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ADVANCED STUDY OF NANO LIQUID CHROMATOGRAPHY AND ITS APPLICATION-A REVIEW

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ABSTRACT

Nano High performance liquid chromatography (Nano HPLC) is an important qualitative and quantitative technique, generally used for the estimation of pharmaceutical and biological samples. This article was prepared with an aim to review different aspects of HPLC, such as principle, types, instrumentation and application.]

KEYWORDS: Important qualitative, quantitative technique.

INTRODUCTION

Russian botanist Mikhail Semenovich Tswett, invented chromatography in 1903 during his research on plant pigments.[1, 2] Chromatography process can be defined as separation technique involving mass-transfer between stationary phase and mobile phase.[3] At present, chromatography is the backbone of separation science, and is being used in all research laboratories and pharmaceutical industries in the world, even chromatography technique's invention crossed 100 years.[1, 2] The reason behind the success of this technique are modernized and novelty. Different techniques are involved in chromatography based on modes of Chromatography, principle of separation, elution technique, Scale of operation and the type of analysis. Among those, High performance liquid chromatography (HPLC) is an established technique that has been used in laboratories universal over the past years. One of the key drivers for the growth of this technique has been the evolution of packing materials used to achieve the separation.[4] After a little modification in HPLC, in 1988 *Karlsson* and *Novotny* were introduced the Nano Liquid chromatography

(NanoLC) technique. The huge increase in miniaturized LC systems has been driven by biological applications and primarily proteomics research.[5] Several definitions have been found in the literature based on column diameter and mobile phase flow rates.[6-8] Table 1 expressed the definition of HPLC systems. Some workers defined NanoLC as chromatographic modality having a mobile phase flow rate at nano milliliters 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 a precise and scientific definition, i.e. a modality of chromatography involving samples in nano liters, mobile phase flow rates in nano milliliters per minute, with detection at nanograms per milliliter.[9, 10] Nano HPLC is also referred to as "*Nano-bore HPLC*" or *"Nano-scale HPLC"*.[11]

ADVANTAGES OVER THE CONVENTIONAL LC (due to miniaturization)

- Significantly reduces solvent consumption and consequent waste production.
- Inner diameter reduction increases sensitivity and/or less sample requirement.
- Decrease in column bead size (packing) narrower the peak width of chromatogram due to enhanced separation efficiency.
- Does not increase system pressure.
- Significantly cheaper than its conventional counterpart.
- Potential portability due to a system size reduction.
- Allows for simultaneous mass separation if LC chips are used.[5, 11]

Table1: Classification of HPLC systems[11]

Description	Internal diameter of Column	Flow Rate
Nano LC	10-100 µm	24-4000 nL/min
Capillary HPLC	100-100 µm	0.4-200 µL/min
Micro HPLC	1.0-2.1 mm	50-1000 µL/min
Normal HPLC	4.0-5.0 mm	1.0 -10.0 mL/min
Preparative HPLC	>10 mm	> 20 mL/min

PRINCIPLE

The fundamental principle of this advancement is governed by the van Deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or 1/column efficiency).

$$H = A + \frac{B}{u} + Cu$$

Whereas, A is Eddy's diffusion; B is longitudinal diffusion; C is Concentration and u is Linear Velocity.[12, 13] Nanoflow LC chromatographic separations are performed using flow rates in the range of low nano liter per minute, which result in high analytical sensitivity due to the large concentration efficiency afforded by this type of chromatography.[14]

THEORY

A reduction in column inner diameter results in a reduced amount of chromatographic dilution and, as a result, the concentration of the injected sample increased on the LC system. The chromatographic dilution (D) of the sample when injected on an LC system, is expressed by the following equation (1):

$$D = \frac{C_o}{C_{max}} = \frac{\varepsilon \pi d_c^2 (1+k)\sqrt{2\pi LH}}{4V_{inj}} \quad \text{----------} \quad 1$$

Where, ***Co*** is the initial compound concentration in a sample (before injection into the LC system); ***Cmax***is the final compound concentration at the peak maximum; ***ε***is the total porosity of the column; dc is the column internal diameter; **k** is the retention factor; ***L*** is the column length; ***H*** is the column plate height and $\mathbf{V_{inj}}$ is the sample volume injected.

D increases proportionally with the square of the column diameter and with the square root of the length of the column. The lower internal diameter in nano-LC promotes a high reduction in **D** value. Thus, downscaling of chromatographic systems means less chromatographic dilution, increasing the mass detectability of the separation **[5, 15].** The gain in sensitivity (*f*) resulting from the use of a LC column with a smaller internal diameter can be approximated by the following relation:

$$Concentration\ Factor\ (f) = (Diameter\ of\ Standard\ column | Diameter\ of\ narrow\ column)^2$$

$$=(4.6\ mm|0.075\ mm)^2 \sim 3800$$

Therefore, down scaling the column used in an analytical method inner diameter from 4.6 mm to 75 µm should result in an almost 3800-fold gain in sensitivity. However, such an increase in sensitivity is not readily achieved because reducing the column inner diameter has practical consequences for the entire setup.[15, 16]

The flow rate (F) in a column is given by

$$F = \frac{u\pi\varepsilon d_c^2}{4}$$

Where **u** is the linear velocity of the mobile phase. The reduction of **dc** leads to a large reduction in the flow rate of the mobile phase, decreasing solvent consumption and waste production in nano-LC separations.[5]

INSTRUMENTATION

Miniaturizing an LC system implies that all system components should be downscaled, including column, Pumps, connecting tubing, connections, injector, and the interface to the detector.

Pump

Nano-LC requires a pump system, which gives reproducible nano flow rates and stability during the separation, and permit gradient elution at nano-scale levels. It requires flow rates of 500 nL/min or less. Two primary systems can be used in nano-LC: split and split less pumps. Spilt systems can be divided into two groups: the passive split system and the active split system. In the passive split system, splitter divides the high flow of the pump between the column and restrictor. The passive split systems are simple and relatively inexpensive, but compromise on flow stability and accuracy. The active split systems have improved flow stability and exhibit better reproducibility than passive split systems, but still the majority of the mobile phase is wasted. The split less systems can be divided into two groups: the "solvent refill" systems and the "continuous flow" systems. Currently, split less systems are widely used in nano-LC. These systems prevent solvent losses and have more reproducible nano flow rates then the spilt systems.[5]

Columns

The commonly accepted standard internal diameter of nano LC columns is 75 µm. This column format provides a good compromise between sensitivity, loadability, and robustness. In the early days of nano LC, researchers had to manufacture their own columns because of the lack of commercially available columns. In the 1990s, the first nano LC columns became commercially available as a result of efficient packing procedures. Throughout the last two decades, the column offering has grown tremendously and nano LC columns are now available. Self-packed nano LC columns are sometimes used by research laboratories, often for economic.

Two types of columns are used in nano LC

1. Packed columns
2. Monolithic columns

Packed columns

The packed columns used in the nano LC columns are made of Polyimide-coated fused silica capillaries. Frits strong enough to withstand pressures are used to retain the stationary phase. The frit commonly used in standard-bore columns, a stainless steel mesh in a connection, will provide too much dead volume, thus impairing the separation efficiency. Therefore, frits in nano LC columns must be prepared inside the fused-silica capillary. Stationary phase particle sizes of nano LC columns are similar to standard HPLC columns: 5 µm, 3 µm, and now down to sub 2 µm.

Monolithic columns

In this type of column, a porous (silica or polymer) structure is formed throughout the column, eliminating the need for frits because the stationary phase is fixed to the column wall. Monolithic stationary phases are single rods of organic or inorganic material that are produced inside the capillary column. No frits are required with monolithic columns and the high porosity of these materials allows higher flow rates of mobile phase, reducing the separation time. These columns are predominantly used in proteomics for the analysis of extremely complex tryptic digests[15]. The chemistry available for stationary phases allows the applicability of nano-LC in a range of analyses. Reversed-phase, hydrophilic interaction chromatography (HILIC), chiral selection, size exclusion, ion exchange and other separation modes are applied to separations, according to the target analytes. Many research groups prefer to prepare their own nano columns specifically for their own purposes.[5] Current state-of-the-art nano LC columns are commercially available in lengths up to 50 cm to provide the separation power required for these complex proteomics samples.[15]

Injection

Typically, the sample is limited, so the injection system should ensure that no sample is lost to waste. Direct injection setups can be used in nano LC setups. The maximum injection volumes for nano columns can be expressed as a function of the column length, plate number, retention factor or some other parameters, and are generally a few nanolitres. Small injected volumes are a major problem in nano-LC, causing loss of detectability, but larger injected volumes produce a band broadening effect, decreasing the efficiency of the separation, especially for poorly retained compounds. Commercial auto-samplers, which usually work at microliter levels, require an instrument adjustment for use in the nanoliter range. This may be overcome by the use of a split valve between the injector and the column. The sample is

injected directly onto the column. Because there is only one column, there is a lower risk of losing analytes, making it popular in some proteomics laboratories. The drawbacks are the possible introduction of salts in the mass spectrometer, the low injection volume (up to 1 µL), and the lack of column protection. Different methodologies can be applied to overcome these problems with direct injection, such as off-line pre-concentration, large-volume injection directly into the separation column using specific injection conditions, and on-line pre-concentration (using a trap column). Off-line pre-concentration can be performed by reducing the sample volume by evaporating or lyophilizing the sample. A pipette-based desalting step can be also incorporated.

Detector

The detection techniques for nano-LC are the same as those employed for HPLC separations. Commonly used detection method in nano-LC is Diode array detection (DAD), because of its low cost, wide range of applicability and use of online detection. Laser induced fluorescence and inductively coupled plasma MS are also used in nano-LC detection, but these are not robust enough to be applied for routine analysis. Biomedical and pharmaceutical applications usually require a universal detection method, such as that provided by MS detection. The nano flow from the column (frequently, 100–500 nL/min) is adequate for MS coupling through various nanospray interfaces.[5] Among all the nanospray interfaces, nanoelectrospray ionization (nanoESI) provides an ideal interface to couple nano LC to MS. The requirements needed for the nanoESI are, obtaining a stable spray for flow rates of hundreds of nanoliters per minute and maintaining the separation efficiency. Typical nanoESI sprayers are made from silica capillaries that do not conduct electricity. The simplest and most economical method of applying voltage is through a liquid junction. An alternative to the liquid junction is to use coated (for example, gold) fused-silica emitters. This solution offers the advantage of eliminating the connection used in the liquid junction, but the metal coatings can deteriorate following electrical discharge. A common alternative is packing nanoESI sprayers with the stationary phase. This was initially performed for on-line concentration and desalting without aiming at separating species. Packing stationary phase in nanoESI emitters for separation purposes has the advantage of eliminating post column dead volume almost completely. Their use can be complicated by Joule heating when columns are very long because part of the voltage applied to form a spray is lost due to the mobile phase electric resistance. A relatively recent development in nano LC–MS is the introduction of chip-based structures. Chip-based systems aim at integrating the connections, columns, and

spray needle in one device to make installation and operation of the nano LC system easier. As a result of the integrated connections and spray needle, extra column volumes are greatly reduced.[15]

Micro fluidic chip method

The study of proteins and nucleic acids in proteomics and genomics requires effective analytical techniques like microchip based LC (Nano-LC). Lab-on-a-chip technology facilitates the performance of several different experimental tasks in combination with automated data analysis in one process on a single instrumental platform. The micro fabricated chip includes a column, frits/filters, an injector, and a detector, fabricated in a process compatible with those conventionally utilized to form integrated circuits. The column can be packed with supports for various different stationary phases to allow performance of different forms of nano-LC, including but not limited to reversed-phase, normal-phase, adsorption, size-exclusion, affinity, and ion chromatography. A cross-channel injector injects a nanolitre/picolitre-volume sample plug at the column inlet. An electrochemical/conductivity sensor integrated at the column outlet measures separation signals. A self-aligned channel-strengthening technique increases pressure rating of the microfluidic system, allowing it to withstand the high pressure normally used in high performance liquid chromatography (HPLC). On-chip sample injection, separation, and detection of mixture of anions in water is successfully demonstrated using ion-exchange nano-LC.[17]

APPLICATIONS

Numerous high sensitivity hyphenated (NanoLC coupled with spectroscopic methods) methods have been reported in the analysis of pharmaceutical and biological compounds at trace level. Nano-LC analyses are now applied for therapeutic and veterinary drugs, doping control, disease diagnosis and the quantitative determination of biomarkers and proteome identification.

Phospholipid determination in human urine

Hanna Kim *et al.*, (2008)[18] were separated and characterized various phospholipids (PL) molecules in human urine using Nano flow liquid chromatography-electro-spray ionization-tandem mass spectrometric (NanoLC-ESIMS-MS) method. MS detection of PL molecules was carried out in both positive and negative ion modes. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was detected by positive ion mode and for Phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), and

phosphatidylglycerol (PG), the negative ion mode was used. 22 PCs, 14 PEs, 15 PIs, 13 PSs, 7 PAs, and 4 PGs were identified during NanoLC-ESI-MS-MS when phospholipids in urine were extracted by ultracentrifugation.

Water analysis:

Steven Ray Wilson *et al., (2007)*[19] were described a method for quantitation of perfluorooctanoic acid (PFOA) and perfluorooctane sulphonate (PFOS) in surface water by using a combination of on-line solid-phase extraction, nano-liquid chromatography, and nano spray mass spectrometry. 0.1-mm i.d. × 150 mm, 3.5 µm Kromasil C_{18} analytical column is conducted using an acetonitrile–10 M ammonium acetate solvent gradient of 30% to 70% acetonitrile for the determination of PFOS and PFOA in urban river water.

Oligosachharide determination in ovarian tissue

Niclas G. Karlsson *et al.*, (2004)[20] were analyzed neutral and acidic O-linked and N-linked oligosaccharide alditols by Negative ion nano-liquid chromatography/mass spectrometry using graphitised carbon as separating medium. They were explored for Comparison to the sensitivity of capillary LC/MS (flow rate of 6mL/min) coupled with a conventional electrospray ionization source, the nano-LC/MS (flow rate of 0.6ml/min) with a nanoflow ion source was shown to increase the sensitivity tenfold with a detection limit in the low-femtomole range. The use of graphitised carbon nano-LC/MS enabled the detection of four sialylated O-linked oligosaccharides on membrane proteins from ovarian tissue.

Proteome application

Edgar Na gele *et al.*, (2003)[21] developed a method for the analysis of complex proteome samples by two-dimensional nano-liquid chromatography–mass spectrometry using a strong cation-exchange and a reversed-phase column in sequence. Xia S*et al.*, (2012)**[22]** developed an integrated multidimensional nano-flow liquid chromatography platform with the combination of protein and peptide separation via online digestion by an immobilized enzymatic reactor and the technique was successfully applied for proteome analysis.

Determination of abused drugs and metabolites in human hair

Kevin Y Zhu *et al.*, (2012)[23] determined the abused drugs and metabolites like cocaine, benzoylecgonine, cocaethylene, norcocaine, morphine, codeine, 6-acetyl morphine, phencyclidine, amphetamine, methamphetamine, and methadone in human hair using a micro fluidic chip based nano-HPLC coupled to tandem mass spectroscopy (nano-HPLC-

chip-MS/MS) simultaneously. The microfluidic chip was fabricated by laminating Polyimide films and its integrated an enrichment column, an analytical column and nano spray tip.

Separation of sulfonamides

Giovanni D Orazio *et al.*, (2012)[24] determined simultaneously 18 sulfonamides by Nano-liquid chromatography coupled with mass spectrometry utilizing a capillary column (Kinetex C18 core shell, 100 µm I.D.) 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).

Histamine determination

Yaru Song *et al*., (2004)[25] was determined histamine by using nano-liquid chromatographic /tandem mass spectrometric (nano HPLC/MS/MS). The method involved pre-column derivatization of histamine with 7-Fluoro-4-nitrobenzoxadiazole (NBD-F). Sodium 1-heptanesulfonate, added as an ion-pairing reagent in the mobile phase for sample loading, was found to facilitate greatly the analyte retention. This method was applied to determine histamine in water, alcoholic beverages and rat brain tissues.

Determination of Aflatoxins (AFs)in peanuts

Hsiang Yu Liu *et al.*, (2013)**[26]** were developed a microfluidic chip-based nano LC (chip-nano LC) coupled to a triple quadrupole mass spectrometer (QqQ-MS) for the sensitive determination of Aflatoxins (AFs) in peanuts and related products. In the developed method by Liu et al., Gradient elution and multiple reaction monitoring were utilized for chromatographic separation and MS measurements.

Determination of cyclotides

H Hashempour *et al*., (2011)[27] were developed a nano-LC Fourier transform mass spectrometry (FTMS) method for the analyzing cyclotides in plants like Viola ignobilis (Violaceae plant family). Cyclotides are macrocyclic knotted peptides originating from plants. Cyclotides are extremely stable and have a range of bioactivities including anti-HIV and insecticidal activity. Varv peptide A, cycloviolacin B2, and cycloviolacin O8 were found in this species.

Nano-LC for glycobioanalysis

Kirsch S et al., (2009)**[28]** developed a method for determination of glycol conjugates and glycans in biological matrices by adapting C18, graphitized carbon and amide-based

stationary phases to nano flow level and on chip format which leads to improve sensitivity of structural analysis.

Analysis of phenolic compounds in olive oil

Garcia Villalba R *et al.*, (2009)[29] developed a Nano LC-ESI-TOF MS for the analysis of phenolic compounds in olive oil. NanoLC analysis was carried out in a fused silica capillary column (75 µm id, effective length 10 µm, 3 µm particle size) packed with C18 stationary phase. The mobile phase was a mixture of water + 0.5% acetic acid and ACN eluting at 300 nL/min in a gradient mode. Phenolic compounds from different families were identified and quantified by using this method. Villalba et al., suggested that this technique useful for the analysis of samples containing low concentration of phenolic compounds, in biological samples.

Analysis of biogenic amines in wine

Hernandez Borges J *et al.*, (2007)[30] developed a simultaneous method for analysis of 10 biogenic amines (ethanolamine, methylamine, tryptamine, 2-phenylethylamine, putrescine, cadaverine, histamine, tyramine, spermidine and spermine) in wines by Nano-LC using UV detection. The 10 selected amines, which are the most important to be determined in wine samples, were derivatized with dansyl-chloride (Dns-Cl) previous to their nano-LC determination. The method was applied to the analysis of this group of amines in white and red wine samples.

Determination of ricin by Nano-LC/MS

Kanamori Kataoka M *et al.*, (2011)[31] developed a Nano-LC/MS method for determination of Ricin, which is a glycosylated proteinous toxin. In this method, lactose-immobilized monolithic silica extraction followed by tryptic digestion and liquid chromatography/mass spectrometry (LC/MS) was developed as a method for rapid and accurate determination of ricin. This method was applied to the determination of ricin from crude samples. It takes about 5 h for detection and identification of more than 8 ng/ml of ricin through the whole handling, and this procedure will be able to deal with the terrorism using chemical weapon.

CONCLUSION

We concluded that Nano LC has shown significantly superior to its conventional counter part by its solvent consumption, speed, sensitivity, resolution and its other advantages.

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