ABSTRACT

Today, chromatography is the backbone of separation science and is being used in all research laboratories and pharmaceutical industries of the world. In these chromatography techniques, HPLC is one of the chromatographic techniques, which is mostly used analytical technique. The current trend in high performance liquid chromatography (HPLC) tends toward the achievement of higher separation efficiency and shorter analysis time. This article represents a brief review of HPLC along with its principle and instrumentation. It describes about new trends in HPLC such as RRLC, UPLC, UFLC and Nano LC. In this article mainly focus on detailed comparison of new developments of HPLC in terms of instrumental operating conditions such as column temperature, flow rate, injection volume and also cover the applications and advantages over HPLC of each technique.

KEYWORDS: Liquid chromatography, HPLC, RRLC, UPLC, UFLC, Nano LC.
is the term used to describe liquid chromatography in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase. An HPLC instrument, therefore, consists of an injector, a pump, a column, and a detector.\textsuperscript{[2]}

**PRINCIPLE**

The underlying principles of this evolution are governed by the van Demeter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or 1/column efficiency).

$$H = A + B/u + Cu$$

Where,  
A- Eddy’s diffusion  
B- Longitudinal diffusion  
C- Concentration  
u- Linear Velocity\textsuperscript{[3, 4]}

**Figure: Diagram of HPLC instrumentation**

**NEW TRENDS IN HPLC TECHNIQUE**

HPLC is compared with the classical techniques are characterized by:

- Rapid Resolution Liquid chromatography (RRLC)
- Ultra Performance Liquid chromatography (UPLC)
- Ultra Fast Liquid chromatography (UFLC)
- Nano Liquid chromatography (NANO LC).\textsuperscript{[5]}
RAPID RESOLUTION LIQUID CHROMATOGRAPHY

RRLC system was designed to provide highest analysis speed, resolution & pressure at a minimum.[6] Fastest and most efficient and flexible LC system in the world. It has become an increasingly useful approach to achieve higher throughput, improve sensitivity and reduce costs.[7] This analysis has become a routine method in the pharmaceutical industry. It holds excellent peak shapes, enhanced reproducibility, high sensitivity, high-speed detection with reduced analysis cost, and is valuable for the quality control of herbal medicines. The separation resolution and reduction of analysis time has continually improved in High Performance Liquid Chromatography (HPLC). Since then, HPLC using smaller particles has become more popular.[8] For further improvement, column efficiency must be increased. The relationship among separation efficiency, the mobile phase linear velocity and particle size was investigated in detail in the early 1970s. This and other systematic investigations have led to high throughput and high resolution HPLC that we know today.

The shortening in analysis time is due to the use of a shorter column length. However, a shorter column may lead to a loss of theoretical plates, hence a decrease in chromatographic resolution that may be required for a complex mixture of compounds. To offset the potential loss of resolution, the use of smaller size particles has resulted in more efficient columns. Long columns packed with smaller particles result in higher efficiency and higher resolution, with new RRLC technology, analysis time can be significantly reduced without losing chromatographic resolution.[2]

The RRLC system enables faster analysis (theoretically up to 20x) than with conventional HPLC while maintaining equivalent resolution. This is achieved by using sub-2 micron column particle chemistry and high flow rates. Often higher temperatures are employed to minimize system back-pressure. With the widespread adoption of RRLC comes the question of HPLC detector compatibility. Presented here is the use of Charged Aerosol Detection with conventional HPLC and RRLC.[7]

Advantages over HPLC

- The major high - throughput RRLC are the increase in throughput.
- The reduction in the analysis cost.
- The shortening in analysis time is due to the use of a shorter column length high resolution separation capability.
• Ultra fast and highest analysis speed.
• It is most improve sensitivity.
• High temperature up to 100°C on certain columns allows more selectivity flexibility.
• It is offers true fast LC analysis.\[^9\]

**Applications of RRLC**

• RRLC-tandem mass spectrometry method for the determination of endocrine disrupting chemicals (EDCs) examples: Bentazone, salicyliacid, silica gel pharmaceuticals and personal care products (PPCPs) in waste water irrigated soils. Analysis for quality control of Rhodiolarosea roots and commercial standardized products.
• It is applicable for Herbal produces examples: Panax and Epimedium species
• It is Applicable for not only pharmaceuticals compound examples: Methanol, vitamins B6, B9, B12, Tri ethylamine but also for chemical compounds example: Acetanilide, Acetophenone, octanophenone, Tubufenozide
• Scalability as a Function of Column Dimensions Using ZORBAX Rapid Resolution HT Columns for the Analysis of the Pharmaceutical Triamcinolone
• Impurity Profiling with the Agilent 1200 Series LC System Part 1: Structure Elucidation of Impurities with LC/MS
• Polycyclic Aromatic Hydrocarbon (PAH) Separations Using ZORBAX Eclipse PAH
• The High-Resolution Reversed-Phase HPLC Separation of Licorice Root Extracts using Long Rapid resolution HT 1.8-um Columns
• The Analysis of Benzodiazepines in Hair Using RRHT LC/MS/MS
• Determination of Benzodiazepines in Oral Fluid Using LC/MS/MS More speed, better resolution and lower LOD using liquid chromatography and fluorescence detection - Comparing the 1100 Series LC to the 1200 Series Rapid Resolution system.\[^10\]

**ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY**

UPLC refers to Ultra Performance Liquid Chromatography. It improves in three areas: chromatographic resolution, speed and sensitivity analysis. It uses fine particles and saves time and reduces solvent consumption.\[^11\-14\] UPLC is comes from HPLC. HPLC has been the evolution of the packing materials used to effect the separation. An underlying principle of HPLC indicates 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 Demeter equation.\[^{15}\] 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.\[^{2}\]

**Advantages over HPLC**

- Decreases run time and increases sensitivity
- Provides the selectivity, sensitivity, and dynamic range of LC analysis
- Maintaining resolution performance.
- Expands scope of Multi residue Methods
- UPLC’s fast resolving power quickly quantifies related and unrelated compounds
- Faster analysis through the use of a novel separation material of very fine particle size
- Operation cost is reduced
- Less solvent consumption
- Reduces process cycle times, so that more product can be produced with existing resources
- Increases sample throughput and enables manufacturers to produce more material that consistently meet or exceed the product specifications, potentially eliminating variability, failed batches, or the need to re-work material.
- Delivers real-time analysis in step with manufacturing processes
- Assures end-product quality, including final release testing.\[^{16}\]

**Applications of UPLC**

- **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.

- **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 decrease.
- **Analysis of amino acids**
  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.

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

- **Analysis of Natural Products and Traditional Herbal Medicine**
  UPLC is widely used for analysis of natural products and herbal medicines. The main purpose of this is to analyze drug samples arise from the complexity of the matrix and variability from sample to sample. UPLC provides high quality separations and detection capabilities to identify active compounds in highly complex samples that results from natural products and traditional herbal medicines.

- **Identification of Metabolite**
  UPLC/MS/MS addresses the complex analytical requirements of biomarker discovery by offering unmatched sensitivity, resolution, dynamic range, and mass accuracy.

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

- **Bio-analysis / Bioequivalence Studies**
  UPLC delivers excellent chromatographic resolution and sensitivity. The sensitivity and selectivity of UPLC at low detection levels generates accurate and reliable data that can be used for a variety of different purposes, including statistical pharmacokinetics analysis. UPLC solutions are proven to increase efficiency, productivity and profitability for bioequivalence laboratories.

- **Dissolution Testing**
  For quality control and release in drug manufacturing, dissolution testing is essential in the formulation, development and production process. UPLC provides precise and reliable automated online sample acquisition. It automates dissolution testing, from pill drop to test
start, through data acquisition and analysis of sample aliquots, to the management of test result publication and distribution.[17-21]

ULTRA FAST LIQUID CHROMATOGRAPHY

It is ten times higher speed and three times better separation than other LC techniques and offers outstanding speed and separation even at normal pressure levels. By maximizing the column and performance of the entire system UFLC minimizes the deviation from the van Demeter theory.[22] The Prominence UFLC series provides ultrafast analysis, while maintaining high analytical precision and reliability.[23]

Advantages over HPLC

- Reduce analysis time by 75% over regular LC system.
- Increased separation performance.[23]

Applications of UFLC

a) Determination of iodiconazole in micro-dialysis samples
b) Determination of podophyllotoxin in dermal and blood micro-dialysis samples of rats.
c) Simultaneous analysis of fluoroquinolones and xanthenes derivatives in serum.
d) Analysis of Isoflavones in Soy.
e) Analysis of Catechins in Green Tea.[2]
f) Analysis of artificial colorants by UFLC- mass spectroscopy.
g) Separation of major components in Panax Ginseng.[24-25]

NANO LIQUID CHROMATOGRAPHY

Some definitions have been found in the literature based on column diameter and mobile phase flow rates.[26-28] Some workers defined NLC as chromatographic modality having mobile phase flow rate at nano ML per minute. But, the detection aspect of this chromatography which is very important in analytical science was not taken into consideration until then. Later in 2009, Ali et al gave an exact and scientific definition i.e. a modality of chromatography involving samples in nano liters, mobile phase flow rates in nano milli liter per minute, with detection at nano grams per milli liter.[29, 30]

This sort of modality is generally carried out in microchips and hence, has also been termed as Lab on chip chromatography.[31]
Advantages over HPLC
- Significantly reduces the mobile phase consumption and subsequent waste production
- Internal diameter reduction increases sensitivity and/or less sample requirement
- Significantly cheaper, quicker than its conventional counterpart
- Increased detection sensitivity in MS because of lower flow rates in smaller columns
- High separation efficiency and possibility to analyses very small amount of solute
- Recent developments have significantly increased the resolution power for complex sample analysis.\[^{32}\]

Applications of Nano LC
- Separation of sulfonamides\[^{33}\]
- Separation of peptides\[^{34}\]
- Discovery of Glycomics towards biomarker Using nano-LC\[^{35}\]
- Nano-LC for glycobioanalysis\[^{36}\]
- In the analysis of Biological and environmental samples.
- Proteomic and genomic research.
- Detection of accumulated drug samples in the body.
  - High throughput screening (HTS) and drug discovery where the limits of detection are very slow.\[^{33}\]

Table 1: Comparison between HPLC, RRLC, UPLC, UFLC AND Nano LC.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HPLC</th>
<th>RRLC</th>
<th>UPLC</th>
<th>UFLC</th>
<th>Nano LC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size</td>
<td>3 to 10 μ</td>
<td>1.8 μ</td>
<td>Less than 2μ</td>
<td>1.7 – 2.2 μ</td>
<td>1.7 – 3 μ</td>
</tr>
<tr>
<td>Analytical column</td>
<td>XTerraC18, Altima C18</td>
<td>ZORBAX Eclipse XDB–C18</td>
<td>Acquity UPLCbeh C18, C8, rp</td>
<td>Shim-pack XR-O DS column</td>
<td>Capillary HPLC, Micro HPLC</td>
</tr>
<tr>
<td>Column dimensions (length x I.D)</td>
<td>150 X 3.2 mm</td>
<td>2.1-4.6mm</td>
<td>150 X 2.1 mm</td>
<td>75mm X 3.0 mm</td>
<td>125 mm X 0.05mm - 4.6mm</td>
</tr>
<tr>
<td>Column temperature</td>
<td>300 C</td>
<td>Up to 1000 C</td>
<td>650 C</td>
<td>400 C</td>
<td>25-350 C</td>
</tr>
<tr>
<td>Injection volume</td>
<td>5μL</td>
<td>1.5 μL</td>
<td>2μL</td>
<td>0.1-100μL</td>
<td>10 nL-125 μL</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.01-5mL/min</td>
<td>0.2-20 μL/min</td>
<td>0.6 mL/min</td>
<td>3.7 nL/min</td>
<td>20-200 nL/min</td>
</tr>
</tbody>
</table>

CONCLUSION AND DISCUSSION
1. RRLC offers improved run times and increased sensitivity over conventional HPLC based methods. In RRLC High Sensitivity - Low limit of detection, Excellent Reproducibility, Broad Applicability, Ease of Use - Easy setup.
2. At a time when many scientists have reached separation barriers with Conventional HPLC, UPLC presents the possibility to extend and expand the utility of chromatography.

3. Columns with small internal diameters and/or short column lengths are more susceptible to extra-column band-broadening for high-speed separation in UPLC.

4. Ultra fast analysis means a significant enhancement in sample throughput (5-10 times) & productivity compared to a conventional HPLC.

5. Nano LC is the latest innovation in separation science in which detections can achieved at nano gram or lower levels.

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