JOURNAL OF PHARMACEUTICAL SCIENCE AND BIOSCIENTIFIC RESEARCH (JPSBR)

(An International Peer Reviewed Pharmaceutical Journal that Encourages Innovation and Creativities)

LC-NMR: A powerful tool for analyzing and characterizing complex chemical mixtures without the need of chemical separation

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ABSTRACT:

The on-line coupling of high-performance liquid chromatography (HPLC) principles to high-resolution NMR spectrometers offers a powerful tool for analyzing and characterizing complex chemical mixtures without the need of chemical separation. LC-NMR promises to be of great value in the analysis of complex mixtures of all types, particularly the analysis of natural products and drug-related metabolites in biofluids. However, the advantages of directly coupling NMR and HPLC instrumentation must be weighed against compromises in performance made to each technique to achieve a hyphenated system. While significant advances have been made in LC-NMR technology, a strong case can be made that HPLC purification of metabolites followed by conventional tube NMR is equally useful.

KEYWORDS: LC-NMR, instrumentation, hyphenated system

Article history:
Received 6 July 2013
Accepted 10 July 2013
Available online 13 July 2013

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INTRODUCTION:

History LC-NMR [1-4]

The first on-line LC–NMR experiments were performed in the late 1970s by Watanabe and Niki who demonstrated stopped-flow measurements of a mixture of known compounds

The conventional NMR probe was transformed to a flow-through probe by the introduction of a thin-walled Teflon capillary within a standard NMR tube and spectra were recorded with sample rotation

The first real sample analyzed by LC–NMR was military jet fuel using normal phase columns and deuterated chloroform and Freon

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

Instrumentation of LC-NMR [1,5]

1) **Direct coupling:** It include direct flow of LC effluent in to NMR flow cell and continuous recording of spectra
   - ✓ post-column splitter
   - ✓ valve-switching interface i.e BNMI (Bruker NMR-Mass Spectrometry Interface)

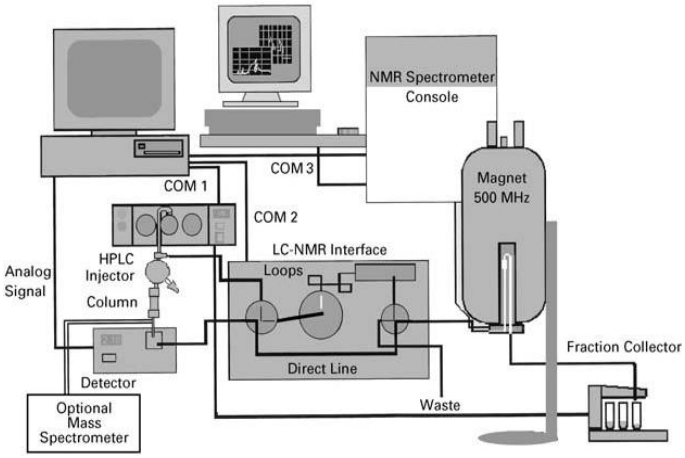

Figure 1 Component of LC-NMR

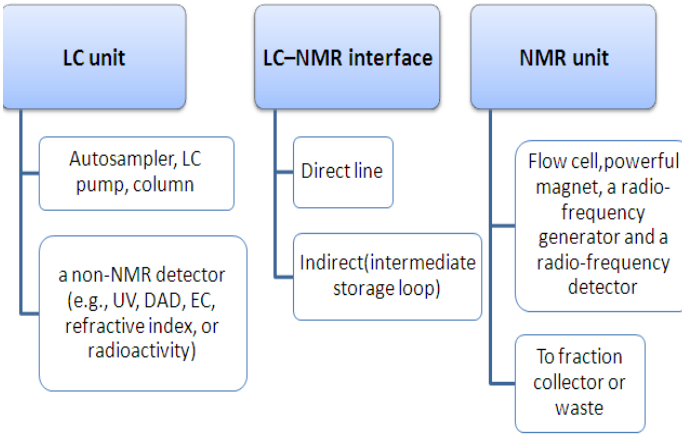

Figure 2 Interfaces of LC-NMR(5,6)

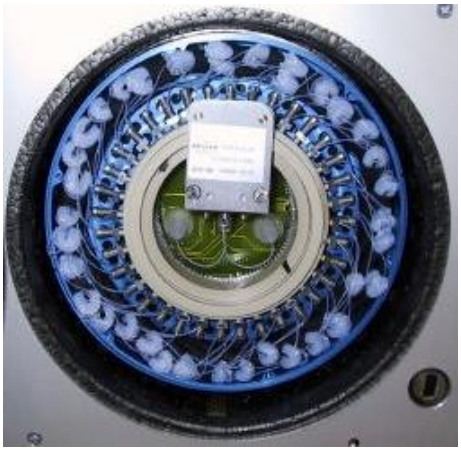

Figure 3 36 Loop Cassette

2) Indirect coupling:

- ✓ intermediate storage loop which transfer outlet of lc to NMR flow cell at specified time interval
- ✓ SPE unit

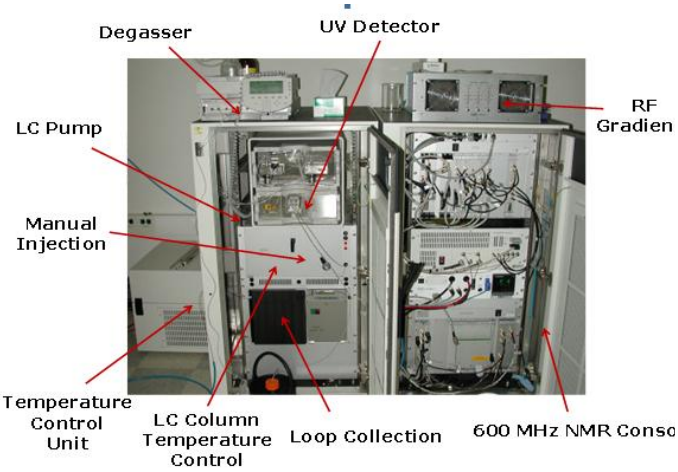

Figure 4

Modes of LC-NMR[3,4,5]

1. **Continuous Flow(on flow):-** Eluent sampled in "real-time" as flowing through NMR Detection Coil
2. **Stopped Flow:-** Pump is stopped at desired location and data acquired
3. **Time Slices:-** Regions, or "time-slices" of interest are analyzed
4. **Peak Parking:-** Peaks of interest are "parked" in off-line sample loops
5. **Peak Trapping:-** Solid Phase Extraction cartridges are used to "re-concentrate" samples

1) On flow mode:

The outlet of the LC-detector is connected directly to the NMR probe. While the peaks are eluting, NMR spectra are continuously acquired

The chromatographic system is used to move the samples/peaks through the NMR cell

Equipment: - Any HPLC system, which delivers a stable pulse free flow.

- LC-NMR Probe
- LCNMR interface not required

With any of the LCNMR interface (BPSU / BSFU) this working mode is also possible, however they are not required.

2) 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 column

When 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

Equipment :- HPLC system

- LC-NMR Probe
- BSFU or BPSU-O
- Controlling station

3) 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)

4) 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

Equipment :- Any HPLC system

- Pump under control for transfer
- LC-NMR Probe
  - BPFU-36, BPSU-12
- Controlling station

5) 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

Equipment :- Any HPLC system

- pump under control for transfer
- LC-NMR Probe
- SPE system
- Controlling station

Technology to improve sensitivity of LC-NMR method[4,5,7-13]

1) LC method
   a) On line SPE method
   b) On line column trapping method
   c) Use of semi micro column
2) NMR method
   a) high strength magnetic field
   b) high sensitivity probe
3) Solvent supression method
   a) presaturation
   b) soft pulse multiple irradiation
   c) WET method

1) LC methods[7]

a) On line SPE method:

It is important to eliminate unnecessary fractions by efficient pretreatment, introducing only the targeted component to the column and controlling overloading.

The SPE cartridge absorbs the desired peak

After the sample is dried with N_2 gas and the contents are finally eluted from the cartridge into the NMR flow probe. [6]

❖ **SPE principle[12,13]**

Solid phase extraction involves the separation of components of samples in solution through their selective interaction with and retention by a solid, particulate sorbent.

The specific hydrophobic organic functional moieties are chemically bonded intimately to a solid surface, such as powdered chromatographic grade silica.

These groups will interact with hydrophobic organic compounds by van der waals forces and extract them from an aqueous sample in contact with the solid surface.

SPE uses the affinity of solutes dissolved or suspended in a liquid (known as the mobile phase) for a solid through which the sample is passed (known as the stationary phase) to separate a mixture into desired and undesired components.

The result is that either the desired analytes of interest or undesired impurities in the sample are retained on the stationary phase.

The portion that passes through the stationary phase is collected or discarded, depending on whether it contains the desired analytes or undesired impurities.

If the portion retained on the stationary phase includes the desired analytes, they can then be removed from the stationary phase for collection in an additional step, in which the stationary phase is rinsed with an appropriate eluent.

SPE DEVICES[12,13]

Several SPE configurations are used which are as following:

- Cartridge
- Disk
- Micropipette tip
- 96-well plate
- Coated fiber

CARTRIDGE

The most popular SPE configuration is the cartridge.

Most SPE is carried out using a small packed bed of sorbent with a nominal particle size of 50-60 µm contained in a cartridge made from a polypropylene syringe barrel, fitted with luer tip, so that a needle can be affixed to direct the effluent to a small container or vial.

The sorbent being retained in position by use of fits. Frits are made of polytetrafluoroethylene (PTFE), polypropylene or stainless steel with a porosity of 10 to 20 µm and thus offer little flow resistance.

The sorbent generally occupies only the lower half of the cartridge, leaving space above to accommodate several milliliters of the sample solution or washing and solvents.

Advantages[6]

1. Highly economical as nondeuterated solvents and HPLC buffers are used
2. The final transfer volume of 200–500 µl is deuterated
3. SPE uses less solvent than liquid-liquid extraction (LLE)
4. SPE is faster (at least 5 times)
5. High capacity
6. Total SPE costs are considerably less than LLE
7. High selectivity: broad choice of bonded phases and solvents
8. Automation much easier

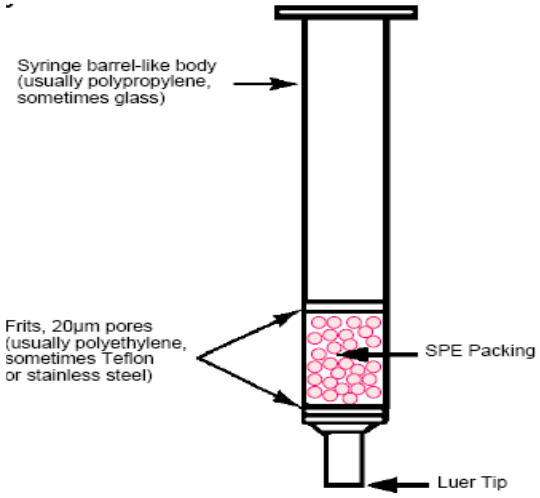

Figure 5 SPE tube

Figure 6 SPEcartridge
(Spark Hyspher Resin GP10, 96 cartridges, volume 30 µL)

b) On line column trapping[3]

In this method, after separation using a conventional column, concentration is first done in a trap column, and the sample is separated again using a semi-micro column then introduced to NMR.

Concentration by this technique is highly effective.

Once sufficient sample has been collected on the trap, the flow reversed and the solute is transported to the NMR for further analysis

c) Use of semi micro column

1) The highest sensitivity is provided when all of the components separated by HPLC are introduced to the flow-cell of NMR

2) However, the peak volume separated by HPLC is greater than the flow-cell capacity **(normally about 30 µL to 120 µL)** therefore, only part of the component is actually the target of measurements

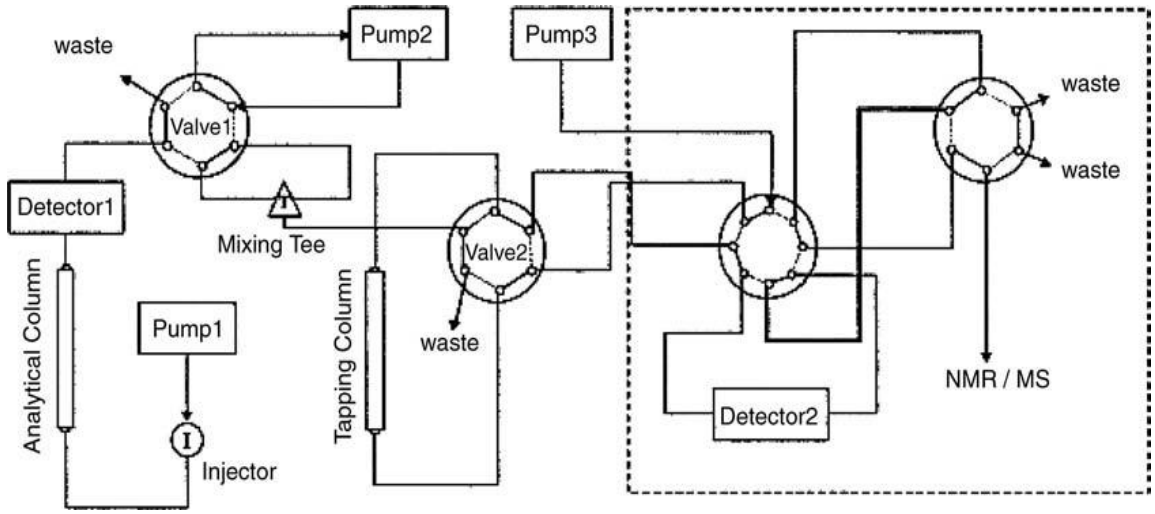

Figure 7 On line column trapping

3) The method of using columns with an internal diameter of around 2 mm, known as semi-micro columns, is a peak concentration method suited to LC-NMR

4) The volume of a semi-micro column is around **1/5** of a conventional column, and since the required amount of solvent is reduced in proportion to the elution, highly concentrated sample solutions can be introduced to LCNMR

2) NMR methods[5,6]

a) High strength magnetic field

NMR detection sensitivity is proportional to the magnetic field strength to the 3/2 power, and the stronger the external magnetic field is, the higher the sensitivity.

Currently, the magnetic field strength has reached 1000 MHz.

Magnetic fields being generated by modern instruments employing cryomagnets, field homogeneity is high and as a consequence the sample need not be rotated.

b) high-sensitivity probe[5]

It is also known as a cryogenic probe that reduces the heat noise arising during NMR signal detection by cooling the coil using superconductor materials.

This will eliminates the thermal electronic noise associated with the initial stages of signal detection and increases the coil quality factor.This leads to an improvement in the S/Nratio by a factor of 3-4.

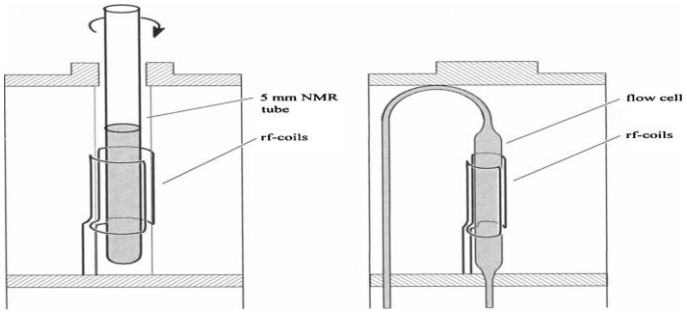

Figure 8 Conventional NMR probe. **Figure 9** Continuous flow NMR probe

Flow Cells – Active Volume

a) 3mm - 60µL
b) 4mm - 120µL
c) 5mm - 240 µL

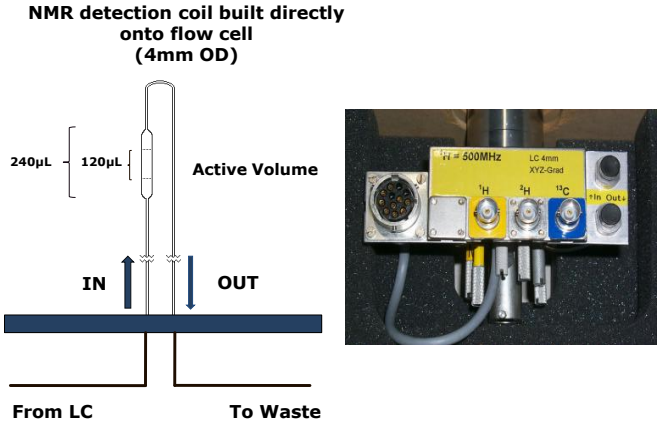

Figure 10 NMR detection coil built directly onto flow cell (Schematic) (4 mm OD)

3) Solvent supression methods[4,5,7]

a) Presaturation

It depend on the phenomenon that nuclei which are unable to relax because their population in ground state and exited state is same, do not contribute to free induction decay after pulse irradiation

Before the data acquisition, a highly selective low power pulse irradiates the desired solvent signal for 0.5 to 2 s. This leading to saturation of solvent signal frequency. During data acquisition, no irradiation should occur. This method is used for stopped flow mode.

b) soft pulse multiple irradiation

Here, presaturation is performed with the use of shaped pulse which has a broader excitation profile. This method is better suitable for supression of multiplets

Advantage:

1. Easy to implement
2. Multiple presaturation can be possible

Disadvantage:

1. Spin with resonance close to solvent frequency will also be saturated and 2D cross peak will be absent

c) WET method

This technique contains NMR difference probe.

This difference probe consists of a dual coil probe that contains two sample coils in a resonant circuit that switches between parallel excitation and serial acquisition to cancel common signals, such as solvent and solvent impurities.

Essentially, this technique is based on a dual beam background subtraction, where the reference signal and sample signal that are collected simultaneously are subtracted from each other automatically.

No software manipulation, pulse sequence modification, or spectrometer alteration is necessary. Hence the technique does not lengthen the pulse sequence but it reduces experimental time.

It takes 50-100 ms. So it is used for on flow method. This method is used for on flow mode.

❖ **Other possible steps for solvent supression[3,4]**

✓ Using eluents that have as few 1H NMR resonances as possible, e.g: H_2O, ACN, or MeOH

✓ Using at least one deuterated solvent, e.g., D_2O (approx \$290/L), ACN-d_3 (approx \$1600/L), or MeOD (approx \$3000/L)

✓ Using buffers that have as few 1H NMR resonances as possible, e.g., TFA or ammonium acetate

✓ Using ionpair reagents that have as few 1H NMR resonances as possible, e.g., ionpairs with *t*-butyl groups create an additional resonance

Sensitivity of LC-NMR[6]

On flow mode:-

sensitivity and resolution are limited by the short residence time of analytes at 0.5–1.5 ml min^{-1}, and typically >10 µg per analyte are needed for quality results at the 1H observation frequency of 500 MHz

Stopped flow mode/loop storage mode:-

The limits of detection at the 1H observation frequency of 600 MHz for analytes are ~100 ng for 60-240µl flow probe cell

For highly concentrated analyts in 1.5 µl NMR active flow probe volume ,detection limit is in 5 ng range

Accuracy limit

^{1}H Sensitivity in 382:1, line shape 2.2/ 4.0, Resolution 0.22 Hz

^{13}C Sensitivity 246:1, line shape 0.7/ 2.3 , Resolution 0.05Hz

Advantages of LC-NMR[5,9]

1. The information between the two (three) techniques is so orthogonal; HPLC methods resolve “complexity of a mixture” by separation, whereas NMR resolves virtually any structure question (especially with different experiments)
2. The NMR can determine if the LC peak impure
3. LC-NMR/MS is “THE” ultimate instrument
4. NMR data can be taken without complete separation of mixture
5. It is nondestructive technique
6. Sample can be stored for analysis by another method

Disadvantages of LC-NMR[5,9]

1. high costs
2. Capital equipment costs; long experiment times; partial use of ^{2}H solvents
3. operator training requirements
4. Doing LC-NMR/MS requires a unique set of skills.
5. Difficulty in solvent selection

6. Eg: TFA, phosphate buffers (great for NMR, but not MS)

   Triethylamine (great for MS, but not NMR)

7. Stopping the pump (for NMR signal averaging) frequenty may affect resolution of method

8. Flow systems can clog up, and get dirty, and be hard to clean

Application[5-14]

1. Separation and characterization of peptide libraries
2. combinatorial chemistry, phytochemical analysis, drug discovery
3. Identification of drug impurities
4. Characterization of isomers of acid glucoronides and vitamin A derivatives
5. Characterization of endogeneous and xenobiotics metabolites directly from biological fluid
6. Combination of LC-NMR and LC-MS
7. Polymer analysis
8. LC-NMR allowed the differentiation of isomers and identification without reference compounds
9. Drug metabolism (to analyze biofluids [I.e., urine or plasma]);

a) ^{19}F (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, carboxyibuprofen, 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.)

10. Identification of nine closely eluting and isomeric aporphine alkaloids in the Taiwanese plant *Litsea* genus using 50 times less material compared with conventional NMR experiments using 5 mm tubes[9]
11. LC–NMR provides rapid multiparametric information on microbial biotransformations 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*[9]
12. Identification and separation of chiral compound : photoisomer of azadirachtin extracted from seed of neem tree which is powerful insect antifeedant is separated and characterized by taking $CH_3CN:D_2O$ ratio of 7:13
13. LC-NMR MS have identified analogoes of vitamin E of palm oil extract