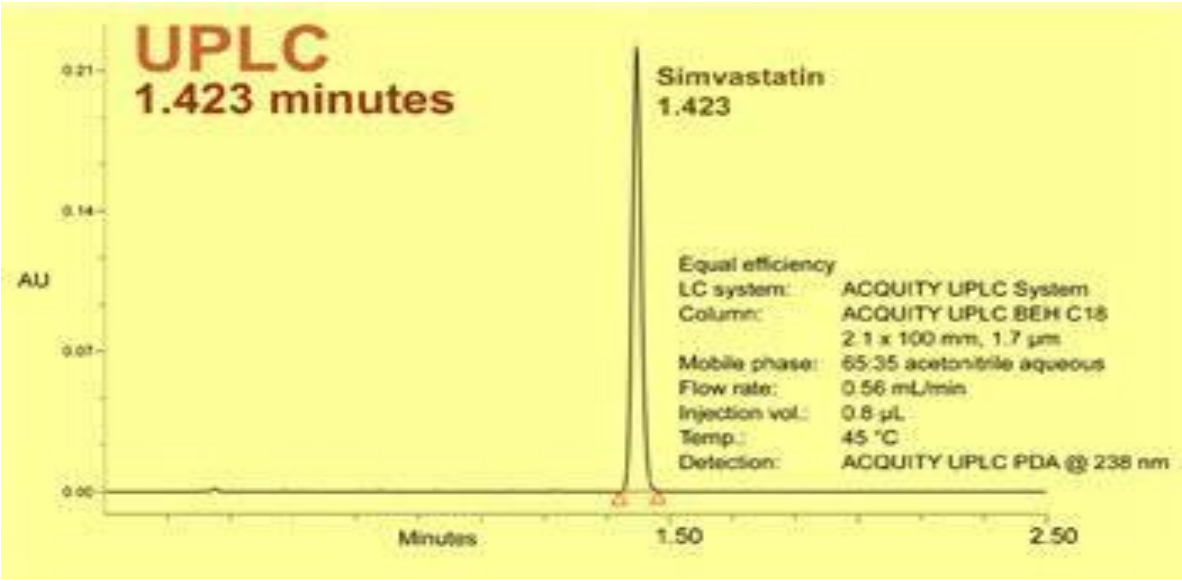
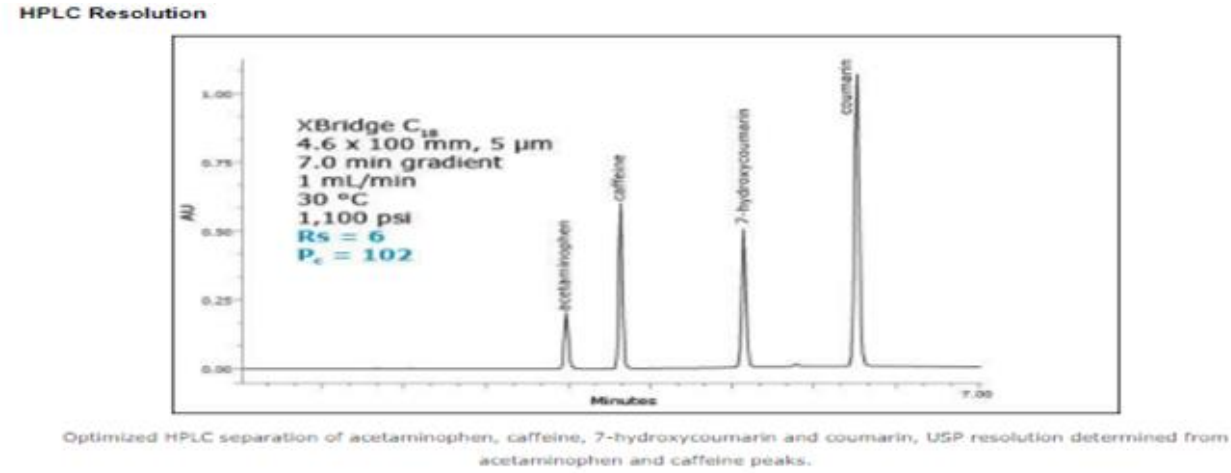


Fig.9. Chromatogram of simvastatin by uplc

Lcsystem-
Column-ACQUITYUPLCBEHC1
82.1×100mm
M.ph-65:35acetonitrileaq.bufferFlowrate-0.56ml/min
Inj.vol-0.8microltr

8.2 HPLC separation of acetaminophen, caffeine, 7 - hydroxycoumarin and coumarin



Optimized HPLC separation of acetaminophen, caffeine, 7-hydroxycoumarin and coumarin, USP resolution determined from acetaminophen and caffeine peaks.

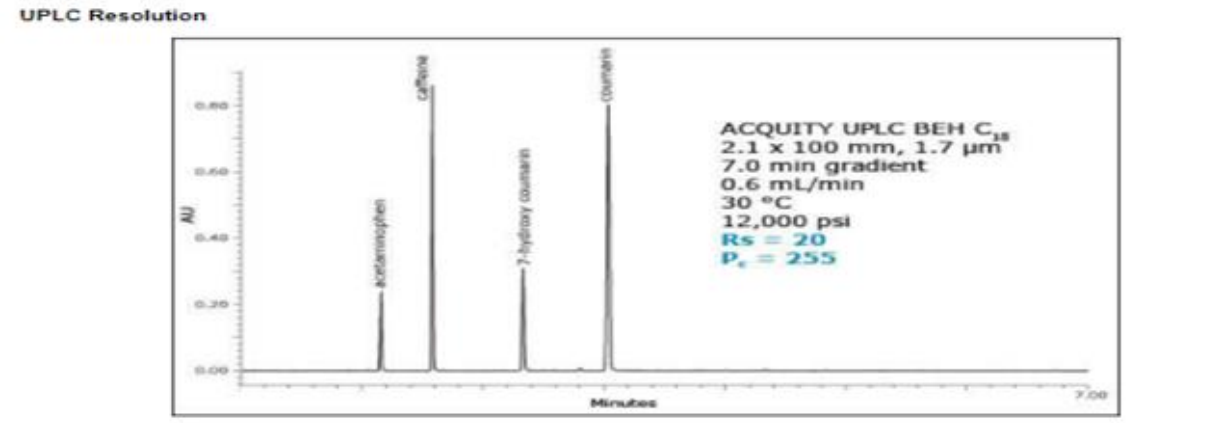


Fig.10. Comparison between hplc and uplc resolution

9. Applications

9.1 Applications of Hplc

- Pharmaceutical Applications: includes controlling drug stability, dissolution studies and quality control.
- Environmental Applications: Monitoring of pollutants and detecting components of drinking water.
- Forensic Applications: Analysis of textile dyes, quantification of drugs and steroids in biological samples.
- Food and Flavour Applications: Sugar analysis in fruit juices, detecting polycyclic compounds in vegetables, analysis of preservatives.
- Clinical Applications: Detecting endogeneous neuropeptides, analysis of biological samples like blood and urine.

9.2 APPLICATIONS OF UPLC

Demand of UPLC analysis is very high, because of its unique features like high resolution in chromatogram, short time analysis which make more analytical work in less time with valuable, reliable and authentic data. More accurate data can be obtained by UPLC in faster way. Method developed by UPLC are accurate and precised. By this method the standard of analysis in every respect like qualitative, quantitative and complexity of sample can be differentiate in very high standard. High resolution and speedy analysis is also very helpful in pharmacokinetic studies like – adsorption, distribution, metabolism and excretion (ADME). ADME studies measure physical and chemical properties of compound. For the drug development and formulation process, profiling, detecting and quantifying drug substances and their impurities can be performed very accurately.

UPLC has following applications

Drug Discovery

- High throughput quantitative analysis

- Analysis of Dosage form
- Analysis of amino acids
- Determination of Pesticides
- Analysis of Natural Products and Traditional Herbal Medicine
- Identification of Metabolite
- ADME (Absorption, Distribution, Metabolism, Excretion) Screening
- Bioanalysis / Bioequivalence Studies
- Dissolution Testing
- Method Development / Validation

10. CONCLUSION:

The HPLC is mostly used analytical technique. It is having several advantages. With the use of HPLC one can produce extremely pure compounds. It can be used in both laboratory and clinical science. With the use of HPLC the accuracy, precision and specificity can be increased. The only disadvantage of HPLC is high cost

UPLC increases productivity in both chemistry and instrumentation by providing more information per unit of work as it increases resolution, speed and sensitivity for liquid chromatography. The main advantage is a reduction of analysis time which also reduces solvent consumption, Analysis time and analysis cost which are very important in many analytical laboratories. This technology thus creates a new opportunity for business profitability in highly efficient manner and allows the product to be introduced to the market in less time.

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