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NANO-LIQUID CHROMATOGRAPHY AND ITS PHARMACEUTICAL APPLICATIONS

SHEABA MARIAM SHAJI* [1] | MRS. RUBY PHILIP [2] | DR. ELESSY ABRAHAM [3]

[1] NAZARETH COLLEGE OF PHARMACY, OTHERA, THIRUVALLA.

[2] NAZARETH COLLEGE OF PHARMACY, OTHERA, THIRUVALLA.

[3] NAZARETH COLLEGE OF PHARMACY, OTHERA, THIRUVALLA.

ABSTRACT

Chromatography may be defined as a method of separating a mixture of components into individual components through equilibrium distribution between two phases. It is a non-destructive procedure for resolving a multi-component mixture of trace, minor or major constituents into its individual fractions. Different variations may be applied to solids, liquids and gases. Chromatography can be used for qualitative and quantitative analysis. Quantitative analysis can be carried out by measuring the area of the chromatographic peak. Nano-liquid chromatography (nano-LC), microchip devices and nano-capillary electrophoresis are miniaturized methods that minimize reagent consumption and waste generation. Nano-LC has great potential to become an indispensable tool for routine analysis of biomolecules.

Keywords:

INTRODUCTION

Chromatography is defined as a procedure by which solutes are separated by dynamic differential migration process in a system consisting of two phases, one which moves continuously in a given direction and in which the individual substance exhibit different mobilities by differences in adsorption, partition, solubility, vapour pressure, molecular size or ionic charge density. It is a non-destructive procedure for resolving a multi-component mixture of trace, minor or major constituents into its individual fractions. Different variations may be applied to solids, liquids and gases. Chromatography can be used for qualitative and quantitative analysis. Quantitative analysis can be carried out by measuring the area of the chromatographic peak.

Russian botanist Mikhail Semenovich Tswett, invented chromatography in 1903 during his research on plant pigments. Chromatography process can be defined as of separation science, and is being used in all research laboratories and pharmaceutical industries in the world, even separation technique involving mass-transfer between stationary phase and mobile phase. 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. 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 types of analysis. Among those, nano-LC is an established technique that has 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.

After a little modification in HPLC, in 1988 Karlsson and Novotny were introduced the nano liquid chromatography technique. The huge increase in miniaturized LC systems has been driven by biological applications and primarily proteomic research. Several definitions have been found in the literature based on column diameter and mobile phase flow rates. Some workers defined Nano-LC as chromatographic modality having a mobile phase flow rate at nano millilitres per minute. Miniaturized separation techniques have emerged as environmentally friendly alternatives to available separation methods. Nano-liquid chromatography (nano-LC), microchip devices and nano-capillary electrophoresis are miniaturized methods that minimize reagent consumption and waste generation. Furthermore, the low levels of analytes, especially in biological samples, promote the search for more highly sensitive techniques; coupled to mass spectroscopy, nano-LC has great potential to become an indispensable tool for routine analysis of biomolecules.

HISTORY

Chromatographic technique was first invented by M. Tswett, a botanist in 1906. In that year, he was successful in doing the separation of chlorophyll, xanthophylls and several other coloured substances by percolating vegetable extracts through a column of calcium carbonate. The calcium carbonate column acted as an adsorbent and the different substances got adsorbed to different extent and this give rise to coloured bands at different positions, on column.

In 1930's chromatography in the form of TLC and ion exchange chromatography was introduced as a separation

technique. In 1941, Martin and Synge introduced partition and paper chromatography. They introduced gas chromatography in 1952. During the next decade the routine use of chromatography as a separation technique become universal and has been extended to several areas of study, especially chemistry, biology and medicine. It is a corner stone of molecular analytical chemistry. Recently used in elemental analysis i.e, coupling with atomic absorption spectroscopy.

LIQUID CHROMATOGRAPHY

"Liquid chromatography (LC) is an analytical chromatographic technique that is useful for separating ions or molecules that are dissolved in a solvent. The mixture to be separated is loaded onto the top of the column followed by more solvent."

NANO LIQUID CHROMATOGRAPHY

"The authors suggested the definition based on the flow rate range rather than the inner diameter of the tubing or its material. They proposed that the **liquid chromatography** method that uses packed microcolumns with 10-150µm ID and flow rates of 10-1000 nL / min to be classified as **nano-liquid chromatography(nano-LC**).

PRINCIPLE

There is no agreement about the terminology of microscale LC. The terms "microbore", "microcolumn" and "capillary" LC are used interchangeably for microcolumns of different i.d. According to Chervet *et al*, separations performed using columns of 0.50-1.0 mm i.d. are described as micro LC; Columns of 100- 500µm i.d. are described as capillary-LC; Finally, separations using columns of 10-100µm i.d. are described as nano-LC. This classification includes separations in microchips because nano-HPLC columns on chips have 20- 100µm as the i.d.

In this work, the classification of nano-LC will be used for separations that operate at nanoliter flow rates, common for columns of 10- 100µm i.d.

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.

- ***Analytical instrumentation of nano-LC systems***
- Internal diameter of column= 10-100µm
- Flow rate = 24-4000 nL/ min

In nano-LC, the conventional instrumentation is all miniaturized. Pumps, connections. Columns, injection loop and detection interface are dimensioned for small volumes and low backpressure. These parameters can greatly influence the chromatographic efficiency of nano-LC and need to be controlled for a successful separation.

Pumps

Pumps for nano-LC needs to present reproducible nano flow rates and stability during the separation, and permit gradient elution at nano-scale levels. Two primary systems can be used in nano-LC: split and splitless pumps, the latter being commercially available.

Tubing and connections

Peak broadening is a significant limitation to nano-LC development. The dispersion of an analyte band in the column is described as a function of the i.d. and the length of the capillary.

Thus, the lower the i.d. and length, the lower the on-column dispersion contribution. Pre-column and post-column dead volumes may lead to significant band broadening, which is critical when using columns with reduced i.d. Inadequate tubing and connections increases band broadening, so that the use of short, tight connections made with low volume tubing is required to reduce this contribution to band broadening. Common connections are made of stainless steel or polyether ether ketone; (PEEK) the latter is especially useful foe fused silica capillaries

Injections

The maximum injection volume 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 nanoliters. 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 separation, especially for poorly retained compounds. However, Heron *et al.*

proved that, when using a weak solvent for the sample, there is an enrichment effect and a gain in efficiency, promoting the concentrations of a sample plug after injection into a stronger mobile phase.

Nano-columns

Although columns of 10 µm i.d. can be employed, nano-LC columns of 75µm i.d. are the most frequently used. This i.d. columns provides a good compromise between detectability, loadability and robustness in nano-LC separations.

DETECTION

The types of detection for nano-LC are the same as those employed for HPLC separations. Diode array detection (DAD) is commonly used in nano-LC, because of its low cost, wide range of applicability and use of online detection. However, due to the short path length of the nano column, detectability is limited when on-column detection is applied. This is overcome by the use of specially configured detection cells that provide longer light paths. 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.

APPLICATIONS

- **Phospholipid determination in human urine**

By using nano flow liquid chromatography-electro-spray ionization-tandem mass spectroscopic methods.

- **Water analysis**

Quantitation of perfluoro octanoic acid (PFOA) and perflouooctane sulphonate (PFOS) in surface water by using a combination of on-line solid-phase extraction, nano-liquid chromatography and nano spray mass spectrometry.

- **Oligosachharide determination in ovarian tissue**

Neutral and acidic O-linked and N-linked oligosaccharide alditols were analyzed by negative ion nano-liquid chromatography/ mass spectrometry using graphitised carbon as separating medium.

- **Proteome application**

Analysis of complex proteome samples by two-dimentional nano-liquid chromatography- mass spectrometry using a strong cation-exchange and a reverse-phase column in sequence.

- **Determination of abused drugs and metabolites in human hairs**

The abused drugs and metabolites like cocaine, benzoylergonine, amphetamine, cocaethylene, norcocaine, morphine, codeine, 6-acetyl morphine, phencyclidine, methamphetamine and methadone in human hair are determined using a micro fluidic chip based nano-HPLC coupled to tandem mass spectroscopy.

- **Separation of sulphonamides**

18 sulfonamides was determined by nano-liquid chromatography coupled with mass spectrometry utilizing a capillary column.

- **Histamine determination**

By using nano-liquid chromatographic/ tandem mass spectrometric methods.

- **Determination of Aflatoxins (AFs) in peanuts**

A microfluidic chip-based nano-LC coupled to a triple quadrupole mass spectrometer was developed for the sensitive determination of Aflatoxins in peanuts and related products.

- **Analysis of N-acetylserine lactones**

A simple method for the simultaneous, rapid and sensitive determination of N-acylhomoserine lactones signalling molecules in bacterial isolates, without prior sample preconcentration and with minimal sample cleanup, is presented.

- **Determination of cyclotides**

A nano-LC fourier transform mass spectrometry (FTMS) method was developed for analyzing cyclotides in plants like Viola ignobilis (Violaceae plant family). Cyclotides are extremely stable and have a range of bioactivities including anti-HIV and insecticidal activity.

- Used for the determination of glycol conjugates and glycans in biological matrices.
- Used for the determination of phenolic contents in olive oil.
- Analysis of biogenic amines in wine.
- Useful for the analysis of polar compounds like aminoacids, sugars, natural products etc.
- Separation of mixtures of drugs and chemicals or biological origin, plant extracts etc.
- Separation of carbohydrates, vitamins, antibiotics, proteins, alkaloids, glycosides, aminoacids etc.
- Identification of drugs.

DRUGS	MOBILE PHASE	DETECTING AGENT
ERYTHROMYCIN ESTOLATE	Isobutyl methyl Ketone	Nutrient agar containing bacillus pumilus.
GENTAMYCIN	Chloroform: methanol: Ammonia:water(10:5:3:2)	Ninhydrin in pyridine-acetone mixture.

- **Identification of impurities.**

DRUG	MOBILE PHASE	DETECTING AGENT

HYDROXO COBALAMIN	s-butyl alcohol: acetic acid: potassium cyanide	Elution and measurement of absorbance at 361 nm.

DRUG	MOBILE PHASE	DETECTING AGENT
PHENFORMIN HCL	Ethyl acetone: ethanol: water(6:3:1)	Potassium ferricyanide, sod.nitroprusside & NaOH.
ERGOTAMINE INJECTION	Chloroform: methanol	P-dimethyl amino benzaldehyde reagent.

- Identification of foreign substances in drugs.
- Analysis of metabolites of drugs in blood, urine etc.
- In the determination of indoles in whole urine and in study of barbiturates, hormones.
- Used in the study of inorganic metal salts and complex ions.
- Separation of cannabinoids (psychoactive drugs) from herbal medicines.
- Separation of antioxidants (tocopherols) from human plasma and commercial preparations.
- Separation of β-blockers from synthetic samples.
- Separation of flavanoids from human urine.
- Separation non-steroidal anti-inflammatory drugs and steroids from synthetic sample and pharmaceutical formulations.
- Separation of anti hypertensives from human plasma.

CONCLUSION

Today, the miniaturization of analytical instrumentation presents an important role in the development of analytical sciences, which is encouraged by studies in many different areas.

Methodologies for pharmaceutical and biomedical applications must be sensitive enough to detect and quantify biologically relevant substances present in minute quantities. Especially for these low-concentration substances, the employed techniques must have excellent detectability and unquestionable identification, as provided by nano-LC-MS and nano-LC-MS-MS hyphenations.

The principle limitation at the present to wider use of nano-LC is the high cost of the analytical instrumentation. However, the rapid development of new equipment is overcoming this limitation, expanding nano-LC to routine laboratories and industries.

The chemistry of commercially available columns for nano-LC is still a limiting factor compared to the many and versatile conventional LC columns, which cover a wide range of analytical possibilities. In the near future, however, nano-LC has the potential to reach a consolidated position in the analysis of biological molecules as a complement to electrophoresis and immunoassays.

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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