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Applications of Hyphenated Analytical Techniques to Pharmaceutical Drug Analysis : A Review

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ABSTRACT

As far analytical evaluations are concerned, today's advanced pharmaceutical organizations are facing increased number of challenges starting from raw materials right upto the finished product. The challenges are related to the accuracy, sensitivity, ease of laboratory demands, the purity, composition and performance of various drugs. Nowadays there is a vast array of scientific techniques available to the analytical scientists that enable this characterization. But the available analytical techniques are lacking to fulfill the increased demand of pharmaceutical analysis. In such contradictory environment, hyphenated technique had opened window of opportunities to the analytical scientists. Hyphenated techniques refer to combination of two or more analytical techniques to characterize the pharmaceutical drugs. These hyphenated techniques offer shorter analysis time, higher degree of automation, higher sample throughput, better reproducibility, reduction of contamination because it is a closed system, Enhanced combined selectivity and therefore higher degree of information. This review discusses the current trends in analytical evaluations of various types of materials which will provide unique path to the researchers to move a step forward.

Keywords : Analytical tools, Hyphenated techniques, Drugs analysis, Pharma Drugs

I. INTRODUCTION

In the past two decades, demands on analytical support for pharmaceutical drugs have intensified. As a result, new technology is continually evolving to meet these challenges. In addition, the use of more established methodologies is being enhanced by incremental improvements in technology and protocol. Hyphenation (combination) of analytical techniques [1, 2] is one such approach adopted by modern pharmaceutical analysts in meeting the needs of today's industry.

A hyphenated technique is combination (or) coupling of two different analytical techniques with the help of proper interface. The term hyphenated techniques range from the combination of separation -separation, separation-identification & identification-identification techniques [3].

The term "hyphenation" was first adapted by Hirsch Feld in 1980 to describe a possible combination of two or more instrumental analytical methods in a single run (Hirschfield, 1980). The aim of the coupling is to obtain an information-rich detection for both identification and quantification compared to that with a single analytical technique [3].

The new dimension in the area of hyphenated techniques that offers some very significant benefits in pharmaceutical analysis is that of multi-dimensional

chromatography. Various set-ups involving coupling GC, HPLC and CE systems together in different configurations have been studied for analyzing many different sample types [4]. Examples include Size exclusion chromatography coupled with RP-HPLC, CE and GC coupled with LC. Since, RP-HPLC and CE techniques are capable of high resolution separation with orthogonal separation mechanism, combining both techniques in a two-dimensional mode can produce very high peak capacities and extremely high resolving power, particularly useful for complex mixtures [5]. This hyphenated technique not only provides appropriate sensitivity but also unique capabilities for identification and confirmation of the species of interest.

In Pharmaceutical analysis mass spectrometric detection is always preceded by some kind of separation that enables the qualitative and quantitative analysis of the different species by separating them from each other and also from matrix interferences [6]. They consist of two main parts: a separation technique (GC, HPLC or Electrophoresis) and a detector (UV, AAS, ICP-MS or ESI-MS) that are connected by an interface. The most frequently applied coupled analytical techniques are HPLC-ICP-MS, HPLC-ESI-Q-TOF-MS and HPLC-Orbitrap-MS [7].

Among the spectroscopic techniques available to date, NMR is probably the least sensitive, and yet it provides the most useful information toward the structure elucidation of pharmaceutical products. Technological developments have allowed the direct parallel coupling of HPLC systems to NMR, giving rise to the new practical technique HPLC-NMR or LC-NMR, which has been widely known for more than last 15 years [8]. Combined CE–NMR can offer the separation capability of CE and the superior detection of NMR [12]. GC-NMR provides the structural information of molecule from the separated components [13, 14].

The FTIR is a useful spectroscopic technique for the identification of organic compounds, because in the mid-IR region the structures of organic compounds have many absorption bands that are characteristic of particular functionalities, e.g., –OH, –COOH but the hyphenation of HPLC with FTIR spectrometry is again brain-storming [9]. Gas chromatography coupled with transform Fourier infrared spectrometry is capable of obtaining Infrared spectra from the peaks as they elute from the capillary columns thus combining the separation power of gas chromatography with the identification power of infrared spectrometry [10].

TLC combined with mass spectrometry is most efficient analytical tools for structural elucidation. TLC-MS technique is used for indirect and direct characterization of analytes on the surfaces of TLC plates [11].

The combination of a Thermogravimetric Analyzer (TGA) with an Infrared Spectrometer (TG-IR) is the most common type of Evolved Gas Analysis (EGA) in use today. The combination of TGA with a Mass Spectrometer (TG/MS) is becoming increasingly popular due to its ability to detect very low levels of impurities [15].

Combining Differential Scanning Calorimetry (DSC) with Raman spectroscopic technique (DSC-Raman) allows us to apply the precise temperature control of the DSC with the ability of Raman to detect the different polymorphic structures, and obtain precise characterization of the material [16].

II. APPLICATIONS OF HYPHENATED TECHNIQUES

1. Identification of drug degradation products
2. Isolation and identification Low-level impurities into drugs
3. Differentiation of isomers and identification without reference compounds

4. Drug metabolism to analyze biofluids such as plasma or urine
5. Separation and characterization of peptide libraries
6. Identification and separation of chiral compound
7. Detection & characterization of bulk drug impurities obtained during drug stability study
8. Analysis of unstable compounds or compounds formed in situ
9. Composition profiling to analyze the content and structure of components in a mixture, thus providing valuable insights of molecular back-bone
10. Identification and quantification of polymorphic forms of drug substance and drug products

III. TYPES OF HYPHENATED TECHNIQUES

1. Double hyphenated techniques
2. Triple hyphenated techniques

1. Double Hyphenated Techniques

- LC-MS
- GC-MS
- TLC-MS
- LC-NMR
- GC-NMR
- CE-NMR
- LC-FTIR
- GC-FTIR
- CE-MS
- TG-MS
- DSC-Raman

2. Triple Hyphenated Techniques

- LC-MS-MS
- GC-MS-MS
- LC-ICP-MS
- GC-ICP-MS
- GC-FTIR-MS

IV. REVIEW OF HYPHENATED TECHNIQUES

1. LC/MS:

Liquid Chromatography/Mass Spectrometry (LC/MS) is a powerful analytical technique that combines the resolving power of liquid chromatography with the detection specificity of mass spectrometry. Liquid chromatography (LC) separates the sample components and then introduces them to the mass spectrometer (MS). The MS creates and detects charged ions. The LC/MS data may be used to provide information about the molecular weight, structure, identity and quantity of specific sample components [17]. A typical automated LC-MS system (Figure 1) consists of double three-way diverter in-line with an auto sampler, LC system, the Mass spectrometer. The diverter generally operates as an automatic switching valve to divert undesired portions of eluting from the LC system to waste before the sample enters the MS [18]. LC-MS is highly selective and sensitive technique. The flow rate of HPLC is around 1ml/min which is difficult to accommodate in mass spectrometry vacuum system also the diluent which is used has to be vaporized which leads to damage of the thermally labile compounds by excessive heating [19]. By hyphenation of these two techniques capabilities of both the techniques are improved.

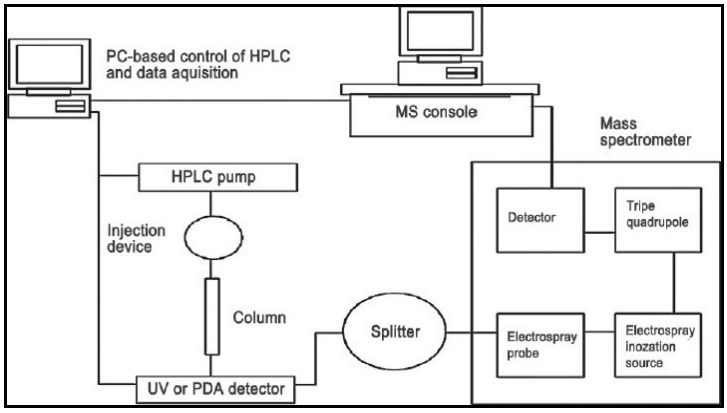

Figure 1. Schematic presentation of LC/MS

The ionization techniques used in LC-MS are generally soft ionization techniques that mainly display the molecular ion species with only a few fragment ions. Nowadays, various types of LC-MS systems incorporating different types of interfaces are available commercially. The interfaces are designed in such a way that they offer adequate nebulization and

vaporization of the liquid, ionization of the sample, removal of the excess solvent vapour, and extraction of the ions into the mass analyser. The two most widely used interfaces are electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The latter is considered as "the chromatographer's LC-MS interface" because of its high solvent flow rate capability, sensitivity, response linearity, and fields of applicability [20].

2. GC/MS:

Gas Chromatography/Mass Spectrometry (GC/MS) is the first of its kind hyphenated technique which became inevitable for pharmaceutical analysis [21]. Mass spectra obtained by this technique offer more structural information based on the interpretation of fragmentations. Compounds that are adequately volatile, small, and stable in high temperature in GC conditions can be easily analyzed by GC-MS. Sometimes, polar compounds, especially those with a number of hydroxyl groups, need to be derivatized for GC-MS analysis. In GC-MS, a sample is injected into the injection port of GC device, vaporized, separated in the GC column, analyzed by MS detector, and recorded. The equipment used for GC-MS (Figure 2) generally consists of an injection port at one end of a metal column (often packed with a sand-like material to promote maximum separation) and a detector (MS) at the other end of the column. A carrier gas (argon, helium, nitrogen, hydrogen etc.) propels the sample down the column. The GC separates the components of a mixture in time and the MS detector provides information that aids in the structural identification of each component.

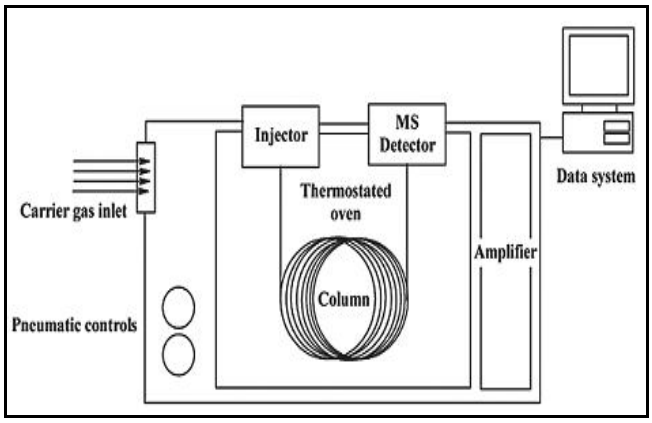

Figure 2. Schematic presentation of GC/MS

The GC-MS columns can be of two types: capillary columns, macrobore columns and packed columns. The process of ionization not only ionizes the molecule but also breaks the molecule into the fragments and detect these fragments with the help of electron impact ionization and chemical ionization. The molecular ion of analyte forms a finger print spectrum which is different from other analytes. The advantage of this technique is sometimes two different analyte will have same mass spectrum but the retention time of both the analytes is different so such type of analytes can be separated or analyses with the help of GC-MS.

Two widely used Ionization techniques in GCMS are the electron impact ionization (EI) and the alternative chemical ionization (CI) in either positive or negative modes [22].

3. TLC/MS:

Many different Thin Layer Chromatography/Mass Spectrometry (TLC/MS) techniques have been reported in the literature. According to differences in their operational processes, the existing TLC/MS systems can be classified into two categories: Indirect mass spectrometric analyses, performed by scraping, extracting, purifying, and concentrating the analyte from the TLC plate and then directing it into the mass spectrometer's ion source for further analysis; Direct mass spectrometric analyses, where the analyte on the TLC plate is characterized directly through mass

spectrometry without the need for scraping, extraction, or concentration processes. Direct TLC/MS analysis is performed under vacuum, but the development of ambient mass spectrometry has allowed analytes on TLC plates to be characterized under atmospheric pressure.

4. LC/NMR:

The first on-line LC/NMR experiment using superconducting magnets was reported in the early 1980s. However, the use of this hyphenated technique in the analytical laboratories started in the latter part of the 1990s. LC/NMR promises to be of great value in the analysis of complex pharmaceutical mixtures. LC/NMR experiments can be performed in both continuous-flow and stop-flow modes. A wide range of bio analytical problems can be addressed using 500, 600, and 800 MHz systems with ^{1H}, ^{13C}, ^{2H}, ^{19F}, and ^{31P} probes. The main prerequisites for on-line LC/NMR, in addition to the NMR and HPLC instrumentation, are the continuous-flow probe and a valve installed before the probe for recording either continuous-flow or stopped-flow NMR spectra [8]. The analytical flow cell was initially constructed for continuous-flow NMR acquisition. However, the need for full structural assignment of unknown compounds, especially during research & development of novel pharmaceutical products, has led to the application in the stopped-flow mode.

Generally, in LC/NMR system, the LC unit comprises (Figure 3) auto-sampler, LC pump, column, and a non-NMR detector (e.g., UV, DAD, EC, refractive index, or radioactivity). From this detector, the flow is guided into the LC-NMR interface, which can be equipped with additional loops for the intermediate storage of selected LC peaks. The flow from the LC/NMR interface is then guided either to the flow-cell NMR probe-head or to the waste receptacle. Following passage through the probe-head, the flow is routed to a fraction collector for recovery and further investigation of the various fractions analyzed by NMR. In most of the LC/NMR operations, reversed-phase columns are used, employing a binary or tertiary solvent mixture with isocratic or gradient elution. To enhance the potential of LC/NMR, it is recommended to use eluents that have as few ^{1H} NMR resonances as possible (e.g., H2O, Acetonitrile or Methanol), at least one deuterated solvent (e.g., D2O), buffers that have as few ^{1H} NMR resonances as possible (e.g., TFA or ammonium acetate), ion pair reagents that have as few ^{1H} NMR resonances as possible (e.g., ion pairs with *t*-butyl groups create an additional resonance).

The major applications of LC/NMR tools are the identification of drug degradation products and isolation and identification of low level impurities.

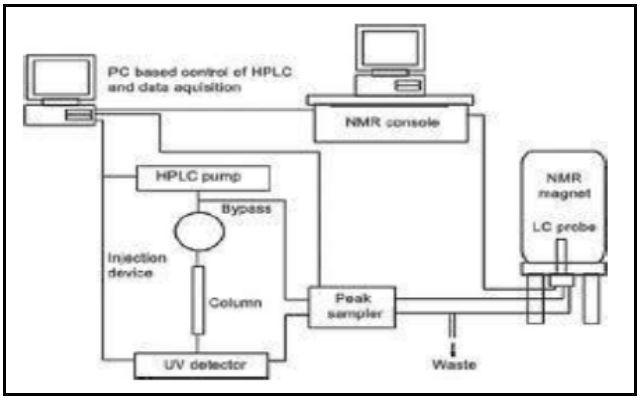

Figure 3. Schematic presentation of LC/NMR

5. GC/NMR:

In GC/NMR technique, NMR perform the identification of the components and GC is used for the separation of the components. A special processing technique was developed to handle the recorded NMR spectrum in the gas phase with very low sample amounts. The chromatographic and spectroscopic conditions were optimized with respect to the ^{1H} NMR detection. The identification of volatile cis/trans-stereoisomers can be accomplished by employing a hyphenated GC-NMR system [23]. The separation of constitutional and configurational isomers is also the major application of GC/NMR technique in pharmaceutical analysis. Also during GC/NMR evaluation of chiral compounds, the

enantiomers show the same spectra at different retention time.

6. CE/NMR:

The low-mass sample requirements with high separation efficiencies and fast separations are keys to the success of CE/NMR technique. Analyte peaks in CE/NMR typically contain low nano-litre volumes. High resolution CE electro-pherograms and NMR spectrum can be obtained using nano-litre volumes. The CE/NMR experiments histidine in phosphate buffer. The NMR microcoil probe can be coupled to the capillary CE system with no major modification to the existing CE instrumentation.

Figure 4 illustrates novel CE–NMR instrumentation with a dual microcoil probe to record continuous flow NMR data under stopped-flow conditions [24].

Using CE–NMR technique the major metabolites of paracetamol in human urine has been demonstrated [25, 26]. In this study CE–NMR successfully analyzed two major metabolites, paracetamol glucuronide and paracetamol sulfate conjugates, as well as endogenous material (hippurate). CE/NMR has been designed and shown to be a promising tool to analyze complex mixtures [27]. Capillary techniques are especially useful for analyzing mass limited samples as required in pharmaceutical analysis.

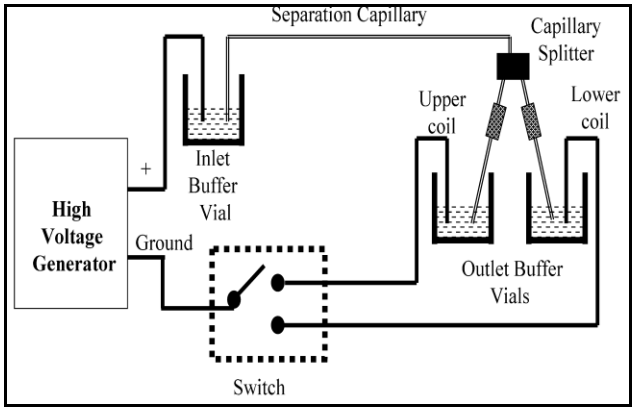

Figure 4. Schematic presentation of CE/NMR

7. LC/FTIR:

The recent developments in LC/FTIR technology have incorporated two basic approaches based on interfaces applied in LC/FTIR. One is a flow-cell approach and the other is a solvent-elimination approach. The approach used with the flow cell in LC/FTIR is similar to that used in UV/Visible and other typical HPLC detectors. In this case, absorption of the mobile phase induces the interference of the detection of sample component absorption bands, but some transparent region of the mid-IR range produces detection possibility. Generally, KBr or KCl salts are used for the collection of sample components in the eluent, and heating up the medium before IR detection eliminates the volatile mobile phase solvents.

Since FT-IR is an absorbance process, the geometry of sample during the measurement process matters. For a fixed mass or volume of the analyte, reducing the diameter by a factor of two creates a deposit with four times the thickness and four times the optical density. Further as the IR detector is total light limited, this deposit diameter reduction of two improves the signal-to-noise ratio by four times. Therefore, to achieve a useful instrument that produces full mid-infrared spectrum, the LC/FTIR hyphenation process must [28].

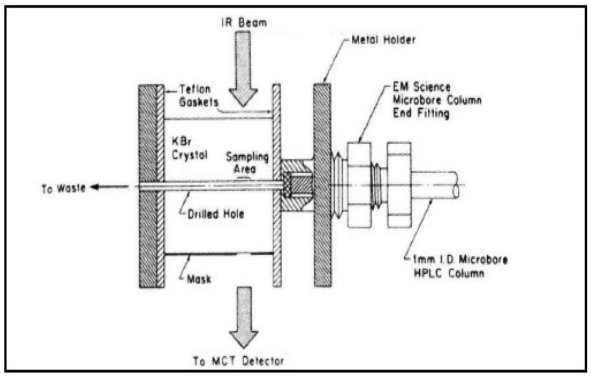

Figure 5. Schematic presentation of LC/FTIR

8. GC/FTIR:

This technique is very sensitive and sample recovery is also possible because IR is non-destructive technique. In this technique the GC does the separation part where as IR perform the function of

identification. The analyte components separated by Gas chromatography travel through the column. These two techniques are linked through glass column or vacuum tubes. Interface used in this technique is internally gold coated small glass pipe connected to column by narrow tubing [29]. Light pipe is heated in order to rid condensation and maximize path length for enhanced sensitivity. Effluent from GC is directly forwarded into the heated pipe of IR at atmospheric pressure. Infrared red spectroscopy identifies the compound by identifying the functional groups.

GC/FTIR technique is particularly useful for distinguishing between structural isomers, such as *ortho*-, *meta*- and *para*-xylene, whose electron-impact and chemical-ionization mass spectra are identical.

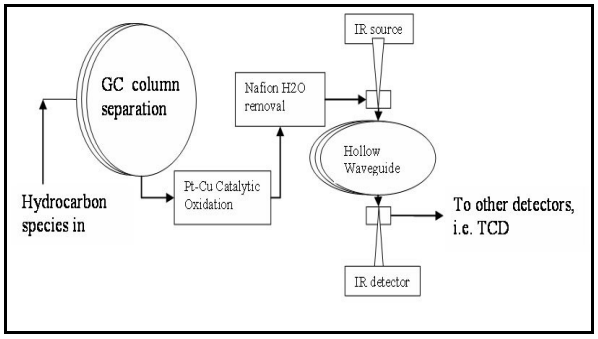

Figure 6. Schematic presentation of GC/FTIR

9. CE/MS:

Capillary Electrophoresis coupled with Mass Spectrometry is an online separation technique in which molecules are distinguished according to their differences in electrophoretic mobilities and structural information [30]. This technique is speedy, efficient and low solvent and sample is required for the analysis. The CE is connected to the MS with the help of the long capillaries which increases the analysis time for better sensitivity. The detectors used for the CE analysis are UV and DAD. The electrolytes used in this technique are inorganic and non-volatile. Ionisation interfaces used are electrospray; fast atom bombardment interface and ion spray ioniser. Detectors used in mass spectroscopy are TOF, ion trap and Quadrupole. Main advantage of quadrupole detector is sensitivity [31].

The major pharmaceutical applications of this technique include the identification of basic and acidic compounds using non-aqueous CE/MS, the analysis of complex arabino oligosaccharides and drug bioanalysis & biomarker discovery.

10. TG/MS:

Heating a sample on the TGA causes a sample to release volatile materials or generate combustion components as it burns. These gases are then transferred to MS for identification. Hyphenating TG/MS is a powerful approach for analysis of an unknown mixture to determine its primary components and identify additives or contaminants. This information may be needed, for example, to evaluate a competitor's product or to determine compliance with regulations [15].

TG/MS technique basically consists of a take-off tube placed very close to the specimen crucible in the TGA, a narrow and heated passage tube for gas transfer without condensation. This tube terminates with a micro metering valve connected to a rotary pump. This acts as the first stage of pressure reduction and gives the desired viscous flow required for faster transfer. The second stage of pressure reduction is achieved through a molecular leak valve. This ensures both a clean sample entry and the low operating pressure at the mass spectrometer [32].

This system is used to study temperature programmed decomposition of many oxyanion based inorganic salts. In conjunction with off-line analytical techniques, the chemical, structural evaluation of the intermediates/ products with complete kinetic/reaction pathways is determined.

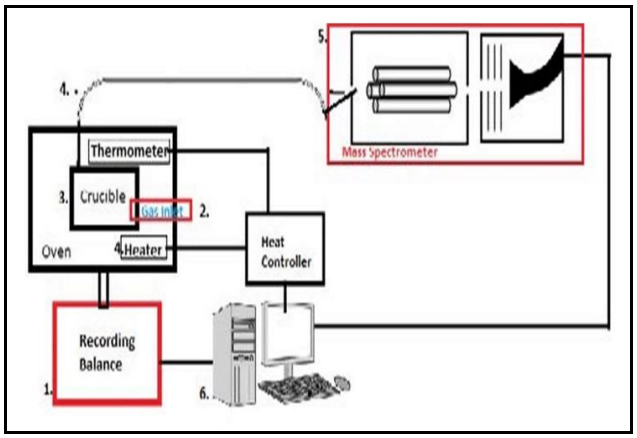

Figure 7, Schematic presentation of TG/MS

11. DSC/Raman:

DSC allows scientists to see the transitions between crystalline forms in a drug but but the forms must be determined by another method. Simultaneous DSC/Raman technique solves this problem. One of the great strengths of DSC/Raman is that a spectroscopist is able to determine structural information from the spectrum. For example, in semicrystalline sample, where the drug exists in mixtures of crystalline and amorphous forms, the ability to understand crystallinity is an obvious advantage.

The Raman spectrometer can collect data during the rapid heating of the material to the melt, although the fast heating will limit the number of spectra collected, and then on both the cooling and isothermal hold stages. After this experiment, the data are more conclusive in the Raman and in the DSC. In other words, DSC/Raman complements each other because Raman spectra can provide qualitative information to supplement the quantitative information from DSC.

In this technique, Raman Station allows the use of a remote probe for collecting spectrum. This makes an optical fibre connection between the spectrometer and the DSC. The lid of the DSC is modified to hold the focusing and collecting optics, with adjustment to align the laser beam with the sample. These modifications serve to protect the remote probe from high temperatures in the DSC, as well as to protect and maintain the controlled thermal environment required for proper DSC operation. The laser illuminates a spot approximately 200 μm across. The sample will normally be in an open pan, although a quartz window can be used for volatile materials [33].

12. LC/MS/MS:

LC-MS/MS can detect the sample components of different class by low injection volume. Minimal sample pre-treatment is required and it also reduces the time of analysis. LC-MS is the first step of LC/MS/MS. This technique is more sensitive and specific than that of the LC/MS. It is around 20-100 times sensitive than LC/-MS. More specific because in this technique second filtering process is also involved.

13. GC/MS/MS:

This technique is sensitive as well as specific and can be used for ultra-trace analysis. For qualitative identification with MS/MS, product ion scan, precursor ion scan and neutral loss with a triple quadrupole or product scan with an ion trap can be used. In recent years the sensitivity of the quadrupole is increased instead of loss. Also the scanning speed is high [22].

V. CONCLUSION

The hyphenated techniques, in which separation techniques are coupled with diverse selective and sensitive detection methods, are widely used in pharmaceutical drug analysis. These techniques create new and ever greater possibilities. Their main advantages include extremely low limits of detection and quantification, insignificant influence of interferences on the determination process, as well as very high precision and repeatability of determinations. Nevertheless, the hyphenated techniques have been constantly developing and gaining more and more importance in pharmaceutical research & development, which is corroborated by the rising number of works pertaining to the subject. Further, the regulatory agencies are also encouraging the pharmaceutical organizations to best utilize the hyphenated techniques.

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