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Review Article

HYPHENATED TECHNIQUES IN CHROMATOGRAPHY AND THEIR APPLICATIONS

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Abstract:

The techniques which combine chromatographic and spectral methods together to exploit the advantages of both are called as hyphenated techniques. They improve resolution and sensitivity along with decreased analysis time. They are widely used in analytical chemistry, and particularly in areas where samples are presented in complex matrices, e.g. environmental, pharmaceutical, biochemical and biomedical analysis. In this article, recent advances related to various hyphenated techniques are discussed with their instrumentation and appropriate examples.

Key Words: Hyphenated Techniques, GC-MS, LC-MS, LC-NMR, CE, Separation Techniques, natural products.

Introduction

Analytical chemistry is the study of separation, identification, and quantification of the chemical components of natural and artificial materials. Chromatography refers to separation of pure or fraction of chemical compound from a mixture, while spectroscopy produces selective information for identification using spectra[1].

Hirshfeld defined the term hyphenated techniques as "the online combination of a separation technique and one or more spectroscopic technique".

In recent years, hyphenated techniques have received a principle attention to solve complex analytical problems. It has been demonstrated over the years for qualitative and quantitative analysis of unknown compound in complex natural product and a complex mixture. To obtain structural information leading to identification of compounds present in a sample a liquid chromatography (LC), like high performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis(CE) is linked to spectroscopic determination technique like fourior

transform infrared(FTIR), photodiode array(PDA), UV absorbance or fluorescence emission, mass spectroscopy (MS), nuclear magnetic resonance technology (NMR) resulting in various hyphenated techniques like CE-MS, GC-MS, LC-MS, LC-NMR[5, 7].

Hyphenation has not been always with only two techniques; it can involve more than one separation or detection techniques.eg. LC-PDA-MS, LC-MS-MS, and LC-NMR-MS. Solid phase extraction (SPE) or large volume injection (LVI) techniques can be incorporated to built a more powerful technique like SPE-LC-MS or LVI-GC-MS in which trace analysis is vital. These techniques helps a lot to natural products researchers as it is a vital process to extract and purify the unknown compound present in the crude.

These techniques serves as important tool for separation of compounds as well as one can know its online spectra. Detailed information on principle, history, instrumentation and methodology has been available in following discussion[2].

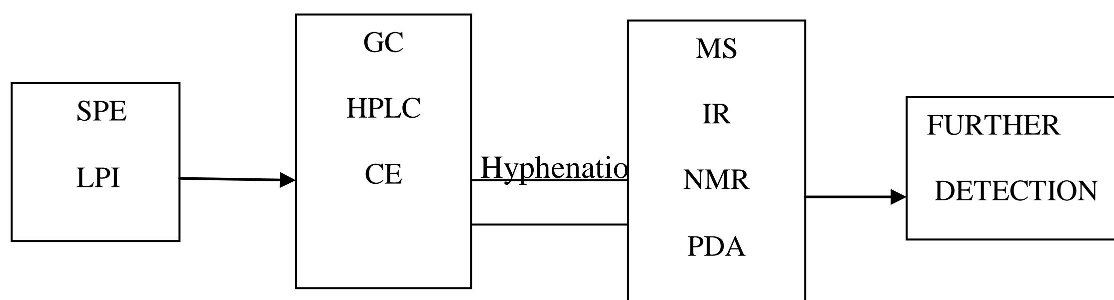

Fig.1: Hyphenated Technique

Available Hyphenated Techniques

GC-MS[4, 6, 16]

Gas chromatography-mass spectrometry (GC-MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. The GC-MS is composed of two major building blocks: the gas chromatograph and the mass spectrometer. The gas chromatograph utilizes a capillary column which depends on the column's dimensions as well as the phase properties. The difference in the chemical properties between different molecules in a mixture will separate the molecules as the sample travels the length of the column.

The molecules take different amounts of time (called the retention time) to elute from the gas chromatograph, and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass to charge ratio.

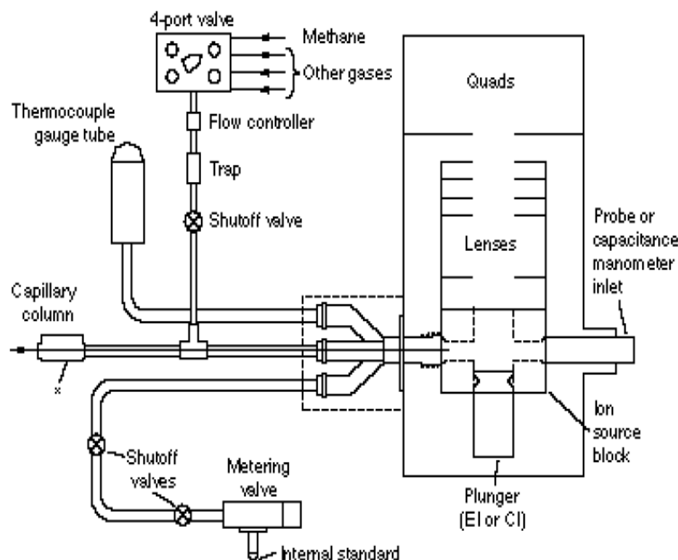

Fig.2: Schematic Diagram of GC-MS.

These two components, used together, allow a much finer degree of substance identification than either unit used separately. It is not possible to make an accurate identification of a particular molecule by gas chromatography or mass spectrometry alone. The mass spectrometry process normally requires a very pure sample, while gas chromatography using a traditional detector detects multiple molecules have the same retention time which results in two or more molecules to co-elute. Sometimes two different molecules can also have a similar pattern of ionized fragments in a mass spectrometer. Combining the two processes reduces the possibility of error, as it is extremely unlikely that two different molecules will behave in the same way in both a gas chromatograph and a mass spectrometer. The most common type of mass spectrometer (MS) associated with a gas chromatograph (GC) is the quadrapole mass spectrometer. Another relatively common detector is the ion trap mass spectrometer. Other detectors may be encountered such as time of flight (TOF), tandem quadrapoles (MS-MS).

A mass spectrometer is typically utilized in one of two ways: Full Scan or Selective Ion Monitoring (SIM). A “full spectrum” analysis considers all the “peaks” within a spectrum. Conversely, selective ion monitoring (SIM) only

monitors selected peaks associated with a specific substance. The typical GC-MS instrument is capable of performing both functions either individually or concomitantly, depending on the setup of the particular instrument. When collecting data in the full scan mode, a target range of mass fragments is determined and put into the instrument's method. Full scan is useful in determining unknown compounds in a sample. It provides more information than SIM when it comes to confirming or resolving compounds in a sample. In selected ion monitoring (SIM) certain ion fragments are entered into the instrument method and only those mass fragments are detected by the mass spectrometer. The advantages of SIM are that the detection limit is lower since the instrument is only looking at a small number of fragments (e.g. three fragments) during each scan.

When molecules travel the length of the column, enters to mass spectrometer where they are ionized. Once the sample is fragmented it will then be detected, by an electron multiplier diode, which turns the ionized mass fragment into an electrical signal that is then detected.

GC-TANDEM MS[11]

When a second phase of mass fragmentation is added, for example using a second quadrupole in a quadrupole instrument, it is called tandem MS (MS-MS). MS-MS can sometimes be used to quantitate low levels of target compounds in the presence of a high sample matrix background.

Another method of analysis measures the peaks in relation to one another. In this method, the tallest peak is assigned 100% of the value, and the other peaks being assigned proportionate values. All values above 3% are assigned. The total mass of the unknown compound is normally indicated by the parent peak. The value of this parent peak can be used to fit with a chemical formula containing the various elements which are believed to be in the compound. The isotope pattern in the spectrum, which is unique for elements that have many isotopes, can also be used to identify the various elements present. Once a chemical formula has been matched to the spectrum, the molecular structure and bonding can be identified, and must be consistent with the characteristics recorded by GC-MS.

PURGE AND TRAP GC-MS[7]

For the analysis of volatile compounds a Purge and Trap (P&T) concentrator system may be used to introduce samples. The target analytes are extracted and mixed with water and introduced into an airtight chamber. An inert gas such as Nitrogen (N_2) is bubbled through the water; this is known as purging. The volatile compounds move into the

headspace above the water and are drawn along a pressure gradient (caused by the introduction of the purge gas) out of the chamber. The volatile compounds are drawn along a heated line onto a 'trap'. The trap is a column of adsorbent material at ambient temperature that holds the compounds by returning them to the liquid phase. The trap is then heated and the sample compounds are introduced to the GC-MS column via a volatiles interface, which is a split inlet system. P&T GC-MS is particularly suited to volatile organic compounds (VOCs) and BTEX compounds (aromatic compounds associated with petroleum).

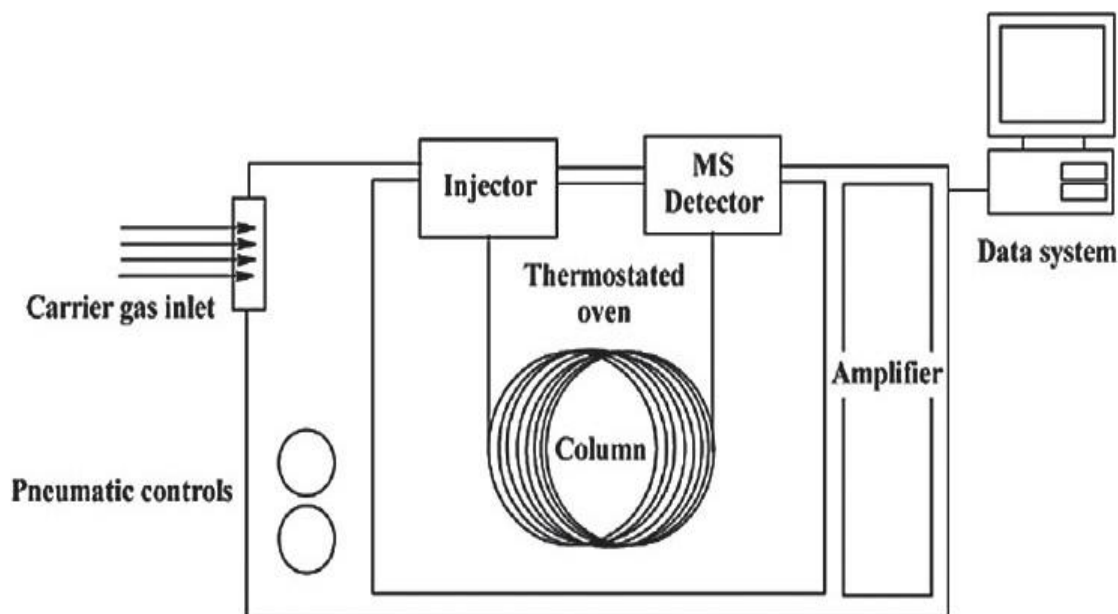

Fig.3: A schematic diagram of GC-MS.

LC-IR[8]

The hyphenated technique developed from the coupling of liquid chromatography and infrared spectroscopy is known as LC-IR. LC IR is an important technique as it shows absorption peaks of functional groups in mid IR region which helps in structural identification of compounds present in a sample. The detection technique of IR is comparatively slow than other techniques like MS or NMR. Two approaches used in these techniques are flow cell approach and solvent elimination approach.

The flow cell approach is as similar to that of UV-visible or other detectors used in HPLC. In this case absorption of mobile phase induces detection of sample component absorption bands. For eg. If one uses mobile phase of deuterated solvent such as heavy water or predeuterated methanol, IR can monitor many organic compounds that have C-H structures in the molecule.

In solvent elimination approach, after the mobile phase solvent has been eliminated IR detection is carried out in such a medium that have transparency for IR region. 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. There are two types of interfaces for the solvent-elimination approach: diffuse-reflectance infrared Fourier transform (DRIFT) approach and buffer-memory technique.

A redesigned interface is reported for use in the detection of microcolumn liquid chromatographic effluents with a Fourier transform infrared spectrometer. The solutes eluting from the column are continuously deposited onto a 50mm diameter KBr disk as a “buffer-memory”. The disk is rotated by a stepper motor with a controlling electronics. After the chromatographic run, the disk is simply transferred to the spectrometer and the transmission spectra are measured as the disk rotates. The use of such a large KBr disk as a substrate permits to obtain spectra of the components having a large capacity factor.

LC-MS[17, 18]

Liquid chromatography-mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and selectivity. Generally its application is oriented towards the specific detection and potential identification of chemicals in a complex mixture.

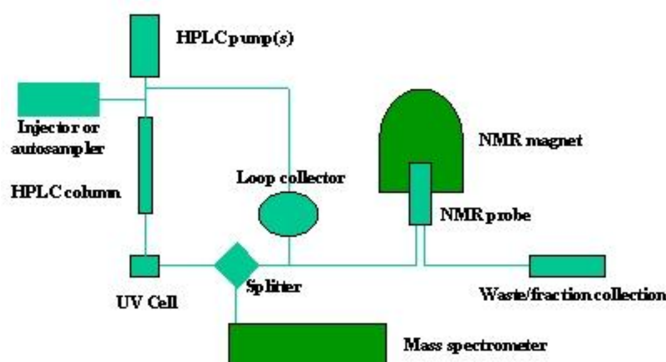

Fig.4: Schematic Diagram of LC-MS.

There are two common atmospheric pressure ionization (API) LC/MS process: Electrospray Ionization (ESI) & Atmospheric Pressure Chemical Ionization (APCI). Both are soft ionization technique. Both of these processes are compatible with most chromatographic separations.

Atmospheric-pressure chemical ionization (APCI) is a form of chemical ionization which takes place at atmospheric pressure. APCI allows high flow rates without diverting the larger fraction of volume to waste. Typically the mobile phase containing eluting analyte is heated to relatively high temperatures (above 400 degrees Celsius), sprayed with high flow rates of nitrogen and the entire aerosol cloud to creates ions. A potential advantage of APCI is that it is possible to use a nonpolar solvent as a mobile phase solution, instead of a polar solvent, because the solvent and molecules are converted to a gaseous state before reaching the mass spectrometer. Typically, APCI is a less "soft" ionization technique than ESI, i.e. it generates more fragment ions relative to the parent ion.

Electrospray ionization (ESI) is a technique used in mass spectrometry to produce ions. It is especially useful in producing ions from macromolecules because it overcomes the tendency of these molecules to fragment when ionized. The liquid containing the analyte of interest is dispersed by electrospray into a fine aerosol. Because the ion formation involves extensive solvent evaporation, the typical solvents for electrospray ionization are prepared by mixing water with volatile organic compounds (e.g. methanol, acetonitrile). To decrease the initial droplet size, compounds that increase the conductivity (e.g. acetic acid) are added to the solution. Large-flow electrospray can benefit from additional nebulization by an inert gas such as nitrogen. The aerosol is sampled into the first vacuum stage of a mass spectrometer through a capillary, which can be heated to aid further solvent evaporation from the charged droplets. The solvent evaporates from a charged droplet. At this point, the droplet deforms and emits charged jets. During the fission, the droplet loses a small percentage of its mass (1.0-2.3%) along with a relatively large percentage of its charge (10-18%).

Ion trap facility has advantage over other tandem mass, as in this method one can select interested ion peak and accumulate for fragmentation. This result in higher sensitivity and less interference. This advantage is exploited for small impurities profiling studies. The interface is most often an electrospray ion source or variant such as a nanospray source; however fast atom bombardment, thermospray and atmospheric pressure chemical ionization interfaces are also used .

LC-NMR[14,]

The combination of liquid chromatography (LC) and nuclear magnetic resonance (NMR) offers the potential of unparalleled chemical information from analytes separated from complex mixtures.

Several other hyphenated NMR techniques have been developed to enhance sensitivity of this technique. LC-SPE-NMR increases sensitivity of the instrument by utilizing a solid phase extraction device after LC column. Capillary LC NMR also practically lowers detection limit to a nanogram range through integration of capillary LC with NMR detection. Further Cryo-LC-probe technology combine the advantage of sample flow and enhanced sensitivity from a cryogenically cooled NMR probe.

Nuclear magnetic resonance (NMR) detection coupled with liquid chromatography (LC) offers great promise in combining the ability to separate complex mixtures into individual components with one of the most structurally rich detection schemes available. In 1978, Watanabe reported the coupling of LC effluent to NMR using a stopped flow approach, and within 1 year, an on-line system had been reported. The major advantages of on-line as opposed to off-line NMR detection of LC are improved chromatographic resolution, consistent response, on-line data analysis, and rapid data acquisition. The drawbacks of continuous flow NMR include poorer sensitivity due to the limited time available to measure each analyze and the flow rate dependence of the NMR line width. Over the past 15 years, numerous groups have reported improved LC-NMR hyphenation methods, improved NMR plus sequences (e.g., to remove the effects of strong solvent resonance and hence alleviate the need for deuterated solvents), and increased chromatographic resolution.

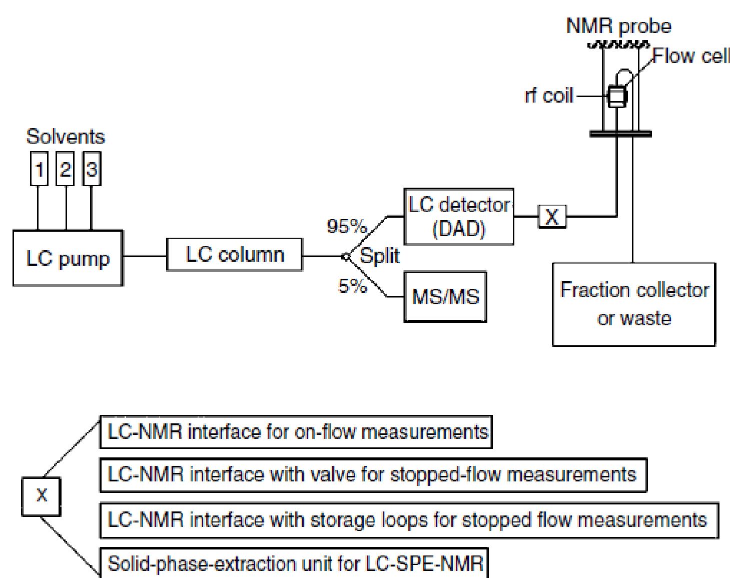

Fig.5: Schematic diagram of LC-NMR.

The experiments can be performed on continuous flow and split flow model. In the closed loop separation identification techniques data can be obtained in 2D and 3D views. Generally in this instrument LC detectors are UV or PDA detectors from which the flow is guided to NMR flow cell interface which is equipped to additional loop. Generally reverse phased LC columns are widely used with isocratic or gradient elution. For enhancement of signaling and detection one has to use 1H NMR or deuterated water as a solvent.

CE-MS[8]

CE analysis can be driven by slectric field performed in narrow tubes which can result in rapid separation of hundreds of compounds. It separates components by applying voltage in between buffer filled capillaries. The components are separated due to production of ions depending on their mass and charge. It is widely used in quantitative determination and the analysis particularly the assay development and trace level determination. When MS is linked to CE then it produces determination of molecular weight of components often termed as CE-MS. Separation is achieved from the etched surface of the capillaries that delivers sample to the ESI MS. This technique runs in full automation and having higher sensitivity and selectivity. The new interface known as coaxial sheath interface has developed which has potential of use of both CE-MS and LC-MS alternatively on same mass spectrometer.

GC-IR[11]

IR spectra were initially obtained off-line, by condensing the eluted solute in a cooled trap, making into a 'mull', or pressing into an alkali halide pellet and the spectrum obtained using standard techniques. Collection of a solute by condensation can be difficult due to the very low concentrations at which each solute is eluted; the partial pressure of the condensed material is often similar to its partial pressure as it leaves the GC column. An efficient method to collect the solute is to use argon as the carrier gas, and condense the argon and the solute simultaneously in a tube cooled with liquid nitrogen. The trapping efficiency can also be improved by trapping the solute on an adsorbent contained in a short length of packed tube and regenerated in a stream of hot gas or by solvent extraction.

The first fully automated on-line GC IR system was developed by Scott et al. Each eluted solute was adsorbed in a cooled packed tube, and then thermally regenerated into an infrared vapor cell. Subsequent to the IR spectrum being obtained, a small sample of the vapor was drawn from the IR cell into a low-resolution mass spectrometer and the mass spectrum was also taken.

This system was not a tandem system but, in fact, the first triplet instrument to be reported (GC/IR/MS). The procedure for analyzing a peak was as follows. As the peak started to elute it was sensed by the detector and the exit carrier gas diverted through the IR cell into a packed trap which concentrated the peak onto the front of the trap packing. After peak elution was complete, the flow of carrier gas was stopped and the solute regenerated back into the IR cell by heating the trap in a secondary stream of nitrogen[20].

APPLICATIONS OF HYPHENATED TECHNIQUES

The various applications of hyphenated techniques are discussed below.

ISOLATION AND ANALYSIS OF NATURAL PRODUCTS[6, 26]

The analysis of crude drugs from the plant extracts is a very difficult task. The above discussed hyphenated techniques are well capable of separating the drugs from natural product sources. They allow analysis of small nonpolar compounds to large polar compounds like oligosaccharides, proteins and tannins. Various alkaloids, coumarins, tannins, resins and essential volatile oil components can be separated by these techniques. Alkaloids are a large group of nitrogen-containing secondary metabolites of plant, microbial, or animal origin.

Various hyphenated techniques have been used in the analysis of several types of alkaloids to date. GC-MS has become the method of choice for the analysis of various pyrolizidine and quinolizidine types of alkaloids. Most of these alkaloids are sufficiently volatile and thermostable under GC conditions to permit analysis vwithout chemical modification.

A number of metabolites, differing in the number and the placement of various oxygen functions on the aromatic rings, have been identified prior to isolation from the cell cultures by LC-NMR and LC-MS. An APCI interface was used in the LC-MS system, and the mass spectra were obtained with selected ion monitoring (SIM) and total ion monitoring (TIM) in the positive ion mode[19].

The coumarins are the largest class of 1-benzopyran derivatives that are found mainly in higher plants. The HPLC-PDA determination of coumarins, where absorption spectra are registered with a PDA detector, provides useful information about the identity of the molecule including oxidation pattern.

The coupling of MS to LC-PDA provides further structural information that is helpful for on-line identification of individual coumarins in any crude extract. Various coumarins together with other oxygen heterocyclic compounds,

e.g., psoralens and polymethoxylated flavones, present in the nonvolatile residue of the citrus essential oils of mandarin, sweet orange, bitter orange, bergamot, and grapefruit, were analyzed by atmospheric pressure ionization (API) LC-MS system equipped with an APCI probe in positive ion mode.

DEREPLICATION[6,22]

The discrimination between previously tested or recovered natural product extracts and isolated single components found is essential to decrease the screening costs by reducing the large collections of isolates that are then subject to further detailed evaluation. Dereplication strategies employ a combination of separation science, spectroscopic detection technologies, and on-line database searching. Thus, the combination of HPLC with structurally informative spectroscopic detection techniques, e.g., PDA, MS, and NMR, could allow crude extracts or fractions to be screened not just for biological activity but also for structural classes. To perform an efficient screening of extracts, both biological assays and HPLC analysis with various detection methods are used. Techniques such as HPLC coupled with UV photodiode array detection and with mass spectrometry provide a large number of on-line analytical data of extract ingredients prior to isolation. The combination of HPLC coupled to NMR (LC-NMR) represents a powerful complement to LC-UV-MS screening. These hyphenated techniques allow a rapid determination of known substances with only a small amount of source material. LC-MS-MS spectra are reproducible. Therefore, the MS-MS databases of natural products can be used for dereplication purposes. For automated on-line dereplication purposes, most of the dereplication protocols available for natural product analysis. The LC-NMR, being able to provide more meaningful structural information, has achieved limited success due to the lack of sensitivity, lack of general access to high-field NMR instruments, and the cost associated with the use of deuterated solvents.

CHEMICAL FINGERPRINTING AND QUALITY CONTROL OF HERBAL MEDICINE[26]

The use of hyphenated techniques, e.g., LC-MS, CE-MS, LC-NMR, or LC-NMR-MS, in chemical fingerprinting analysis for quality control and standardization of medicinal herbs has attracted immense interest in recent years. Generally, fingerprinting method is used to highlight the profiles of the sample matrix, which is often sufficient to provide indications of the source and method of preparation.

In herbal medicines, the profile depends not only on the preparation processes but also on the quality of the crude herb source material. The uniformity and stability of the chemical profiles represent the quality of the raw herbs. In

both good agricultural practice (GAP) and good manufacturing practice (GMP), fingerprinting analysis is used to appraise the quality of the herbal material.

In this process, the fundamental objective is to develop links between marker compound-based chromatographic or spectroscopic profiles and the efficacy of herbal products. GC-MS or LC-MS can be used to detect and confirm the identity of these trace marker compounds. In fingerprinting analysis, it is imperative to optimize all laboratory instrumentations and methodology to avoid any artifacts in the results. The relative intensity of the peaks is important, and chromatographic fingerprints must be specific for the substance being analyzed. Hence, it is necessary to check fingerprints obtained from related botanical products and known adulterants to ensure that, the method developed can distinguish true from false identifications. Several analytical protocols based on LC-MS fingerprinting have been developed and integrated into a high-throughput analytical program incorporating standard methods, template structure determination, and structural libraries. For eg, LC-MS was used to characterize mixtures of taxanes from *Taxus brevifolia* extracts and to develop a taxane database.

Medicinal properties of herbs used in traditional systems of medicine, for eg, traditional Chinese medicine or Ayurveda, are attributed to the presence of various types of biologically active molecules. Any variation, either qualitative or quantitative, in the chemical profile of the herb can lead to the total loss of medicinal properties, decreased potency, or even increased toxicity. Therefore, it is essential, for quality control purposes, to ascertain the presence of certain molecules in the herbal preparation or extract, and also to determine the quantity of each of the active principles by applying a suitable method, which allows on-line detection of molecules present in the herbal extract. Nowadays, with the advent of modern hyphenated techniques, it is possible to obtain comprehensive chemical profiles of herbal medicine preparations or extracts. GC-MS and LC-MS are now being used quite extensively for direct on-line analysis of components present in the herbal preparations and for ensuring the quality of the herb. These techniques have been used in the traditional Chinese medicine.

The mass spectra of various components present in the extracts of Chinese medicine have been obtained on-line from the LC-MS run and matched with known standards for structural confirmation. Integrated MS databases have also been useful for identification of these compounds. In this way, GC-MS, LC-MS, and MS-MS fingerprinting profiles of the active ingredients of various Chinese herbal extracts have been obtained, and information has been stored in

the form of an electronic database, which can be used for routine comparison of chemical profiles of individual herb extracts for quality control purposes. The GC or LC retention time and mass spectral data are reproducible, provided the chromatographic and spectroscopic conditions are kept constant. LC-NMR and LC-NMR MS have also been used to this purpose.

STRUCTURAL IDENTIFICATION OF COMPOUNDS

The uniqueness of LC/MS is the combined high resolving power of HPLC and superior mass detection capability of MS. The LC/MS and LC/MS/MS techniques provide solutions to a wide range of structural characterization problems in pharmaceutical research. They include identification of trace level impurities and degradants in bulk drug substances, identification of metabolites in drug metabolism studies, unknown identifications in synthetic reaction products as well as determination of molecular weight of these agents. An important aspect of pharmaceutical development is the identification of impurities in bulk drug substances. The use of LC/MS and LC/MS/MS techniques can rapidly provide structural information of unknown impurities found in production batches.

For example, mometasone furoate (MW 520 Da) is a highly potent synthetic dichlorinated corticosteroid. It has been widely used in the treatment of dermatological disorders as topical formulations of ointments, seasonal and perennial allergic rhinitis as an aqueous intranasal spray, and in asthma as a dry powder. Several impurities were found to be present in the course of large-scale production of the drug substance, as shown in Fig. 6(a). There are two co-eluting components detected in peak D, corresponding to molecular ions at *m/z* 535 (containing two chlorine atoms) and 581 (containing one chlorine atom). The impurity ion at *m/z* 535 in peak D was established as a 6-keto structure, although the complete characterization of the ion at *m/z* 581 was not carried out. To fully characterize all the impurities in the sample, we utilized mass spectrometer to obtain the structural information. As latest MS instrumentation has the capability of performing high-resolution LC/MS and LC/MS*n* experiments with high resolving power (up to 100 000), excellent mass accuracy (<3 ppm with external calibration) and large dynamic range. Several groups have demonstrated low ppm mass accuracy on mixture analysis, including human urinary proteome studies with identifications of more than 1500 proteins, top-down protein sequencing, and structural identifications of drug metabolites in doping control analysis, as well as in human liver microsomal metabolism.

In the case of the impurity ion at *m*/*z* 581, high-resolution LC/MS experiments (at a resolution of 30 000) were performed to determine its elemental composition. Based on its isotopic patterns and likely possible element combinations, the best possible elemental composition was determined to be C8H34O9ClS for *m*/*z* 581 with a mass accuracy of 0.18 ppm. Comparing with the elemental composition of C27H31O6Cl2 for mometasone furoate, the net addition for the unknown is the moiety of CH3O3S with removal of one chlorine atom. Further high-resolution LC/MS/MS experiments on *m*/*z* 581 suggest its structure as the addition of sulfur-moiety at the 20-keto position, as supported by accurate mass measurements of product ions (Fig. 7). One of the possible mechanisms for formation of the proposed structure for this impurity (*m*/*z* 581) involves reactions with CH3SO2Cl (reagent). Other impurities in the sample are also characterized and their proposed structures are shown in Fig. 6(b). The 6-keto structure in peak D (which is a mixture of two impurities) and the 6-methyl impurity structure for peak F have the same nominal MWs of 534 Da. Their structural differences can be differentiated using accurate mass data. Thus, different elemental compositions can be obtained to facilitate structural identifications.

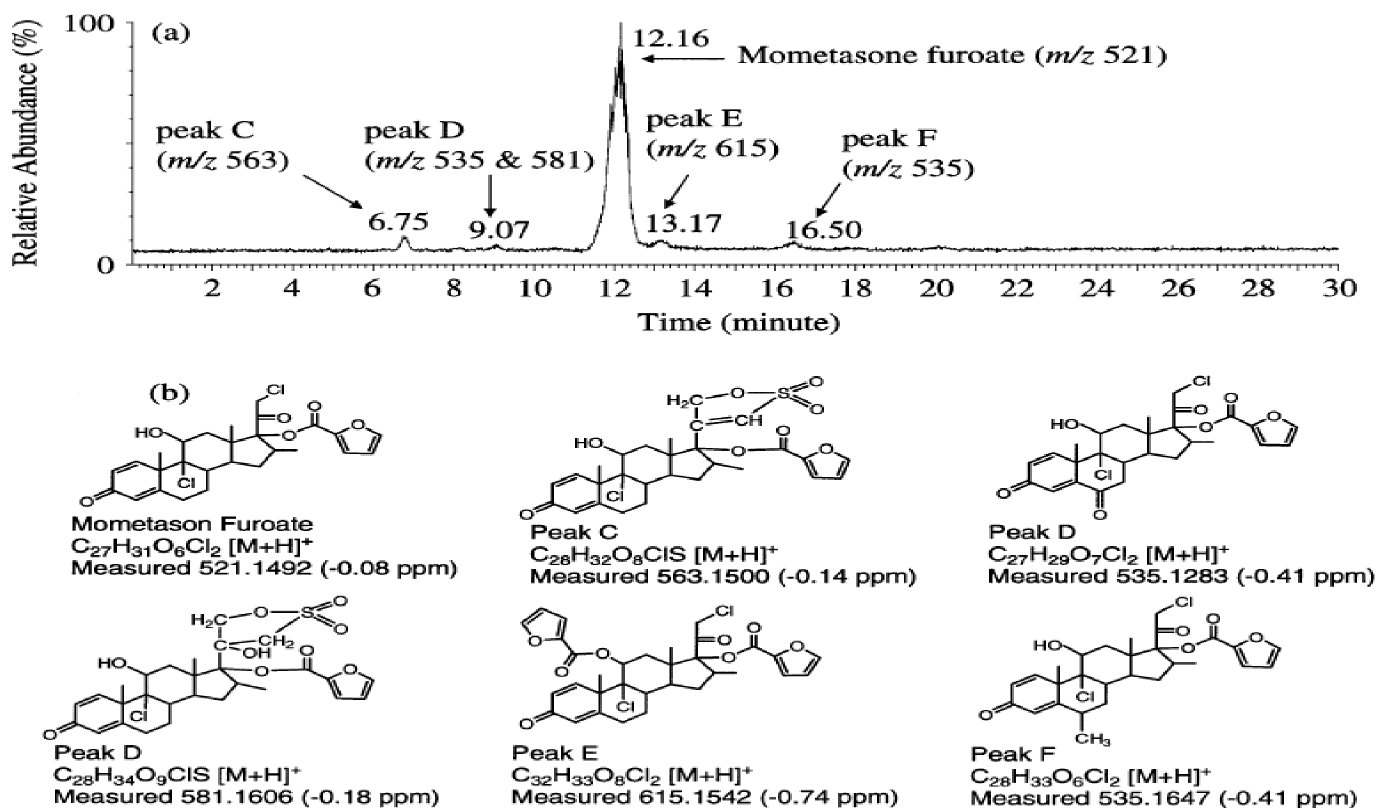

Figure 6a: LC/MS total ion chromatogram of the bulk drug substance mometasone furoate.

6b: Proposed structure of impurities.

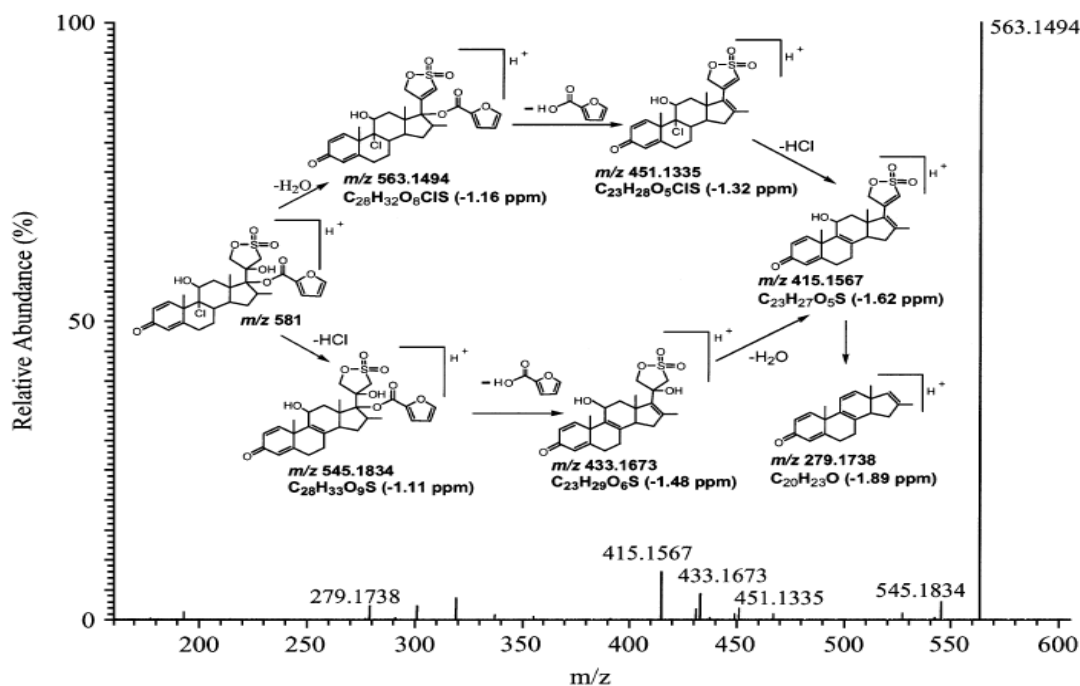

Figure 7: LC/MS/MS Product ion mass spectrum.

HIGH-THROUGHPUT ANALYSIS[23]

One important aspect of the drug discovery efforts is the lead compound generation that involves screening thousands of compounds for biological activities. These compounds are derived from different sources, including traditional synthetic organic compounds collection, combinatorial chemistry, and natural products isolation. With accumulation of large number of compound collections in research laboratories, it is imperative to have accurate analytical methodologies for analysis of these compound collections with high speed. The LC/MS technique is well suited for this task. There are different approaches to high-throughput analysis by LC/MS, depending on specific needs. For samples from medicinal chemistry, the sample analysis can be carried out by chemists using open access MS system composed of an auto-sampler, a HPLC system and a mass spectrometer.

The advantage of LC-MS analysis is the quick generation of MW information on reaction products with a very short analysis time (1–2 min), although it does not provide the sample purity information, and ionization suppressions can occur without LC separation of individual components. On the other hand, the GC/MS mode generates the MW and sample purity information with minimized ionization suppressions. The run time can vary from 5 to 10 min, depending on the specific methods. All data analysis is automatically performed by the workstation. This approach

only needs minimum user training on sample submission, and expertise in carrying out MS experiments is not required. The chemists can monitor chemical reaction products in real time and make quick decisions about synthetic strategies. This has become a part of daily activities for medicinal chemists, resulting in tremendous increase in productivity.

In some cases, the reaction products can be complex mixtures, which require the coordinated use of LC/MS and LC/MS/MS methods. It is also common to include a UV detector in the LC/MS system to generate LC/UV chromatograms. Based on UV responses, compound purity can be assessed as well. For large compound libraries derived from combinatorial chemistry, a parallel LC/TOF-MS system with multiple channels (four or eight HPLC columns) can be utilized to analyze multiple samples almost at the same time, increasing the throughput by four to eightfold. For example, samples in a 96-well plate may take 8 h for the analysis using a conventional single quadrupole mass spectrometer, while a four-channel parallel LC/TOFMS system can reduce the run time to 2 h. The key to the success in high-throughput analysis by LC/MS is the automation and integration. The entire system has to be robust and automatic for all processes, including sample submission, MS analysis, and data analysis. Fortunately, with the development of advanced instrumentation and software, automation is highly achievable in generating reliable data. Another critical component in high-throughput analysis is the data management. With thousands of spectra produced every day, an effective instrument data management system is essential to handle daily data storage and archiving, and organize the data for easy access by other researchers.

DRUG DEVELOPMENT[23]

Liquid chromatography/mass spectrometry (LC/MS)- based techniques provide unique capabilities for pharmaceutical analysis. LC/MS methods are applicable to a wide range of compounds of pharmaceutical interest, and they feature powerful analytical figures of merit (sensitivity, selectivity, speed of analysis, and cost effectiveness). These analytical features have continually improved, resulting in easier to use and more reliable instruments. These improvements were timely and coincided with the aforementioned developments in the pharmaceutical industry.

An important perspective of these events, improvements in LC/MS technology and industry change, is how LC/MS techniques became so widely accepted within every stage of drug development. It can be argued that the proliferation

of LC/MS occurred not by choice but by need. For example, if a nuclear magnetic resonance (NMR)-based approach existed for the quick, sensitive, and efficient analysis of combinatorially derived mixtures in the early 1990's, then LC/MS would certainly have had a limited role in this area of drug development.

Along with timing and perception issues, there are four technical elements that have been critical for the recent acceptance of LC/MS-based techniques in the pharmaceutical industry. The first is separation sciences. Simply put, the chromatographic method defines the pharmaceutical analysis. Chromatography provides analytical criteria to compare, refine, develop, and control the critical aspects of developing and manufacturing high quality drug products. Thus, it is common in industry to see LC/MS methods distinguished by the chromatographic technology and features rather than by mass spectrometry performance and capabilities. Indeed, the effective combination of a wide variety of HPLC technologies and formats with mass spectrometry played a vital role in the acceptance of LC/MS.

It is highly dependent on software to integrate key analysis elements that deal with sample preparation, real-time analysis decisions, and the distribution of results. The pharmaceutical industry has benefited from this trend and, as a result, the derived information has been easily translated into a form that many professionals can understand, interpret, and base their decisions

DATABASES

One strategy for taking advantage of (leveraging) processed information is to categorize it into databases. Although a modest amount of time and resources is required to implement this strategy, databases have two important benefits. First, it provides a reference-friendly format to search data. This feature is essential for the rapid identification of known compounds. For example, the identification of a metabolite may require only retention time and molecular weight information via LC/MS analysis when compared to the metabolite structure database compiled from previous LC/MS/MS studies. The process of identifying a natural product in a plant extract, when the natural product has been previously identified and recorded in the database, is often referred to as ``dereplication''.

A second benefit of databases is the efficient extraction of information. The database may be ``mined'' to detect trends that may not be otherwise noticed. This information is useful for candidate selection and development planning activities. Once established, a database is transferred to other laboratories that are participating in the specific drug

development activity. The resulting databases are readily accessed via information intra-nets. Information is coordinated within the database, and a variety of scientists ``pool'' their information.

SCREENING

LC/MS emerged as an advantageous technique for screening. Screening-based strategies are incorporated into quantitative bioanalytical assays to provide a highly selective approach for high throughput analysis. Highly automated methods featuring fast chromatographic separations in combination with either one or two dimensions of mass spectrometry analysis provide powerful methods for quantitative analysis. The two approaches are referred to as selected ion monitoring (SIM) and selected reaction monitoring (SRM). In experiments that use SIM, the mass spectrometer is set to detect a selected ion mass that corresponds to the drug molecule. Sensitivity is enhanced by using this selected ion drug screening approach as opposed to sampling a range of ions via a conventional mass spectrum[25].

BIOMEDICAL APPLICATIONS

Hyphenated techniques are often used for structural identification of biomedical compounds.

Nucleosides and Nucleotides

Nucleic acids are well known as difficult to analyze materials. Attempts to develop the technique for the analysis of nucleic acid constituents have been made and the method has been used to analyze human urine for nucleosides. Enzymic hydrolysates were analyzed using thermospray and with the same technique, adducts of carcinogens to guanosine and alkylation mechanisms were investigated. Modified nucleobases and analogs were determined, and with continuous flow FAB nucleotides and their metal complexes were studied.

Peptides and Proteins

For the structure analysis of proteins it not only necessary to determine the molecular weight, but also to analyze fragments made with enzymatic digests and even the amino acid composition, if necessary. The last **was** mentioned already, but the determination of molecular weight can be achieved either by electrospray coupled with microHPLC or, even more efficient with capillary electrophoretic techniques.

When it becomes possible to extract the information on peptide sequence from the electrospray LC/MS/MS experiments made with multiply charged ions, that technique will be a very valuable tool for the protein chemists.

Steroids

The analysis of body fluids to determine the steroids has several aims; the most important ones are the detection of steroid drugs and the profiling of endogenous steroids. Both can be done quite effectively using LC/MS techniques. Among the drugs, dexamethasone and its metabolites have been determined, stilboestrol analogues using thermospray, trenbolone and testosterone esters in tissue and injectables

The use of thermospray LC/MS in profiling steroid conjugates in body fluids has been demonstrated successfully, in particular steroid sulphates can be detected with high sensitivity. In another study, plasmaspray was used to analyze saliva of patients with congenital adrenal hyperplasia for steroid hormones.

Amino Acids

Amino acids were among the first compounds analyzed by LC/MS. A moving belt interface combined with SIMS and laser desorption and thermospray was used.

Saccharides

The most often used technique for the sugars and oligosaccharides is continuous flow FAB, which appears to be sensitive enough.

Bile Acids

In the past, procedures using static FAB methods were developed for the analysis of bile acids, but with API LCMS interfaces and with thermospray they have been determined successfully **as** well.

DRUG METABOLITES

The analysis of drug metabolites is one of the most important applications for LC/MS, since most of the metabolites are either chemically or thermally labile, and usually the isolation and purification involves some sort of liquid chromatography.

Therefore, the development of analytical procedures is straight forward, if a suitable interface and MS technique is available. Depending on the nature of the drug and the metabolite, moving belt technique has been used; recently the thermospray and increasingly the API techniques have shown great potential for the analysis of labile and highly polar metabolites. Metabolic profiling using thermospray LC/MS/MS has proven to be a rapid and successful technique for the search for new metabolites.

As a few examples, studies shall be mentioned concerning the metabolism of ranitine, betamethasone, doxylamine, 8-methoxypsoralen and almitrine in body fluids and the determination of N-methylformamide-glutathione adducts.

Glucuronides

Conjugates of glucuronic acid are candidates for thermospray mass spectrometry due to their high polarity and several studies have been published. Steroid glucuronides were characterized as well as those from methapyrilene and from several phenolic compounds. Using CE/MS drug glucuronides derived from formamides have been identified.

Coniugates

Among the metabolites discussed earlier are **a** broad range of conjugates, but a few classes should be mentioned here separately. Steroidal conjugates have been studied extensively, normally employing thermospray as mentioned already. Likewise, glutathione and its conjugates especially with methylformamide and monomethylcarbamates were assessed using thermospray[28].

ENVIRONMENTAL MONITORING

GC-MS is becoming the tool of choice for tracking organic pollutants in the environment. The cost of GC-MS equipment has decreased significantly, and the reliability has increased at the same time, which has contributed to its increased adoption in environmental studies. There are some compounds for which GC-MS is not sufficiently sensitive, including certain pesticides and herbicides, but for most organic analysis of environmental samples, including many major classes of pesticides, it is very sensitive and effective.

The examples from application in environmental analysis have to be very diverse, because the matrices and the analytes span over a wide range of physically and chemically different materials. Matrices can be soil, sand or sludge, drinking or waste water and air (gaseous analytes or particulates). The analytes range chemically from nonpolar hydrocarbons to ionic organometallic species, all requiring an appropriate means of concentration, separation and detection.

Pesticide

For the analysis of several herbicides, LC/MS and in particular thermospray LC/MS, appears to be the method of choice. Triazine derivatives have been determined using LC/MS/MS. The chlorophenols and the phenoxyalkanoic acids, rather widely used herbicides, can be determined as well as the sulfonylurea herbicides using thermospray and

organophosphorus compounds. Organometallic compounds are usually determined either with atomic detection methods such as AAS, giving information about the element only, or, after derivatization, by GC/MS of the fully alkylated species. It has been shown that not only the organo lead species derived from tetraethyllead in gasoline can be identified using thermospray LC/MS, but some tin compounds such as fentinacetate (triphenyltin acetate), a fungicide, and marine paint additives such as di- and tributyltin chlorides have been measured with thermospray as well.

METABOLOMICS

LC-MS is also used in the study of proteomics where again components of a complex mixture must be detected and identified in some manner. The bottom-up proteomics LC-MS approach to proteomics generally involves protease digestion and denaturation (usually trypsin as a protease, urea to denature tertiary structure and iodoacetamide to cap cysteine residues) followed by LC-MS with peptide mass fingerprinting or LC-MS-MS (tandem MS) to derive sequence of individual peptides. LC-MS-MS is most commonly used for proteomic analysis of complex samples where peptide masses may overlap even with a high-resolution mass spectrometer. Samples of complex biological fluids like human serum may be run in a modern LC-MS-MS system and result in over 1000 proteins being identified[27].

Conclusion

The techniques developed from the coupling of separation technique and online spectroscopic detection is known as hyphenated techniques. In the last two decades there has been tremendous use of these techniques in analytical detection. These techniques have wide variety of applications but mostly they are used in separation of natural products. Its various applications include isolation and analysis of natural products, chemical fingerprinting and quality control of herbal medicine, Chemotaxonomy Metabolomic and proteonomic studies. The various applications are discussed in the article itself.

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