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Overview an Nano Liquid Chromatography

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ABSTRACT
Miniaturized separation technology is emerging as an environmentally friendly alternative to available separation processes. Nano-liquid chromatography (Nano-LC), microchip devices, and nanocapillary electrophoresis are miniaturized methods that minimize reagent consumption and waste generation. Additionally, low levels of analytes, especially in biological samples, will facilitate the search for more sensitive techniques. Combined with mass spectrometry, nano-LC has great potential to become an indispensable tool for the routine analysis ofbiomolecules. This brief overview introduces fundamental aspects of analytical nano-LC instruments, discusses practical considerations, and highlights key differences between miniaturized and conventional instruments. We discuss some theoretical aspects to better explain both the potential and the main limitations of nano-LC. Recent pharmaceutical and biomedical applications of this separation technique are also presented, demonstrating the satisfactory performance of complex matrices, especially proteomics analysis, achievedwith nano-LC.

I. INTRODUCTION

The development of miniaturized systems is nothing new. In the 1950s, the use of capillary columns for gas chromatography was introduced by his Golay (1) and Horva´th et al. used small inner diameter (id) columns for liquid chromatography (LC) separations (2) in the 1960s. The presently known nano-LC technique was first introduced by Karlsson and Novotny in1988 (3) to test very small id packed columns. Recently, the proliferation of miniaturized LC systems is primarily driven by biological applications in proteomics research. Mixtures of proteins or peptides need to be analyzed and the amount of sample available and the low concentrations of target analytes in the sample matrix are not compatible with conventional LC systems. Conventional high-performance liquid chromatography (HPLC) analysis is performed using columns from i.d. from 3.5 – 4.6mm. The typical flow rate for these analytical columns is

(I)mL/min. Columns with smaller dimensions (20–100 mm id) using flow rates of nanoliters per minute are called nanocolumns (4) and are used in nano-LC. Nano LC is an alternative to conventional LC and offers more options for chemical analysis. Virtually any sample analyzed with conventional LC can be analyzed with the miniaturized technique. In this context, capillary electrophoresis and capillary electrochromatography also complement and compete with nano-LC as miniaturized liquid phase separations. The development of nano- LC has been associated with many advantages that this technology has over conventional HPLC analysis. Some good pointsHere are the sidesA sharp reduction in the consumption of mobile and stationary phases containing toxic reagents.

(ii)small samples required; (iii) high efficiency separation while maintaining the same retention behavior; (iv) Easy coupling to mass spectrometry (MS). One of the most important advantages today is the reduction of waste generation following the principles of green chemistry (5–7). However, the analytical equipment used in nano-LC is still very expensive, limiting its widespread use. In addition, significant technical knowledge of nano- LC details is required to avoid experimental problems, especially those related to instrumentation. The number of publications focusing on nano-LC applications has increasedin recent years. However, neither theoretical nor instrumental aspects were reported in these publications. Therefore, in this review, we describe the main aspects of nano-LC technology and its recent pharmaceutical and biomedical applications,

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especially the most commonly used biological research areas. A major application of nano-LC, proteome analysis, is also introduced.

PRINCIPAL OF NANO LIQUID CHROMATOGRAPHY

There is no consensus on micro LC terminology. The terms "microbore," "microcolumn," and "capillary" LC are used interchangeably for microcolumns with different internal diameters. (The 8th). Correspondence sherbet and the like. (9), separations performed on 0.50–1.0 mm columns I would. Called micro LC. Columns with an inner diameter of 100 to 500 mm are called capillary LC. Finally, separate on columns with 10–100 mm id. Called nano-LC

THEROTICAL ASPECTS OF NANO-LC

During the chromatographic process, the injected analytes can become diluted within the column, altering the separation efficiency. This dilution event, called chromatographic dilution (D), is expressed as where Co is the initial concentration, Cmax is the final concentration of the analyte during the chromatography process, dc is the column inner diameter, l is the column porosity, L is the column length, Vinj is the sample injection volume, k is the H is the chromatographic parameter retention factor and plate height, respectively. According to the formula, D decreases proportionally as the square of the column diameter decreases. Compared to conventional HPLC, lower i.d. nano-LC facilitates a significant decrease in D-values. A smaller chromatographic system therefore means less chromatographic dilution, which improves the mass detectability of the separation (6). The flow rate (F) in the column is given by where u is the linear velocity of the mobile phase. Lowering dc significantly reduces the mobile phase flow rate, reducing solvent consumption and waste generation in nano-LC separations. Theoretically, miniaturization of LC systems is very beneficial for liquid phase separations. However, some practical separation issues must be considered as they contribute to reduced separation efficiency.

Analytical instrumentation of nano-LC systems

1. PumpPumps for nano-LC should have reproducible nano-flow rates and stability during separations and enable nano-scale gradient elution. Two main systems are available for the Nano LC: There are split pumps and splitless pumps, the latter being commercially available. Split systems use a flow restrictor between the pump and a miniaturized column to separate high flow rates (mL/min) from conventional HPLC pumps. These systems can use standard HPLC pumps with a simply designed nano flow restrictor (10). However, split systems can lead to variable split ratios, poor nanoflow reproducibility, and poor separation reproducibility (10). Reproducible gradient elution is very difficult to achieve, especially with homemade split devices. Different mixed solvent viscosities can lead to variations in backpressure, limiting this mode of elution (11). Currently, splitless systems are widely used in nano LC. These systems eliminate solvent loss and deliver highly reproducible nanoflow rates. Syringe pumps using a single limited-volume reservoir are superior to split systems, but continuous-flow pumps, similar to conventional piston pumps with two pistons per channel, are currently the most common. Pump model. Continuous-flow pumps can be used for both isocratic and gradient nanoflow elutions, and tuning to the desired nanoflow rate is easily achieved.

2. Tubing & ConnectionsPeak broadening is a significant limitation of nano-LC development. The variance of the analyte band within the column is calculated as a function of the inner diameter. And capillary length (7, 12). Therefore, the lower the ID and length, the lower the variance contribution of the column (12). Pre- and post-column dead volumes can lead to significant band broadening, which is important when using small id columns. Poor tubing and connections increase band broadening, so short, tight connections with small amounts of tubing should be used to reduce their contribution to band broadening (7). Common connectors are made of stainless steel or polyetheretherketone. (PEEK) The latter is particularly useful for fused silica capillaries. Gaps formed by imperfect connections can also promote reduced separation resolution. Noga et al. (13) reviewed some common limitations in nano-LC separations. This includes connectivity issues and their practical solutions. They performed a bovine serum albumin (BSA) digest separation and compared missing and sufficient compounds. Figure 1 shows the effect of dead volume on the quality of chromatographic separations. According to the authors, poor fitting within the system resulted in severe mixing within the system and peaks werenot detected until 35 minutes.

3.InjectionThe maximum injection volume for nanocolumns can be expressed as a function of column length, number of plates, retention factor, or other parameters, and is typically a few nanoliters (9). Small injection volumes are a major problem in nano-LC, causing loss of detectability, whereas large injection volumes produce band-broadening

effects that reduce separation efficiency, especially for poorly retained compounds. Heron et al. Using a weaker sample solvent has been demonstrated to have a concentration effect and increased efficiency that facilitates concentration of the sample plug after injection into a stronger mobile phase (14). Commercial autosamplers that typically operate in the microliter range require device adjustments for use in the nanoliter range. This can be resolved by using a split valve betweenthe injector and column (9).

Nano-Columns

Nano LC columns with 10 mm id but 75 mm id can be used. Is most commonly used. This ID column offers a good compromise between detectability, loadability, and robustness in nano-LC separations (10). Nano LC columns are generally made of polyimide-coated fused silica capillaries, featuring flexibility, high mechanical strength, and varying internal dimensions, although stainless steel and titanium tubing are also used in nano columns. Will be It can be filled with silica-based particles, filled with monolithic beds or, less commonly, walls coated with suitable organic or inorganic materials. The most common particle size for filled nanopillars is 3–5 mm. Particle-filled small-diameter row Difficult to prepare. A retaining fritis required to prevent leakage of the stationary phase. Fabrication of frits is also poorly reproducible and often inefficient (15). An uneven bed after packing also degrades chromatographic performance (15). Monolithic stationary phases are individual rods of organicor inorganic material that occur within capillary columns. No frit is required for monolithic columns. In addition, the high porosity of these materials results in higher mobile phase flow rates and shorter separation times (16). Monoliths can be made organically or inorganically using a variety of synthetic routes, and biocompatible materials are an interesting alternative in biospecific analyses (17). Dolmann et al. (18) We compared the performance of packed and monolithic stationary phases for BSA separations and evaluated carryover effects on different capillary columns (Figure 2). BSA was chosen as a model for a typical proteomics sample because it contains both hydrophilic and hydrophobic peptides. Better separation efficiency for 11 peptides is attributed to the monolithic silica column, which is very similar to the efficiency of the 2.7 mm fused core packed silica column. Carryover effects, which can affect peptide analysis, were less with the fused core 2.7 mm silica column than with the polymer monolith. The chemistries available for theStationary phase make nano-LC applicable to a wide variety of analyses. Reversed-phase, hydrophilic interaction chromatography (HILIC), chiral selection, size exclusion, ion exchange, and other separation modes are applied for separation depending on the analytes of interest. Many research groups prefer to fabricate their own nanopillars for specific purposes. A chiral stationary phase for nano-LC was reported by Fanelli et al. developed. (19). In this study, silica particles coated with cellulose tris (3-chloro-4-methylphenylcarbamate) were used to separate six of his neutral drugs, including the teratogenic drug thalidomide. Separation of the enantiomers using the 100 mm id column was achieved in less than 10 minutes and was particularly good for the separation of thalidomide, which cannot be sold as a racemic mixture.

Detection

Nano-LC detection types are the same as HPLC separations. Diode array detection (DAD) is widely used in nano-LC due to its low cost, wide application range, and use of online detection. However, the short optical path length of nanocolumns limits the detectability when applying on-column detection. This is overcome by using a specially configured detection cell that provides a longer optical path (11). Laser-induced fluorescence (20) and inductively coupled plasma MS (21) are also used for nano-LC detection, but these are not robust enough for routine analysis. Biomedical and pharmaceutical applications typically require good detectability and universal detection methods such as MS detection. Nanoflow from the column (often 100-500 nL/min) is sufficient for MS coupling through various nanospray interfaces, especially electrospray ionization (ESI), requiring only a small amount of eluent from the LC column and not because you can succeed (22).

Enrichment in Nano liquid Chromatography

Theoretically, nano-LC allows for greater concentration of analytes than conventional HPLC. Decreasing ID columns reduce chromatographic dilution, resulting in increased immediate concentration of injected analytes as they pass through LC instrument components. This concentration factor is due to the lower dilution factor and is proportional to the square of the column radius and the amount of analyte injected, as reported by Rieux et al. (Ten). Smaller column radii result in lower dilution ratios and lower analyte detectability. According to Cutillas (4), an analyte concentration factor of 5,000 is obtained compared

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to using a 4.6 mm ID column with a 75 mm id. However, this enrichment factor is not easily achievable. B. Excessive connector tubing, connector dead volume, nanoflow turbulence. Several experimental observations have shown that the small amount of injected sample reduces the detectability of nano-LC compared to conventional HPLC, especially when using ultraviolet (UV) detection (11). Applying MS detection, multidimensional (nano) LC, or on-column traps greatly improves detectability in nanoLC

Hyphenation in Nano liquid Chromatography

MS is the most common nano-LC separation. The coupling of nano-LC with MS or tandem mass spectrometry (MS-MS) has been applied in a variety of fields to solve a variety of analytical science problems. For example, nano-LC separation combined with online MS (or, rarely, offline MS) has improved the diagnosis and treatment of several human diseases and improved quality of life (23– 25). Nanocolumns are also useful in combination with secondary separation techniques to enable two-dimensional (2D) chromatography systems. Luo et al. (26) proposed a 2D separation for complex proteomic analysis of cervical cancer cells using strong cation exchange and wall-coated reversed-phase nanopillars combined with MS detection. The authors concluded that the orthogonality of the open tubular column improved the separation performance compared to the one-dimensional reversed-phase. Separation can also be applied using two miniaturization schemes, such as biological microanalytical systems (27) or separate (orthogonal) nanocolumns (26, 28) coupled in the second dimension of 2D separations. I can do it. However, few reports of this type of hyphenation have been published so far. This is probably due to equipment limitations.

Recent Pharmaceutical and Biomedical Applications of Nano-LC

Molecules of biological interest need to be identified quickly with reliable results. In this regard, recent advances in analytical tools and sample preparation methods have advanced biological analysis to identify these molecules of interest. Nano-LC analysis is currently used for therapeutic and veterinary drugs, doping control, disease diagnosis, biomarker quantification, and proteome identification. The latter is the main field of application mainly due to the very small amount of sample required. Proteomic research Arguably, proteomics research is a major application of nano-LC separations (29–34). Protein sequencing of complex biological samples is required for biomarker identification, disease control, and clinical therapy, primarily from plasma and tissue samples. HPLC-based methods overcome classic problems of protein analysis such as gel electrophoresis and immunoassays. Both are limited by some pre- analytical steps. Diversity in proteome complexity demands speed and uncontroversy Identification techniques facilitated by the advent of nano-LC in combination with MS and MS-MS. These allowed the accurate determination of the amino acid sequence of proteins or peptides, supported by a complete identification database. However, conventional methods are still used in nano LC-MS. This is because much of the information for protein sequencing and peptide mapping is obtained by combining two or more identification strategies.Without proper diagnosis and treatment, periodontitis can lead to acute tooth loss and systemic complications. Choye et al. (35) proposed to identify proteins in gingival samples from healthy and periodontal patients while searching for specific biomarkers for this inflammatory disease. Or The authors used nano LC-MS-MS for proteomics analysis and immunoassays for test confirmation. 305 proteins identified In both sick and healthy patients, of which he had 45 directly related periodontitis. Azurocidin was chosen as the best biomarker and its levels were significantly increased in periodontitis In these cases, patients inhibiting bone differentiation. The main conclusion of this study was to propose an early diagnosis of periodontitis by direct measurement of azurocidin. Enrich complex oral samples with nano LC-MS-MS to prevent complications from untreated disease. Proteomic analysis of synovial fluid from rheumatoid arthritis patients was performed using nano LC-MS-MS (36). Both osteoarthritis and rheumatoid arthritis are devastating joint diseases characterized by the gradual deteriorationof joints. Cartilage by defense cells, followed by inflammatory disease. Mateo et al. (36) Identification of Related Peptides Both in joint disease and other peptides, each is exclusive. Knowledge of the proteome from synovial fluid has been critical to identify protein fractions that serve as biomarkers and facilitate efficient clinical control of patient treatment. Table I shows other proteomic analyzes performed by nano-LC reported in the last two years.

1.Biomarkers

Biomarkers are defined as endogenous indicators of a particular biological state, usually

peptides or carbohydrates. They are experimentally measured and can be evaluated for normal or disturbed processes. In biomedicine, biomarkers are specifically associated with health or disease states. Biomarker It can also be a substance introduced into an organism to assess normal or abnormal function (44, 45). Nano-LC plays an important role in biomarker analysis. The low analyte concentrations from biological samples require highly sensitive separation techniques, and nano-LC combined with MS or MS-MS easily demonstrates this property.Garcı´a-Villalba et al. (46) nano-LC-MS was used to assess polyphenol metabolism in human breast cancer cells. Polyphenols are found in extra virgin olive oil and their metabolites have been shown to have antitumor activity. The authors quantified polyphenol metabolites according to their uptake time by cancer cells and concluded that these biomarkers can be readily measured by nano LC-MS. A search for brain injury biomarkers in cerebrospinal fluid was performed by Sjo¨din et al. (47). You measured some A protein that can indicate the extent of brain trauma after the post-traumatic period by nano- LC-MS-MS. To prevent protein degradation The autosampler was kept at 108°C. biomarker Enriched and quantified over a wide dynamic range using commercially available ligands. However, the chromatographic run time was too long, even with gradient elution. This was probably due to strong interactions between the stationary phase and protein analytes.

2.General drugs

LC is established as an analytical tool in pharmacy Targets in different matrices. Validated LC methods from drug discovery to pharmaceutical formulation quality assurance It is used in the pharmaceutical industry, R&D centers, and for residue analysis in wastewater (49). Nano LC can be used as an alternative to regular LC, but the penetration rate of this new technology is currently low. The reason for this is the high initial acquisition cost. However The use of nano- LC is gradually increasing due to the apparent decrease in the amount of solvent required and the associated waste. Disposal costs. Shay et al. (50) identified eight common penicillin dosages in pharmaceuticals. The authors also measured these active ingredients in milk and tissue samples, proving the applicability of this method. In various biological matrices. C18 packed columns were manufactured with high reproducibility of separation replicates. Various polymer frits were evaluated to pack the column and a polystyrene-based frit was chosen as the superior one. Separation resolution better than other polymer frits tested. We compared the performanceof nano-LC using UV and MS detection for the simultaneous measurement of penicillin compounds (Figure 3). As expected, the limits of detection (LOD) and limits of quantification (LOQ) were higher for UV than for ion trap MS, but peak area reproducibility for MS was lower than for UV detection. Method validation using both detectors was performed and penicillin residues were found in several commercially available tissue samples.

3.Forensic analysis

Analysis of toxic substances and their metabolites in wastewater You can determine population access to these substances and public health requirements to control them. Steroid hormones, hallucinogens, cannabinoids, opioids, and various prescription drugs are listed as commonly used drugs by the US National Institute on Drug Abuse (58). Urine, sweat, blood (plasma), and saliva can be tested for current drug use. However, due to the need for non-invasive sampling, hair seems to be the sample of choice. Compared to Hair samples are much less likely to be contaminated and provide information over a longer detection period compared to other samples. History of substance abuse, if any (59). Hair samples from detox center patients were collected for analysis of cocaine, amphetamines, and morphine. And related drugs (59). The authors developed a simple and validated nano-LC method as an alternative to inconclusive immunoassay techniques using dedicated nanochip LC instruments. We also significantly reduced the sample preparation steps and required sample volume (less than 10% of typical volume). Despite being an excellent surveillance tool, nano-LC is not typically used to identify and measure drugs of abuse. Probably due to the lack of nano LC devices. Routine analytical lab. Gas chromatography and conventional LC are the most important instrument choices due to their properties. While ubiquitous in forensic centers, immunoassay testing is the most common analytical strategy for first drug Detection in biological samples because it is quick and easy to implement.

4.Enzymes

Nano-LC has not yet been widely used for enzymatic analysis (60, 61). In many cases, nano-LC stationary phases change the conformation of enzymes, reducing their catalytic activity (62). Other miniaturization techniques such as capillary electrophoresis (CE) It is preferred over nano-LC as it does not promote changes in the actual

morphology of the enzyme. However, the reproducibility The higher CE for Nano LC (63) is likely due to the more stable pressurized flow than the generated electroosmotic flow. In CE capillaries. Krˇı´zˇek and Kubı´cˇkova´ (63) reviewed state-of-the-art methods for kinetic enzyme assays and showed that CE and its isolation methods are widely used for enzymatic analysis, whereas nano-LC , only used in a few papers. How to Overcome the Limitations of Enzyme Analysis Nano LC uses bioaffinity columns. These special microparticle or monolithic stationary phases immobilize enzymes in an accessible conformation without significant loss of original enzymatic activity (64). According to Tetala and van Beek (65), bioaffinity columns for nano-LC can be easily made from organic or inorganic materials. It can be used not only for enzymes, but also for other biomolecular analyzes involving immobilized enzymes.

II. Conclusion And Outlook

Today, miniaturization of analytical instruments plays an important role in the development of analytical science, and analytical science is being researched in various fields. Pharmaceutical and biomedical applications Sufficient sensitivity is required to detect and quantify biologically relevant substances present in trace amounts. Especially for these low concentration substances, the technique used should have good detectability and unambiguous discrimination as provided by nano-LC–MS and nano-LC–. MS-MS separation. A major current limitation to the widespread use of nano-LC is the high cost of analytical equipment. However Rapid development of new devices is overcoming this limitation and extending nano-LC into routine laboratory and industry. Also, the chemistries of commercially available columns for nano-LC remain a limiting factor compared to many versatile columns. A conventional LC column that covers a wide range of analytical possibilities. Stationary phase preparation, with a focus on novel nanocolumns such as monolithic and sub-2 mm particle separation columns, is still a nascent area of development. Owever, nano-LC has that potential in the near future. As a complement to electrophoresis and immunoassays, it has established a strong position in the analysis of biomolecules.

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