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R E V I E W A R T I C L E

Review on Advancements of LC-NMR

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A B S T R A C T

High performance LC-NMR is an advanced analytical technique for the identification of components in pharmaceutical and biological mixtures without the need of chemical separation. The advantages of this technique NMR directly coupling with HPLC instrumentation must be weighed against compromises in performance made to each technique to achieve a hyphenated system. The coupling of LC separation with NMR characterization has been employed in a wide range of applications. The implementation of mass spectrometry in LC–NMR is also useful on account of the molecular weight and fragmentation information that it provides, especially when new plant species are studied. The present review describes that history and development of LC—NMR, Instrumentation along with applications. LC-NMR was also coupled with other techniques such as two-dimensional NMR measurements (LC-2D NMR), Mass spectroscopy or MS (LC-NMR/MS) and solid phase extraction or SPE (LC-SPE-NMR) to give rise to new range of separation and characterization of applications have a high sensitivity.

Keywords: LC-NMR, HPLC, Chemical separation, Instrumentation, LC-2D NMR, LC-NMR/MS, LC-SPE-NMR

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C O N T E N T S

1. Introduction

The chromatographic techniques and the spectrometric methods are combined online, they are termed as the hyphenated techniques. LC-NM, is a hyphenated technique which is the combination of the high performance liquid chromatography (LC) and nuclear magnetic resonance (NMR) spectrometers. NMR and MS techniques provide unequivocal structural information for the individually isolated compounds. The traditional way of studying natural

products includes fractionation of a crude mixture or extract, separation and isolation of the individual components using liquid chromatography and structure elucidation using various spectroscopic methods (UV, IR, NMR, MS)[1,2]. In spite of the fact that this approach is time-consuming and technically demanding, both LC and NMR have been and are still routinely used in mixture analysis. In theory, the physical coupling of LC with NMR could save a lot of time and was already proposed over 20 years ago. However, a successful and practical LC–NMR coupling has been achieved only in the last decade[3].

Advantages of LC-NMR

- The information that is provided by this techniques are orthogonal to each other which means they works very differently without interfering with other techniques i.e. LC methods is helping in separation of the complex mixtures whereas NMR helps in determination of the structure (through different experiments).
- The NMR can determine whether the peak is for pure compound or impure compound.
- NMR data can be taken without complete separation of mixture.
- It is non-destructive technique.
- Sample can be stored for analysis by another method.

Disadvantages of LC-NMR

- This technique is involving high costs.
- Capital also include high equipment costs,
- Longer time required for experimental works.
- It also include the use of deuterated solvents (partial use).
- Skilled professionals required therefore operator training requirements.
- Difficulty in solvent selection.

History of LC-NMR

In spite of the fact known that this approach is time consuming and technically demanding, both the LC and NMR have been and are still routinely used in the mixture analysis. In theory, the physical coupling of LC and NMR could save a lot of time and was proposed over 30 years ago. Even then the successful and applicable coupling of LC-NMR was achieved in past three decade. The first on-line LC-NMR experiments were performed in late 1970s by Watanabe and Niki who demonstrated stopped-flow measurements of mixture of known compounds. The conventionally used NMR probes was converted to the flow-through probe by the use of the thin-walled Teflon capillary within a standard NMR tube and spectra were recorded with sample rotation[4]. The first real sample to be analyzed by LC-NMR technique was a military jet fuel using the normal phase columns and deuterated chloroform and Freon. After the advances made the combination of LC-NMR was made. LC-NMR and LC-MS are considered to be the most valuable techniques for the structure elucidation of theunknown compound in wide field of application[6]. This technique is essential for analysis of products obtained from natural sources because, various closely related substances are present in their extracts, which are difficult to separate. It is important to note that substances derived from plant origin are almost containing 40 % of newly registered compound present in the drug discovery program. Thus, there is the need for development of new innovative technique that can describe the profile of each and every component of complex mixture and that to in a very simple way as well as fast procedure, this has become a challenge and this is to be looked forward into.Recently, there are various LC-NMR system available, and data acquisition can be accommodated with the help of different modes depending on the status of the sample during investigation[7,8].

2. LC-NMR Coupling

The proper learning and developing skills of a conventional NMR spectrum necessitates the dissolving of the sample to be tested in the deuterated solvent. This sample solution is introduced in a cylindrical sample tube and placed in a conventional NMR probe within the NMR magnet. As already described, that it requires a probe that must be modified to allow the continuous flow of the solution that is under study. The LC-NMR coupling technique should involve the appropriate interface of LC and NMR, flow through sampling probe design and many other factors such as solvent suppression, NMR sensitivity, LC and NMR compatible solvents and volume of chromatographic peak verses the volume of the NMR flow cell[9,10].

Modes of LC-NMR

Different modes of operations for LC-NMR are used which can be distinguished based on the status of the samples during measurement[11].

Continuous Flow(on flow): Eluent sampled in “real time” as flowing through NMR Detection Coil.

Stopped Flow: Pump is stopped at desired location and data acquired.

Time Slices: Regions, or “time-slices” of interest are analyzed

Peak Parking: Peaks of interest are “parked” in off-line sample loops

Peak Trapping: Solid Phase Extraction cartridges are used to “re-concentrate” samples

Continuous Flow(on flow):

In this mode, the NMR spectrometer acts similar to a UV- or MS detector in a chromatographic system since the sample is measured without stopping the flow. The result is typically displayed as a two-dimensional (2D) time–frequency plot consisting of a set of one-dimensional spectra (frequency domain) versus retention time, similar to an LC–DAD (liquid chromatography-diode array detection) plot The optimum flow rate for continuous-flow NMR is usually chosen as a compromise between the rate required for the best chromatographic resolution and the best NMR sensitivity. The measurement time for each analyte is limited by the short residence time within the rf coil at the flow rates normally used, and this often results in a poor S/N ratio for the NMR spectra. Reduction of the flow rate by a factor of 3–10 increases the residence time and hence the measurement time and S/N for each component, but diffusion at slow flow rates may reduce the chromatographic separation of the individual peaks eluting from the LC column or the NMR flow-cell. In spite of that,

on-flow measurements with very low flow rates (0.05 ml/min) have been described and preformed as overnight experiments, allowing a number of up to 128 scans to be recorded per spectrum[12.13].

Stopped Flow method: The outlet of the LC-detector is connected directly to the NMR probe. A LC-detector (normally UV) is used to detect peaks eluting from the columnWhen a peak is detected, the flow continues until the peak arrives in the NMR cell. At this time, the chromatography (pump, data acquisition, gradient) stops and the NMR experiments are performed. Once the NMR experiments are completed, the chromatography resumes until the next peak is found. This process can be repeated several times within one chromatogram[14].

Time slice method:

It include to stop the flow at short interval over the chromatography peak to time slice different part of chromatography run. It is useful if there is poor chromatography separation or if compound under study have poor or no UV chromophore or if the exact chromatography retention time is unknown. The data from such a time slice experiment referred as a total NMR chromatogram (tNMRc).

Peak Parking method:

The outlet of the LC-detector is connected to the sample loops of the BPSU-36 or BPSU-12. A LC-detector (normally UV) is used to detect peaks eluting from the column. A detected peak is moved into one of the sample loops without interrupting the chromatography. When the chromatography is completed, the HPLC pump is used to transfer the peaks from the loops into the NMR probe[15].

Peak trapping method: The outlet of the LC-detector is connected to the SPE unit. A LC-detector (normally UV) is used to detect peaks eluting from the column. A detected peak is moved trapped on a SPE cartridge without interrupting the chromatography. When the chromatography is completed, the chromatography solvents are removed and the peak is transfer with fully deuterated solvents into the NMR probe[16].

Instrumentation of LC-NMR

Principle: That combines high-performance liquid chromatography (HPLC) and nuclear magnetic resonance spectrometers (NMR), they have been applied widely in the analysis of complex mixtures that contain unknown components, such as impurities and metabolites in pharmaceuticals, natural products and synthetic polymers, ever since they were first reported in 1978(5)[19].

Instrumentation:

The common online LC-NMR system consists out of a standard LC-device connected to a NMR-detection device where a flow probe is inserted. The LC device consists out of a pump system that pushes liquid solvent through the system, a column where the separation takes place and a detector with flow cell that utilizes light to measure the components as they elute. This light-detector can either be an Ultraviolet-/Visible light (UV/VIS)-detector, a refractive index (RI)-detector or Infrared light (IR) detector. Most common nowadays is the use of Diode Array Detector (DAD), a type of UV/VIS-detector that can measure multiple light wavelengths at once. Basically, any type of detector can be used, as long as it does not alter or destroy the sample. The NMR system consists out of a huge radiofrequency (RF) - magnet where a non-rotating flow cell has been put oriented vertically. This orientation allows for laminar flow and gets rid of bubbles in the mobile phase easily. The RF coil is wrapped around the cell so that a good filling factor is obtained and the difference in detection volume and coil volume is only the glass that comprises the flow cell[20].

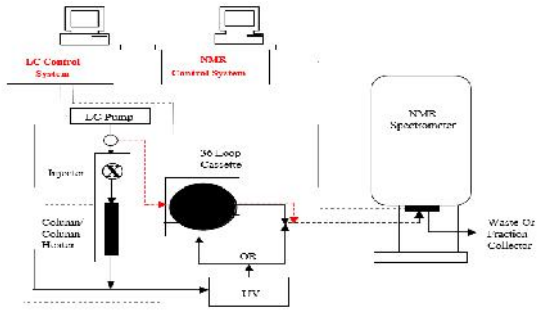

Fig 1: Schematic representation of a LC-NMR system

Other methods in LC-NMR

LC-NMR was also coupled with other techniques such as two-dimensional NMR measurements (LC-2D NMR), Mass spectroscopy or MS(LC-NMR/MS) and solid phase extraction or SPE (LC-SPE-NMR) to give rise to new range of separation and characterization of applications have a high sensitivity[21].

LC-2D NMR (two-dimensional NMR measurements using LC-NMR)

There are many compounds, such as heterocycles and ring-fused compounds, with a small number of protons that causes a less connected spin system. Two-dimensional NMR (HSQC, HMBC, etc.) spectra, which give observations of 1H-13C correlations are useful for analysis of these compounds. HSQC spectra are a method for observing correlation between carbons (1JCH) that directly bond protons to protons, and HMBC spectra for observing correlation between carbons (2JCH and 3JCH) that are more remote. The carbon shift information and bonding information that is obtained is extremely useful for structural analysis. In particular, since correlation is observed even when sandwiching a heteroatom, HMBC is used for connecting partial structures to each other. However, these methods have lower sensitivity than 1H NMR, so they have not been popular in LC-NMR[21].

LC–NMR/MS

This technology takes advantage of the rapid and sensitive screening capabilities of MS, which can pinpoint analyte peaks of interest in complex mixtures for further structural analysis by NMR spectroscopy. The most common way of interfacing LC to both MS and NMR is the parallel mode, where the eluent is split to give two parallel flows. The balance between the two split flows can be adjusted using a splitter. Since NMR is less sensitive compared to MS, a typical splitratiois 95:5for NMR vs MS. The LC–NMR/MS technique can be used in both on-flow and static conditions; although in the latter case the mass spectrometer would be idle most of the time while waiting for NMR data

acquisition to finish. Because of the high sensitivity and rapid scanning abilities of the mass spectrometer, sometimes MS data can be acquired on-line during the chromatographic run. The first application of LC–NMR/MS in the field of natural products was presented in 1999.44 Since then, this new, extended hyphenation has been used several more times in studying plant metabolites, and it was soon realized that the combination of the NMR and MS might be the most powerful analytical tool in plant-products analysis. Parallel to the necessary hard ware developments, a variety of auto mation procedures and software packages have become available for LC–NMR and LC–NMR/MS, allowing such analyses to be performed in a convenient, reproducible and precise manner[22,23].

LC–SPE–NMR

To further increase LC–NMR detection sensitivity one can concentrate the samples of interest, to achieve the highest possible analyte concentration in a minimum volume, and employ small-volume NMR rf coils. Solid-phase extraction (SPE) is a powerful technique for reproducible, rapid and selective sample preparation. There are a number of studies on on-line coupling of SPE with LC–UV, LC–MS and LC–NMR aiming at concentrating the sample before the separation. Alternatively, the use of a guard column after the LC separation to concentrate the eluted compounds prior to NMR analysis,54 as well as the use of a disposable SPE cartridge connected to an NMR flow probe55 have been described in order to increase the sensitivity of LC–NMR. The first automated on-line LC–SPE–NMR measurements by our group have been carried out on an extract of Greek Oregano.

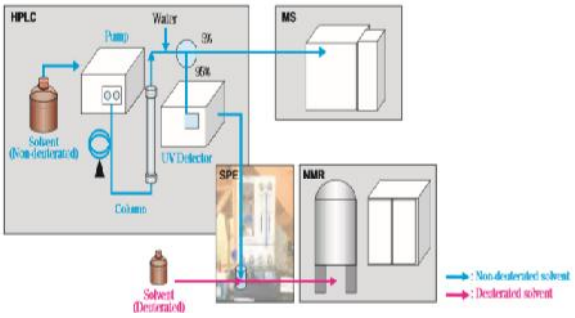

Fig 2: Schematic diagram of LC-SPE-NMR/MS

The separated peaks were diluted post column with water, and trapped automatically on SPE cartridges. A drying step with nitrogen followed, to remove all solvents that were used in the chromatographic separation. Finally, the analytes were transferred with the deuterated solvent of choice (e.g. methanol, acetonitrile or chloroform) to the NMR flow-cell probe and spectra were acquired. Online LC–SPE–NMR for 'peak parking' allows the use of normal protonated solvents for the LC run and thus the need for solvent suppression is strongly reduced or even no longer necessary. Potentially, the LC–SPE–NMR coupling provides an increase in sensitivity relative to conventional LC–NMR. Extension of the technique to LC–SPE–NMR/MS could lead to the unequivocal assignment of a complex mixture. Since chromatography is performed with standard LC solvents, the interpretation of the maas information becomes easier. Moreover, multiple trapping of the same analyte from repeated LC injections to the same cartridge can be utilized to solve the problem of low concentration. LC–SPE–NMR has recently been used to characterize a paracetamol metabolite present in human urine, and to identify antioxidants in a commercial rosemary extract and extracts of Rhaponticum carthamoides[22,23].

3. Applications of LC-NMR

- Separation and characterization of peptide libraries
- Combinatorial chemistry, phytochemical analysis, drug discovery
- Identification of drug impurities
- Characterization of isomers of acid glucoronides and vitamin A derivatives
- Characterization of endogeneous and xenobiotics metabolites directly from biological fluid.
- Analysis of ring fused and heterocyclic compounds that have a small number of protons - the LC- 2D- NMR technique provides carbon shift and bonding information for these compounds, which is very useful for analysis of their structure.
- Polymer analysis
- LC-NMR allowed the differentiation of isomers and identification without reference compounds
- Drug metabolism (to analyze biofluids [I.e., urine or plasma]); a) 19F (a selective tracer; minimal background); 19Fobserve of 19F-containing drugs is very selective and clean by NMR b) We were able to identify 2-hydroxyibuprofen, carboxy ibuprofen, and unmetabolized ibuprofen molecules from a small urine sample after a therapeutic dose of ibuprofen. (Used a micro-coil NMR probe, with an active volume of 3 microliters.)
- LC–NMR provides rapid multipara metric information on microbial biotransformation's as illustrated by the identification of novel warfarin metabolites from Streptomyces rimosus and the identification of the antibiotic aristeromycin from broth supernatants of Streptomyces citricolour.
- Studying uncharacterized, complex, non- living natural organic matter (NOM) present in the atmosphere, oceans, soil and sediments. LC- NMR and LC- SPE- NMR have been used to study dissol ved NOM from fresh water and alkaline soil extracts for the separation and characterization of components in the complex mixture.
- Analysis of crude extracts of natural products and plant- derived compounds - the technique is optimized for rapid i dentification of potential drug candidates in plant products.
- LC-NMR MS have identified analogoes of vitamin E of palm oil extract.

4. Conclusion

The LC-NMR technique has advanced technique with respect it is sensitivity and practically. LC–NMR coupling

is well established with a series of available options for solving a variety of analytical problems. These techniques can be used for the characterizations of many new upcoming molecules, detection of the impurities, determination of the unknown compounds from unknown sources, degradation products, etc. Coupling LC-NMR with other analyitical techniques such as MS has provided novel insights into the Structure of different compounds. The use of spectral databases and softwares for structure analysis will help speed up structure elucidation.

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