



Comparative Study of New Trends in HPLC: A Review

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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. Recent developments in chromatographic supports and instrumentation for liquid chromatography (LC) are enabling rapid and highly efficient separations. 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, applications and advantages over HPLC of each technique.

Keywords: Liquid chromatography, HPLC, RRLC, UPLC, UFLC, Nano LC.

INTRODUCTION

Today, chromatography is the backbone of separation science and is being used in all research laboratories and pharmaceutical industries of the world. The journey of chromatography was started first by Mikhail Tswett. (Russian botanist) in 1903¹. Chromatographic process can be defined as separation technique involving mass-transfer between stationary phase and mobile phase. In these chromatography techniques, HPLC is one of the chromatographic techniques, which is mostly used analytical technique. In This method stationary phase can be a liquid or a solid phase. HPLC utilizes a liquid mobile phase to separate the components of a mixture. High-performance liquid chromatography (HPLC) 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 detector².

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).

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

A- Eddy's diffusion

B- Longitudinal diffusion

C-Concentration

u- Linear Velocity^{3,16}.

New Amendments 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)

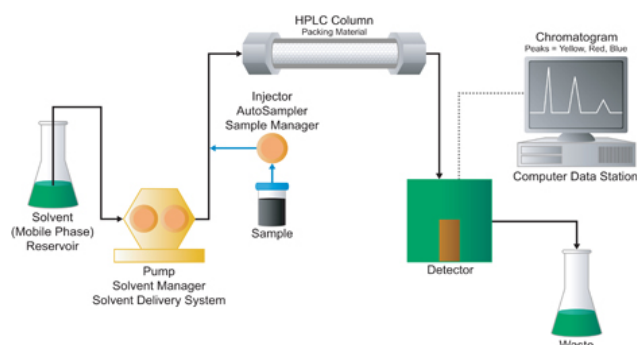


Figure 1: High-Performance Liquid Chromatography [HPLC] System⁴.

RAPID RESOLUTION LIQUID CHROMATOGRAPHY

RRLC system was designed to provide highest analysis speed, resolution & pressure at a minimum⁵. 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 medicines⁶. 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 popular⁶. 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 1970 s. 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 resolution³.

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

Characteristics	HPLC	RRLC	UPLC	UFLC	Nano LC
Particle size	3 to 10 μ	1.8 μ	Less than 2 μ	1.7 – 2.2 μ	1.7 – 3 μ
Analytical column	XTerraC ₁₈ , Alltima C ₁₈	ZORBAX Eclipse XDB–C18 RRHT	Acquity UPLCbeh C ₁₈ ,C ₈ ,rP	Shim-pack XR-ODS column	Capillary HPLC, Micro HPLC
Column dimensions (length x I.D)	150 X 3.2 mm	2.1-4.6mm	150 X 2.1 mm	75mm X 3.0 mm	125 mm X 0.05mm - 4.6mm
Column temperature	30 ^o C	Up to 100 ^o C	65 ^o C	40 ^o C	25-35 ^o C
Injection volume	5 μ L	1.5 μ L	2 μ L	0.1-100 μ L	10 nL-125 μ L
Flow rate	0.01-5mL/min	0.2-20 μ L/min	0.6 mL/min	3.7 nL/min	20-200 nL/min

Advantages over HPLC:

❖ Faster chromatography

There are several advantages of having shorter run times. High Throughput labs now have higher capacity and can analyze more samples in less time. More samples in less time also mean lower costs³.

❖ Higher resolution

It having higher efficiency and higher resolution^{5,7}.

Applications of RRLC:

a) Rapid determination of paeoniflorin from *Paeonia sinjiang* K. Y. Pan.⁸

Paeonia sinjiang K. Yaphank root is a very important drug substance in Chinese herbal medicine for heat-clearing, blood-cooling, activating blood, absorbing clots and eliminating carbuncle. A rapid, effective, binary reverse phase rapid resolution liquid chromatographic method has been developed for the determination of Paeoniflorin extracted from *Paeonia sinjiang* K. Y. Pan. With short run time. RRLC separation was achieved by using Agilent (Zorbax XDB-C18 4.6 mm \times 50 mm, 1.8 μ m) column with mobile phase composed of methanol and 0.05 mol/l potassium phosphate monobasic.

b) RRLC-MS/MS method for the determination of EDCs and PPCPs in waste water irrigated soils⁹

A multi residue analytical method was developed for the determination of 9 endocrine disrupting chemicals (EDCs) and 19 pharmaceuticals and personal care products (PPCPs) including acidic and neutral pharmaceuticals in water and soil samples using RRLC-MS/MS. Analyzed by using Agilent 1200 rapid resolution liquid chromatography coupled to Agilent G6460A triple quadruple mass spectrometer. The chromatographic separation of each group was performed on an Agilent

SB-C18 column (3.0 \times 100 mm, 1.8 μ m) with a RRLC in-line filter kit (4.6 mm, 0.2 μ m filter) (Germany). The column temperature was maintained at 40^oC. The chromatographic mobile phases were run at a flow rate of 0.3 mL/min.

c) RRLC analysis for quality control of *Rhodiola rosea* roots and commercial standardized products¹⁰

A simple, sensitive and reliable reversed phase Rapid Resolution Liquid Chromatography method was developed and validated for six biologically active compounds (salidroside, tyrosol, rosarin, rosavin, rosin and rosiridin) in *Rhodiola rosea* L. roots and powder extracts. The method uses a Phenomenex C18 (2)-HST column at 40 degrees C with a neutral gradient system mobile phase (H₂O and acetonitrile), a flow rate of 1.0 mL/min, and UV detection wavelengths set at 205 and 254 nm, simultaneously. Baseline separation of the six active compounds was achieved within 8 minutes. The average percentages of rosavins (rosarin, rosavin, and rosin) in authentic *R. rosea* roots and root powder extracts were quantitatively determined and a characteristic *R. rosea* roots RRLC profile was established. The RRLC method is accurate and sensitive.

d) Fast Analysis of Phenolic Antioxidants and Erucamide Slip Additives in Polypropylene Homopolymer Formulations Using 1200 RRLC with RRHT Columns and Method Translator¹¹

Vitamin E (tocopherol), phenolic antioxidants and erucamide slip additives in polypropylene homopolymer formulations were resolved and detected using liquid chromatography with ultraviolet/visible detection, under guidelines suggested by ASTM Method D6042. Using the Agilent 1200 Rapid Resolution LC system with Agilent ZORBAX RRHT columns, the antioxidants could be rapidly separated with the same or improved resolution.

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 consumption¹²⁻¹⁵. 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 equation¹⁶. 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.

Advantages over HPLC^{17, 18}:

- ❖ Decreases run time and increases sensitivity
- ❖ Provides the selectivity, sensitivity, and dynamic range of LC analysis
- ❖ Maintaining resolution performance
- ❖ Operation cost is reduced
- ❖ Less solvent consumption
- ❖ Reduces process cycle times, so that more product can be produced with existing resources

Applications of UPLC¹⁹⁻²¹:**a) 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.

b) High throughput quantitative analysis

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

c) 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.

d) 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.

e) Determination of Pesticides

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

f) 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.

g) Identification of Metabolite

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

h) 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.

i) 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 bio equivalence laboratories.

j) 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

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 theory²². The Prominence UFLC series provides ultrafast analysis, while maintaining high analytical precision and reliability²³.

Advantages over HPLC²³:

- ❖ Reduce analysis time by 75 % over regular LC system
- ❖ Increased separation performance

Applications of UFLC:**a) Determination of iodiconazole in micro-dialysis samples²⁴**

Iodiconazole is a very potent antifungal agent used to treat serious fungal infections. After transdermal administration, several factors affect the exposure of iodiconazole, resulting in large variability and demanding further elucidation of drug distribution. For determination of iodiconazole in dermal microdialysate, ultra-fast liquid chromatography (UFLC, Shimadzu) assay using UV detection at 230 nm has been used. Iodiconazole was separated on a Shimadzu Prominence UFLC C18 column (2.2 micron, 50 mm x 2.0 mm i.d.) using acetonitrile-0.025% tri-ethylamine solution, adjusted to pH 3.6 with phosphoric acid (65:35, v/v), at a flow rate of 0.5 ml/min.

b) Determination of podophyllotoxin in dermal and blood micro-dialysis samples of rats²⁵

The micro-dialysis samples were prepared by liquid-liquid extraction using ethyl acetate with etoposide as the internal standard (IS). Podophyllotoxin was separated with an Agilent ZORBAX XDB-C18 column (2.1 mmx50 mm, 3.5 micron). The mobile phase consisted of acetonitrile : 10 m mol/L ammonium acetate (40:60, V/V) at a flow rate of 0.3 ml/min and the analysis was performed at the ambient temperature. The UFLC-MS/MS system was operated in the mode of multiple reactions monitoring using the electro spray ionization technique in positive mode.

c) Simultaneous analysis of fluoroquinolones and xanthenes derivatives in serum²⁶

For selective extraction of fluoroquinolones and xanthenes derivatives from human serum samples, a new molecularly imprinted polymer was synthesized using ofloxacin and theophylline as template and methacrylic acid as function monomer and it was employed as a special dispersant of matrix solid-phase dispersion. For Simultaneous analysis of these derivatives in serum is done by molecularly imprinted matrix solid-phase dispersion coupled with liquid chromatography.

d) Analysis of Isoflavones in Soy²⁷

Analysis of Isoflavones in Soy was done by using prominence UFLC. For this analysis Shim-pack XR-ODS (75 mm L x 3.0 mm i.d) column is used. Mobile phase used are 0.1 % formic acid aqueous solution and formic acid acetonitrile at the flow rate of 1.2 mL /min, and sample injection volume is 5 µL at 40⁰ C temperatures.

e) Analysis of Catechins in Green Tea²⁷

Analysis of Catechins in Green Tea was done by using prominence UFLC. For this analysis Shim-pack XR-ODS (50 mm L x 2.0 mm i.d) column is used. Mobile phase used are 0.1 % formic acid aqueous solution and acetonitrile at the flow rate of 0.5 mL /min, and sample injection volume is 2 µL at 50⁰ C temperatures.

NANO LIQUID CHROMATOGRAPHY

Some definitions have been found in the literature based on column diameter and mobile phase flow rates²⁸⁻³⁰. 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^{31,32}.

Advantages over HPLC³³:

- ❖ 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

Applications of Nano LC:**a) Separation of sulfonamides³⁴**

Nano-liquid chromatography coupled with mass spectrometry was used for the simultaneous determination of 18 sulfonamides by utilizing a capillary column (100 µm I.D.) stationary phase, used is Kinetex (®) C (18) core-shell. A binary mobile phase, consisting of water and acetonitrile and both containing 0.1% (v/v) formic acid, was employed in a gradient mode at a low flow rate (190 nL /min).

b) Separation of peptides³⁵

An integrated multidimensional nano-flow liquid chromatography platform with the combination of protein and peptide separation via online digestion by an immobilized enzymatic reactor was established, and successfully applied for proteome analysis.

c) Discovery of Glycomics towards biomarker Using nano-LC³⁶

Nanoflow LC, or nano-LC, significantly provides a highly sensitive and quantitative method of separating and profiling glycans.

d) Nano-LC for glycoanalysis³⁷

Structural heterogeneity of glycoconjugates and glycans in biological matrices. C18, graphitized carbon and amide-based stationary phases were adapted to nanoflow level and on chip format, leading to improved sensitivity of

structural analysis and superior level of information on highly complex glycans and glycol conjugate mixtures

e) Other applications³³

- ❖ In the analysis of Biological and environmental samples: When small amounts of samples are available such as blood of infants, cerebrospinal fluid, hormones enzymes and xenobiotics at nano levels.
- ❖ High throughput screening (HTS) and drug discovery where the limits of detection are very low.
- ❖ Proteomic and genomic research.
- ❖ Detection of accumulated drug samples in the body.

CONCLUSION

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. 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. Columns with small internal diameters and / or short column lengths are more susceptible to extra-column band-broadening for high speed separation in UPLC. Ultra fast analysis means a significant enhancement in sample throughput (5-10times) & productivity compared to a conventional HPLC. Nano LC is the latest innovation in separation science in which detections can achieved at nano gram or lower levels.

ABBREVIATIONS

- HPLC-High performance liquid chromatography
- RRLC-Rapid Resolution Liquid chromatography
- UPLC-Ultra Performance Liquid chromatography
- UFLC-Ultra Fast Liquid chromatography
- Nano LC - Nano liquid chromatography
- MS - Mass Spectrometry
- LC - Liquid chromatography
- MS/MS - Tandem mass spectrometry

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