

# A Review: Recent Trends in Analytical Techniques for Characterization and Structure Elucidation of Impurities in the Drug Substances

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## Parmar *et al.*: Recent Analytical Techniques for Characterization of Impurities

Impurity is an unwanted substance present in the active pharmaceutical ingredients that form during the synthesis process of active pharmaceutical ingredients or any unwanted constituent that is produced besides the active ingredient during the formulation or the aging of active pharmaceutical ingredients. Even the insignificant quantity of impurity existing in the medicinal product may harm the patient life and compromises the purity and superiority of the medicinal product. According to International council for harmonisation guidelines, analytical monitoring of impurity is a prerequisite and mandatory requirement for approval of market authorization of the new drug substance. Any pharmaceutical product would be capable to serve their intended therapeutic activity when they are free from impurity. Thus, an impurity existing in an active pharmaceutical ingredient needs to be identified and quantify with the help of modern analytical approaches. This review explores the basic information concerning impurity profiling, highlights the advantages of an analytical technique and also focuses on the limitation of different analytical methods for impurity profiling with possible ways to overcome the limitation.

**Key words:** Impurity profiling, active pharmaceutical ingredients, drug substance, hyphenated technique, chromatographic separation techniques

The therapeutically active product comprising of active pharmaceutical ingredients (API) and excipients, the API is responsible for producing pharmacological effects after absorption in systemic flow in the living body. But in some circumstances, the active constituent or excipients could not be 100 % pure and may contain some other component that may arise in the medicinal product from different sources, i.e., from synthesis, an excipient, residual solvent, degradation product. These unwanted components other than API and excipients are known as impurities. Many definitions for impurity and impurity profiling have been well-defined in different reviews and guidelines. Impurity is the product or substance form in the synthesis includes intermediate or the side product of intermediate that forms during the side reaction or unwanted chemical reaction<sup>[1,2]</sup>.

In any pharmaceutical product or drug substance, if the impurities are expected to be present, then they need to be identified and characterized by using appropriate analytical methods and this procedure is known as impurity profiling. Impurity profiling is a

systemic process to identify the unknown impurity and to isolate the impurity to elucidate the structure. It is an important approach designed for identifying and quantifying the impurities existing in the medicinal substance<sup>[3]</sup>. Impurities are needed to be recognized and characterize, should be there in acceptable limits which do not produce any toxicological effect in living body thus, to synthesis the medicinal product of best quality and efficacy demands for impurity reporting in upcoming days. Impurity profiling requires highly sensitive, selective and efficient analytical techniques to regulate the trace quantity of impurity. The highly accurate, precise and sensitive technique is demanded in the current era for impurity profiling because the impurity may exist in a very trace amount in the drug

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substance that is very difficult to isolate and identify with lower sensitivity and accuracy techniques and in most of the cases the structure of impurities are derivatives or degradants of the parent drug molecule thus, hyphenated analytical techniques is a vital requirement for impurity identification. Various current approaches in investigative techniques are now available for identification, characterization and the structure elucidation of impurity<sup>[4]</sup>.

### Impurity profiling significance:

In assuring the high standard quality of drug products reached into the market, it's important in screening impurities existing in the medicinal product throughout manufacturing. As compared to the online spectrum obtained during the earlier impurity profiling study that is required for registration of the drug master file, the standard impurity spectrum is of better purity and superiority. Synthesized impurity is used for toxicological studies and also use as the standard for determining impurity<sup>[5]</sup>. The impurity existing in the pharmaceutical product may vary the dissolution and solubility of the drug constituent and may affect the systemic circulation thus, it not only affects patient safety but also alter the biopharmaceutical behaviour of the drug substance. Thus, impurity profiling is essential for assuring the quality, efficacy and safety of pharmaceutical products<sup>[6,7]</sup>.

### Types of impurities:

Impurity may be divided into three types such as an organic, inorganic and residual solvent. The further classification of impurity is shown in the fig. 1<sup>[1]</sup>.

## CHARACTERIZATION OF IMPURITY

The detected impurity is further characterized by diverse spectroscopic techniques, before the characterization sufficient amount of impurity essential be isolated by suitable separation techniques. As stated in the International Council for Harmonisation (ICH) standard that when the level of impurity is greater than 0.1 % then it should be recognized and characterized appropriately. The various techniques for impurity profiling are given in fig. 2 and the examples of analytical techniques use in structure elucidation of impurity in different API are shown in Table 1.

## SEPARATION TECHNIQUES

### Accelerated solvent extraction:

The accelerated solvent extraction technique is unique and extensively uses a technique that involves the extraction of a chemically active constituent with the help of solvent which penetrates within the pores of the solid matrix for extracting the desire chemical constituent. It has found its application in various pharmaceutical fields. Most widely applied for extraction of a natural chemical constituent from herbal plant materials. The application is not only restricted for the extraction of natural constituents but has also found its application in the impurity profiling of drug substances. The technique has been extensively employed in screening micro-organism, dietary supplements, insecticide residue, an examination of environmental samples and organic contaminants. This technique has several benefits over the traditional method (soxhlet extraction, maceration, purification, turbo-extraction and sonication).

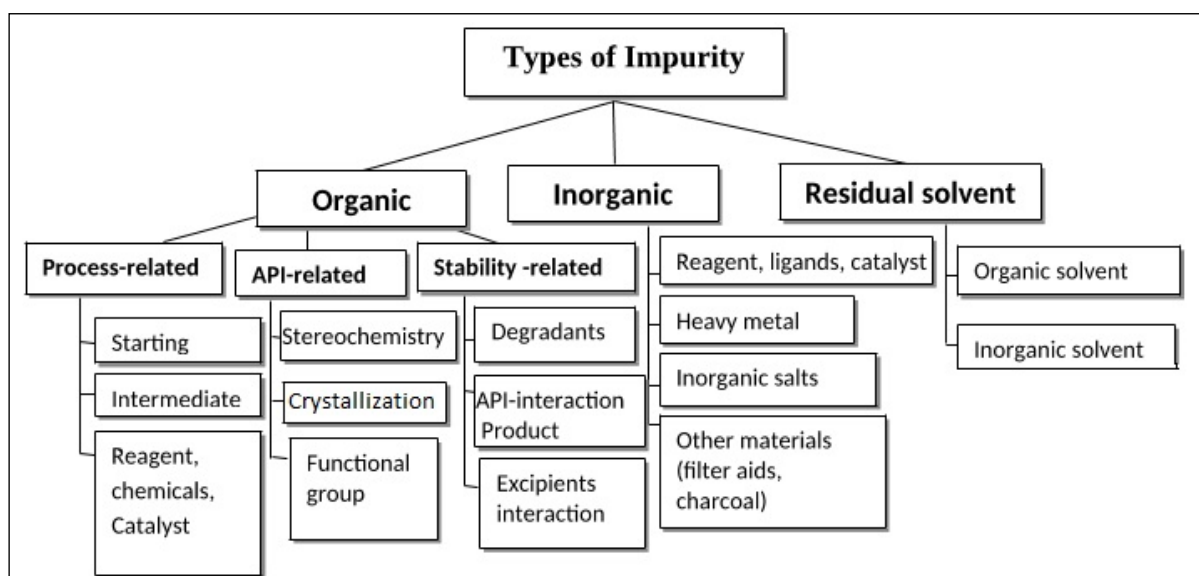


Fig. 1: A flow chart of types of impurity

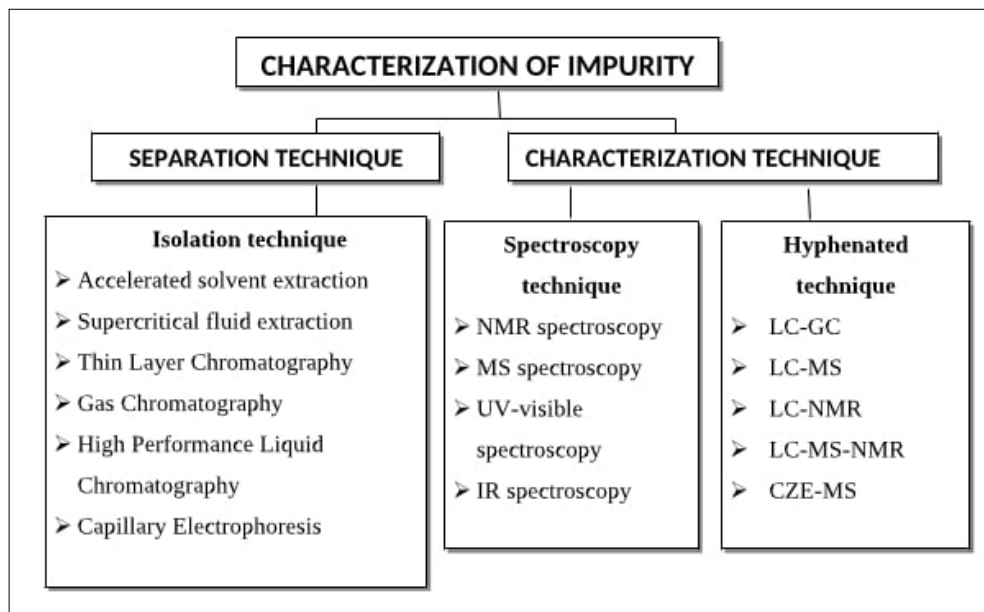


Fig. 2: Techniques for impurity profiling

TABLE 1: ANALYTICAL METHOD USED TO DETECT AND IDENTIFY THE STRUCTURE OF IMPURITY IN ACTIVE PHARMACEUTICAL INGREDIENTS

Sr No	Drug	Impurities	Method to detect impurities	Method to identify impurities	References
1	Ezetimibe	A. 2-(4-hydroxybenzyl)-N,5-bis(4-fluorophenyl) Pentanamide B. 1-(4-fluorophenyl)-3(3-(4-fluorophenyl)propyl)-4-(4-hydroxyphenyl)azetidin-2-one	HPLC (Waters Alliance 2695 separation module)	MS,NMR,FT-IR,TLC	64
2	Halobetasol propionate	A. Diflorasone diacetate B. Diflorason acetate 17 propionate C. Halobetasol D. Diflorasone acetate 21 propionate E. Diflorasone17 propionate21 mesylate F. Clobetasol propionate	UPLC (ACQUITYUPLC System)	LC-MS/MS	65
3	Icatibant	4,4(5,5(1E,1E)-3,3(4,4methylenebis(thiophene-4,2-diyl))bis(2-carboxyprop-1-ene-3,1-diyl)bis(2-butyl-1H-imidazole-5,1-diyl))bis(methylene)dibenzoic acid	Agilent 1260 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped	NMR,MS-MS	66
4	IIIM-290 (preclinical candidate)	A. Rohitukine, B. IIIM-290-NO	HPLC (LC-6AD HPLC system)	NMR, FTIR, and ESI-MS	67
5	Tolterodine tartrate	A. N-(3-(2-hydroxy-5-methylphenyl)-3-phenylpropyl)-N,N-diisopropyl hydroxylammonium trifluoro acetate salt. B. 3-(2-hydroxy-5-methyl phenyl)-N-isopropyl-3-phenyl propane-1-amine oxide	HPLC (Waters ACQUITYT UPLC system)	MS, NMR	68
6	Olanzapine	A. 2-methyl-4-(4-methylpiperazin-1-yl)-10-((methylthio)methyl)-thieno[2,3-b][1,5] benzodiazepine B. 10-(3-(1H-benzo[d]imidazol-2-yl)-5-methylthiophen-2-yl)-2-methyl-4-(4-methylpiperazin-1-yl)-thieno[2,3-b][1,5] benzodiazepine	HPLC(Shimadu LC-20AD)	UV,FT-IR, LCMS/TOF, NMR and X-ray diffraction analysis	69
7	Isoproterenol hydrochloride	A. isoproterenone or (1-(3,4-dihydroxyphenyl)-2(isopropylamino)ethanone hydrochloride) B. 4-[2-(propan-2-ylamino)ethyl]benzene-1,2-diol	UHPLC(Nexera-X2)	NMR, IR, LCMS/ESI	70
8	Meprobamate	Carbamic acid 2-carbamoyloxymethyl-2-methyl-pent-3-enyl ester	SFC(Waters Acquity UPC)	LC-MS	71

9	Metoprolol tartrate	$C_{27}H_{45}NO_{13}$ -adduct of lactose and Metoprolol formed by Maillard reaction	HPLC (Waters Model Alliance 2695 Separation Module)	NMR, IR	72
10	Dapoxetine		TLC	IR, NMR, and MS	73
11	Rosuvastatin		UPLC (Waters Acquity system)	UPLC-MS	74
12	Simvastatin	<p>A. 1-(2E)-cinnamyloxynaphthalene B. 1-(2Z)-cinnamyloxynaphthalene</p> <p>A. Anti-isomer impurity: (3R,5R)-7-[4-(4-fluorophenyl)-6-isopropyl-2-methyl(methylsulfonyl amino)pyrimidin-5-yl]-3,5-dihydroxy-6(E)-heptenoic acid. B. Lactone impurity: N-{4-(4-fluoro-phenyl)-5-[2-(4-hydroxy-6-oxo-tetrahydro-pyran-2-yl)-vinyl]-6-isopropyl-pyrimidin-2-yl}-N-methyl-methanesulfonamide.</p> <p>A. 7-[7-(2,2-dimethyl-butryloxy)-2,6-dimethyl-1,2,6,7,8,8a-hexahydro-naphthalen-1-yl]-3-hydroxy-5-hydroxymethyl-heptanoic acid A. 5-ethyl-7-methyl-6-(2-chlorophenyl)-8-methyl-3,4,6,7-tetrahydro-2H-1,4-benzoxazine-5,7-dicarboxylate</p>	HPLC(Waters Acquity system)	MS/MS	75
13	Amlodipine Maleate	<p>A. des carboxy ofloxacin B. ofloxacin-N-oxide C. N-des methyl ofloxacin D. 9-methyl piperizine E. difluoro pyrido benzoxazine carboxylic acid</p>	HPLC(Agilent 1100 series)	LC-MS/MS NMR, IR	76
14	Ofloxacin		HPLC(Water Breeze)	-	77

To improve the extraction process; this technique utilizes high temperatures and pressure. The elevated temperature will enhance the extraction kinetics thus, it leads to a decrease in the viscosity of the sample medium, which enhances the diffusion of the liquid into the sample medium. High pressure will force the solvent inside the sample medium pore and hence ease the extraction process<sup>[8,9]</sup>.

#### Benefits of accelerated solvent extraction technique over other techniques<sup>[10]</sup>:

The accelerated solvent extraction technique is a quick and competent technique for extracting chemical constituents from the solid sample. The extraction technique is faster. A smaller volume of solvent consumption, thereby reduce environmental pollution.

Extraction yield increases, by decreasing the inclusive extraction cost. It is a reproducible technique

**Limitations:** Thermo labile compound might be susceptible when exposed at the higher temperature, thus when they are exposed to high temperature it undergoes degradation.

### **Supercritical fluid extraction:**

A substance over its critical temperature and pressure is known as a supercritical fluid (SCF). It has a property of both fluid and gas, it has gas like mass transmission and fluid-like solvating power hence provide an additional advantage over other methods. The supercritical fluid has wide applicability over the extraction of the natural component from plant materials besides also remained active in impurity profiling<sup>[11]</sup>.

### **Advantages of supercritical fluid extraction over other techniques<sup>[12,13]</sup>:**

The supercritical fluid extraction technique has a property of both liquid and gas thus can diffuse inside the sample medium identical to gas and dissolve the solid similar to a liquid and lead to accelerating the mass transfer process. Increase the efficiency of extraction. A chiefly employed supercritical fluid is carbon dioxide as it's cheaper and easily available. Supercritical fluid CO<sub>2</sub> is eco-friendly and identifies as safe by the Food and Drug Administration (FDA) and European Food Safety Authority (EFSA). The critical condition of CO<sub>2</sub> is at 30.9° temperature and 73.8 bar pressure. Similarly, used for thermo labile samples or easily oxidizable compounds as it remains operated at low temperatures.

**Limitations:** Supercritical fluid CO<sub>2</sub> has low polarity; this can be overcome with the addition of co-solvent (methanol). By adding co-solvent, it will be alternating the polarity of SCF and enhance the solvating power.

### **Thin-layer chromatography (TLC):**

TLC is a unique and most reliable analytical tool for testing the purity and identity of any compound. Thin-layer chromatography would need shorter development time, visualization of the separated compound is easy, separation procedure is quicker and TLC is the cheaper technique and minor sample size is required and reduced the quantity of solvent consumption. TLC can test a wider polarity range, so all the contamination can be detected. By using the newly hyphenated Liquid chromatography-mass spectrometry (LC-MS) technique identification of unknown impurity can be achieved,

avoiding time-consuming isolation through simply scrapping off followed through mass spectrometry (MS) determination for clarifying the structure of unknown impurity. Thin-layer chromatography (TLC) /High-performance thin-layer chromatography (HPTLC) and other planar chromatography techniques used for various applications in the pharmaceutical field in the analysis of numbers of drugs<sup>[14,15]</sup>.

**Limitations:** Results of TLC are not completely reproducible and reliable as it's an open structure. Critical factors like environmental changes and analyst variation may influence the result. It provides only qualitative data, quantification is not possible. It's a non-automated technique, the manual error may also influence the correctness and exactness of the result. To resolve the weakness of TLC recent advances have been made that permit quantitative assessment through high precision densitometer and automatic multiple developments (AMD) is a distinct developing chamber that improves the selectivity of the method.

### **Advantages of HPTLC over TLC<sup>[16]</sup>:**

The limitation of TLC can be overcome with the advance and automated High performance thin layer chromatography technique. It provides reproducible results and optimizes the analysis condition. HPTLC has a high resolution, sensitivity and speed; as compared to TLC. Quantification can be accomplished automatically by scanning the plate with the densitometer. Simultaneously, two analysts can complete the analysis at a time. Less solvent consumption in the analysis.

### **Gas chromatography (GC):**

Gas chromatography is a unique investigative tool, most applicable for impurity profiling. The chief benefit of GC is that it is capable of isolating the volatiles from non-volatile medium over the specially employed sample pre-treatment. A residual solvent can be determined by GC since no other method can be employed for residual solvent determination. GC has high separation power, good selectivity and a wide choice of flame ionization detector (FID). GC renders the highest separation potential and for detection of the sample; linearity can be achieved up to 10<sup>7</sup> units. The dynamic headspace mode of sample pre-treatment provides the highest sensitivity<sup>[17]</sup>.

**Limitations:** It cannot be practice for the study of the non-volatile and thermally unstable compound. GC is

a unique separation technique where ultraviolet (UV) spectra cannot be taken. A direct injection mode of sample pre-treatment decreases the sensitivity.

#### **The significance of derivatization technique<sup>[18,19]</sup>:**

Derivatization technique is required for analysis of the compound which undergoes degradation when exposing to higher temperatures. The derivatization technique replaces the polar functional group, to increase its volatility and detection ability. Trimethylsilyl is most widely used as a derivatizing agent for derivatization in GC, which results in the silylation of -OH and -COOH group and enhances the thermal stability and volatility.

Derivatization also uses to furnish the information of the functional group present in the compound, through making a comparison of retention time of the derivative compound and un-derivative substance and additionally mass spectra acquire by GC/MS.

#### **High-performance liquid chromatography (HPLC):**

The HPLC is an automated, separation technique that provides high sensitivity, selectivity and high-resolution power. The technique is fast and efficient, for testing purity and the separation of impurities existing in the drug substance. Determination of impurities in biological material, the reverse phase HPLC technique is extensively used. When HPLC is employed as a separation technique, UV-detector can produce good quality UV spectra. Sample preparation is easy in this system, even the error is also minimized<sup>[20]</sup>.

**Limitations:** To detect the compound in HPLC, the compound should possess, following structural features such as UV chromophore and the fluorescence element or electrochemical activity, to attain the appropriate detection power. The drug substance that does not contain any structural elements like UV chromophore and a fluorescence element or electrochemical activity is analyzed by derivatization of the drug substance<sup>[21]</sup>.

#### **Capillary electrophoresis (CE):**

Capillary electrophoresis is a very valuable technique in impurity profiling. Impurity determination is an issue, since the impurity may be relatively complex and usually, impurities may be structurally analogous to the core drug substance. High peak efficiency is the benefit and strength of CE over other separation techniques. CE can be operated in diverse modes, thus there is an increase in the separation capability<sup>[22]</sup>.

#### **Advantages of capillary electrophoresis over liquid chromatography (LC)<sup>[23]</sup>:**

The analyte ranging from smaller ions to larger protein molecules can be analyzed using the identical capillary and provides higher separation efficiency. The instrument operated under a wide range of pH of the solution using aqueous or non-aqueous conditions deprived of resetting of the instrumental setup. Most extraction techniques make use of an organic solvent for the extraction, but CE is the only technique that mainly utilizes the aqueous buffer. Thus, the global scarcity of organic solvent mainly acetonitrile can be overcome. CE is an eco-friendly technique and reduces waste production. A pseudo-stationary phase is used in the micelle electrokinetic chromatography (MEKC) mode, which is employed for the extraction of neutral molecules. The pseudo-stationary phase is a design by accumulating a desirable surfactant in the background electrolyte. For derivatization reactions of the analyte, sample preparations need to be performing within the capillary.

**Limitations:** Due to the low optical path length of the UV detector, capillary electrophoresis has a low sensitivity. There is overheating of the sample, therefore limited to the use of low voltage. In terms of instrumentation, a few options available. As compared to the liquid chromatography technique, CE is more complicated.

### **CHARACTERIZATION TECHNIQUE**

#### **UV- Visible Spectroscopy:**

To regulate and determine the purity of the drug material, unique and easiest methods are UV-VIS spectroscopy. The spectrophotometric active drug constituent can be controlled using UV-VIS spectroscopy. It's also used for the impurity detection within the drug substance. The gradient HPLC technique is most widely used for impurity profiling, where the peaks are tracked using the retention time<sup>[24]</sup>.

But the problem to track and differentiate between the impurity and drug substance peak is the retention time may shift or when the peaks are not properly resolved. Thus, to resolve this issue UV detection using a diode array detector that provides the UV spectrum of the unknown peak and to perform the matching using some algorithm, to a spectrum in a spectral library. The spectral library is prepared by using the reference standard. The investigation is mainly focused on the

major peaks and the minor peaks, generally at less than 0.1 UV area percent, have not been characterized<sup>[25]</sup>.

**Advantages of UV-Visible Spectroscopy<sup>[26]</sup>:** This technique is cost-effective over other analytical techniques and sample preparation is easy and quick. Require lesser analysis time and provide higher precision and accuracy. Provide a wide range of alternatives for the selection of solvents and chemicals used in the analysis of the sample. Sample can be reuse and recover for further analysis, as it does not undergo any destructive effect during analysis.

**Limitations:** The drug structure must have a UV chromophore in its structure, to absorb the UV radiation. The UV cut-off is considered as an essential parameter for the selection of the solvent for the preparation of a stock solution. Thus, the absorption maxima of the drug substance and solvent used for the preparation of the stock solution should not be identical<sup>[27]</sup>.

### **Infrared (IR) spectroscopy:**

The drug material will absorb a specific wavelength when exposed to electromagnetic energy, a specific bond present in the structure will absorb at a characteristic's wavelength. Thus, this technique can be active to detect the sample structure by identifying the functional group prevailing in the sample. A lot of time is consumed in the sample preparation in the chromatographic technique, even though it has many advantages such as the better resolution of the impurities even in the multi-component sample. A new advance in the impurity profiling method is the use of a spectroscopy method coupled with chemometric as a replacement for the chromatographic method. The *Fourier-transform infrared spectroscopy (FT-IR)* technique is a fast, less expensive technique, but for separation and determination of impurity, this technique cannot be applied directly. Hence chemometric technique is requiring chiefly multivariate regression for impurity profiling. The chemometric technique seems to be crucial in the extraction of information from complex data sets. The combination of IR with chemometrics will simplify and improve the quality control method of the drug substance in the manufacturing process<sup>[28]</sup>.

The method has been reported that has applied the FT-IR method along with the chemometric method for the identification of impurity in the simvastatin. Two methods were applied such as cluster analysis and principal component analysis for the data obtained from FT-IR spectroscopy. The partial least square

model was built to predict the relative content of the lovastatin, the main impurity of simvastatin and the sum of statin like impurities. The method was able to predict the impurities content in the drug product containing simvastatin with good prediction ability ( $R^2 > 0.95$ ). Each molecule has a unique fingerprint spectrum thus use to analyze the drug and its impurities<sup>[29]</sup>.

**Limitations:** Sample preparation is very time-intense. It can't give detailed information as nuclear magnetic resonance (NMR) spectrometry. The method is destructive; the sample cannot be reused for further analysis. For detection in the IR range, require IR active sample.

### **Mass spectrometry (MS):**

Mass spectrometry offers high reproducibility, specificity and it's a highly sensitive technique in the analysis of a trace compound and for elucidating the structure. The identification of biomolecules or protein molecules present in a biological sample can be completed using mass spectroscopy and the study of high molecular mass, non-volatile and thermally susceptible compound can be possible by the introduction of a soft ionization technique. The parent molecule undergoes ionization to produce ions or fragments and they travel to the analyzer compartment of the instrument where ions are resolved according to their mass to charge ratio. The mass spectrum will provide data concerning the molecular composition of the parent drug compound. MS is coupled with different chromatographic techniques. This hyphenated technique has wide application in the determination of impurity structure<sup>[30]</sup>.

One of the drawbacks in mass spectrometry techniques that the excipients present in the formulation may also contribute to the mass spectrum that resulting in a complex mass spectrum since it also suppresses the analyte signal. Whereas, in the UV spectrometry technique, excipients do not interfere in the UV chromatogram. This problem in mass spectrometry analysis can be overcome by using tandem mass spectrometry which reduces solvent interference<sup>[31]</sup>.

### **Mass spectrometry without chromatographic separation:**

Tandem mass spectrometry (MS-MS) is an advanced analytical method that utilizes a broad range of mass analyzers in a sequential arrangement. Each of these

analyzer configurations has certain characteristic features that offer a particular advantage over the other configuration. Tandem mass spectrometry can have a detection limit up to ppm-level. Thus, due to the high sensitivity of this technique, it does not demand prior tedious chromatographic extraction of the compound on a preparative scale. The tandem mass spectrometry can provide the online spectra; therefore, minimize the time for extraction and also reduce the solvent interference in the mass spectrum<sup>[32,33]</sup>. An example of the enormous specificity and sensitivity attain by tandem mass analysis is the characterization of clindamycin in which six impurities were detected with the online mode of analysis<sup>[34]</sup>.

#### **Limitations of Mass spectrometry without chromatographic separation:**

The method is restricted to the differentiation of the isomers with the same molecular weight. The differentiation between the stereoisomer and positional isomers can be made possible by the prior separation of the sample by chromatographic technique followed by mass spectrometry analysis.

#### **Mass spectrometry with chromatographic separation:**

Different chromatographic methods are employed in the isolation of impurities and the fraction isolated can be analyzed in mass spectrometry. The differentiation between the stereoisomer and positional isomers can be made possible. An example of differentiation of stereoisomer, described in discrimination of eight chloramphenicol isomers by LC-MS/MS sequentially to examine the natural occurrence of chloramphenicol. In mass spectrometry with chromatographic separation, the analyte of interest can be isolated and pre-concentrated for further detailed analysis and the structure elucidation has been possible using this offline approach<sup>[35]</sup>.

**Limitations:** The drawback of this approach is that the solvent contamination may interfere in the chemical examination of the analyte of attention. The solvent interference or contamination may suppress the analysis of the compound of interest; this can be observed in the fast atom bombardment (FAB) ionization where the compound is more easily ionized. The resolution in the above situation, attain by using a continuous flow of fast atom bombardment ionization, electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) techniques. MS does not use for samples

comprising of inorganic salts along with buffers.

#### **Nuclear magnetic resonance (NMR) spectrometry:**

The field of NMR has proved to be unique to the prosperous and rewarding sources of hypothetical and theoretical research in spin physics; now with new advances, NMR spectroscopy has been developed into a powerful tool and multidisciplinary in its own right. NMR spectrometry provides the magnetic property of the atomic nuclei, hence a useful tool in structure elucidation of unknown molecules. <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy are mainly employed for structure elucidation. Other additional methods are two-dimensional experiments such as heteronuclear single quantum coherence (HSQC) and double quantum filtered correlation spectroscopy (DFC-COSY) which are widely employed for structure elucidation<sup>[36]</sup>. The final and conclusive method for structure elucidation is NMR spectroscopy. The innovation in this technique involves modification of pulse-field gradient, solvent suppression, advancement in probe tools and high magnetic field configuration which will provide the driving energy for the structure elucidation of the unknown impurity<sup>[37]</sup>.

NMR plays an important task in detecting insignificant impurities devoid of or following chromatographic separation. It is a great technique in determining the configuration and structure elucidation of synthetic and organic molecules, providing that they must be accessible in acceptable purity and amount including molecular mass, not more than 50 kDa<sup>[38,39]</sup>.

**Limitations:** The sensitivity is a critical problem in the effective application of NMR spectroscopy. While the unequivocal determination of trace quantity of analyte or impurities is of key significance concerning a figure of essential industrialized welfare, such as in-market approval application, quality control and quality assurance of the formulations, regulatory features and protection of the patent right. After the separation of impurity by using the regular TLC plate and analytical HPLC column, it is impossible to acquire the NMR spectra. The preparative scale separation requires isolating enough quantity of impurity for NMR spectroscopy. Another disadvantage of NMR spectroscopy is that it requires a long time to understand the spectra and the NMR instrument is very expensive.

#### **HYPHENATED TECHNIQUES**

The hyphenated technique is a recent approach in



the field of analytical science, where the two or more technique is a couple with the help of an interface. The standard of impurity is often unavailable for the characterization of impurity, thus; to generate the fingerprint of that unknown impurity, the hyphenated technique plays an important role.

The key idea behind the coupling of MS with the other chromatography method is that there is an increase in the signal-to-noise ratio, hence the noise decrease even faster which increases the selectivity of the analyte, due to an increase in the number of coupling.

Now due to lacking funds and less often use of this technique, some research laboratories cannot afford the installation of hyphenated techniques in their premises, as this technique is very expensive. In this laboratory the structure elucidation of the chromatographically separated fraction followed by the mass spectroscopy analysis for structure identification of the eluted sample fraction; which is very tedious and time-consuming<sup>[40]</sup>.

#### **Liquid chromatography-gas chromatography (LC-GC):**

LC-GC is an extremely powerful practice; it combines the wide separation mechanism of LC and the high efficiency of GC. It is especially appropriate for a pure sample where high sensitivity and selectivity is required. LC-GC can exploit high sample capacity. Extensively used in offline mode. But there is some drawback in operating in an offline mode such as time-consuming analysis, exhaustive in operation and low reproducibility<sup>[41]</sup>.

#### **Advantage of LC-GC in online mode:**

An analysis of the small quantity of a sample, it is difficult to perform the analysis in offline mode, therefore analysis in online mode is a quick, reliable and efficient technique for providing higher sensitivity and analysis of the various sample. The technique is faster, completely automated, additional sensitive and extremely reproducible. Less solvent consumption and also minimize the problems related to the sample manipulation or artifact formation due to atmospheric air interference. The different applications of the LC-GC technique, specified in the reference.

**Limitations:** LC-GC technique requires a complex instrument that used at different interface. Require trained users for careful optimization of various parameters.

#### **Gas chromatography-Mass spectrometry (GC-MS):**

GC-MS is a powerful method for structural elucidation and chemical analysis of a thermally stable and volatile compound. Analyte separations can be achieved through coupling and spectral information with the help of GC and MS provided sensitivity. It has some significance like progressive chromatographic resolution and increases peak capability, requires a single mobile phase in separation, fewer problems with the solubility and separation that have been achieved through electronic controls such as heat programming<sup>[42]</sup>. The GC-MS combination is a compact instrument greatly cheaper and well-known in current laboratories of the pharmaceutical industry. In the determination of the residual solvent, GC-MS holds a vital function. The impurity profiling can be completed besides, the structure elucidation can also achieve, in a case where the peak is associated with solvent interference, it can be detected, thus providing information on the presence of the toxic and hazardous solvent of class I category<sup>[43]</sup>. Solid-phase micro-extraction of more interfering volatile from the sample in addition to the environment sample can be identified with static headspace; hence it is essential to combine solid-phase micro-extraction with MS detection. MS-MS is another alternative for detecting unknown volatile in a highly complex sample. Mass fragmentography is a prevailing means for precise and sensitive detection of residual solvent<sup>[44]</sup>.

Online GC-MS has a great benefit it provides data simultaneously on some impurity at a level of less than 0.01 %. The key benefit of GC-MS is that it can give molecular mass evidence using a chemical ionization method and to elucidate the composite structure it provides information on fragmentation using electron impact (positive and negative charges) ionization technique<sup>[45]</sup>.

**Limitations:** During the ionization procedure some compound gets extensively fragmented, hence provide more complex spectra which are difficult to interpret. However, in various cases, ions are less precise or fragment are too widespread which reduce the sensitivity<sup>[46]</sup>.

#### **High-performance liquid chromatography-Mass spectrometry (HPLC-MS):**

The combination of LC-MS has several advantages over the individual instrumentation LC and MS spectrometry. Owing to the high selectivity, sensitivity,

dynamic range and ruggedness of LC-MS, it has been extensively employed in the pharmaceutical research area. It has excellent sensitivity for the small amount of impurity and degrading products. The innovation in the current era, increase the use of the LC-MS/MS, which is also known as tandem LC-MS. It has wide applicability in the quantitative analysis and structure elucidation of the unknown impurity<sup>[47]</sup>.

The LC can be skilfully couple with the MS, as there is less risk for the solvent and flow rate interference in the LC-MS interface. The important benefits of HPLC-MS are that it can be coupled with a photodiode array UV detector. To furnish all information for the structure explication of the impurity the most extensively use is HPLC/UV/MS/MS. Operating the LC-MS in the multiple reaction monitoring modes that will repeat the experiment (n) number of times, where n in (LC-MS<sup>n</sup>) is the number of MS-MS experiments. This technique is extensively used for structure elucidation<sup>[48]</sup>.

**Limitations:** The first-generation instrument employs a soft ionization method that gives molecular mass records only. In the LC-MS excipient interference is observed repeatedly. The drug product in solution form generally causes contamination of the tip of the capillary. The confirmation of the final structure of the compound can be incomplete without an NMR spectrometry study. Some of the factors that should be considered for optimization of the method such as mobile phase composition, flow rate, additives like a buffer or any ions pair and should be considered the ionization techniques factors such as spray voltage, the temperature of nebulizer, the voltage of cone in MS, nature of gas and its pressure should be considered as an essential factor to be considered.

### **Liquid chromatography-nuclear magnetic resonance spectrometry (LC-NMR):**

LC-NMR is a viable commercial technology, since about the middle of 1990. LC-NMR has sensitivity issues; here sensitivity is the capability of the NMR spectrometer to acquire sufficient data to allow for the unambiguous structure elucidation of the trace intensity in a compound mixture analyzed by HPLC. Previously many limitations of LC-NMR had obstructed its widespread application; however, this limitation has been recently overcome. Since the cryoprobes are highly sensitive as compared to the regular probes<sup>[49,50]</sup>.

The signal-to-noise ratio for the cryoprobes is four times that of regular probes, therefore regular probes

require 0.5 µg of sample whereas; cryoprobe requires only 0.1 µg of the sample. Thus, it reduces the time for analysis, as well as reduce the sample required for the analysis<sup>[51,52]</sup>. The practice of LC-NMR would reduce the time and labor exhaustive isolation step, thus the identification and analysis become faster and easier<sup>[53,54]</sup>.

### **The limitations of LC-NMR:**

The sensitivity issues of the NMR are the main limitation in the application of LC-NMR<sup>[55]</sup>. In LC-NMR, the protonated solvent cannot be used as a mobile phase for HPLC. The protonated solvent shows a resonance signal that dominates <sup>1</sup>H-NMR spectra and swamps the moderately weak signal from a small amount of analyte. Thus, to compensate for this issue deuterated solvent can be used, but it is cost expensive, result in peak broadening and R<sub>i</sub> shift may be observed. Need solvent suppression technique, for using the deuterated solvent, this result in suppression of analyte proton signals near the suppression solvent line and result in damage of spectral information<sup>[56]</sup>.

As HPLC contains metal and moving metal portions, the problem may be encountered if it is appearing close to the superconducting magnet of the NMR system. For unshielded magnet require the HPLC to be a minimum of 1.5-2 m away and 30-50 cm is requiring for the shielded magnet. The HPLC system should be overloaded with the analyte in demand to confirm that a suitable quantity of analyte is in the NMR active region<sup>[57]</sup>.

### **LC-MS/NMR:**

The limitation of on-flow LC-NMR is the sensitivity issue, at the magnetic field strength of 500 MHz and 1ml/min flow rate the residence time of the analyte is reduce and the detection limit is 10 ppm. Thus, to increase the analyte residence time the flow rate should be reduced but this may increase the analysis time and also affect the resolution. Hence one of the recent advancements to increase the NMR sensitivity is LC-MS/NMR<sup>[58]</sup>.

Directly coupled LC-MS/NMR is used universally in pharmaceutical laboratories. Coupling of the HPLC with that of MS and NMR is known as LC-MS/NMR, in this type of hyphenated technique the eluent from the HPLC column is split into the relatively small portion, from that one portion going to MS (ESI) and the remaining portion going to the NMR spectrometer as MS has advanced sensitivity as compared to that of NMR spectrometer. The coupling of both data

types allows an unambiguous correlation of NMR spectra with a particular trace level analyte. Many a time the MS spectra are not sufficient for the full structural elucidation. Thus, by coupling of LC, MS and NMR will give additional information on the drug structural configuration, functional group and NMR-silent heteroatoms (N, Cl, O) present in unknown impurities<sup>[59]</sup>.

#### Advantages:

Minimal sample requirement and it is a fully automated technique. Reduce the time for analysis with minimal sample degradation chances and sample can be recovered for further analysis.

#### Capillary zone electrophoresis-Mass spectrometry (CZE-MS):

The main reason to couple the capillary zone electrophoresis with the mass spectroscopy technique is that the ideal separation power of the capillary electrophoresis (CE) has been achieved whereas; mass spectra will provide sufficient structural information. There should be a high grade of orthogonality between the methods during the separation of impurity, to accomplish better resolution of separated impurities<sup>[60-65]</sup>. Different techniques for impurity profiling, include coupling of CE with varying MS ionization systems such as electrospray ionization (ESI-MS), atmospheric pressure chemical ionization (APCI-MS), atmospheric pressure photoionization (APPI-MS) and thermospray ionization (TSI-MS). The ESI-MS and TSI-MS are useful for detecting the ionic compound whereas; the APCI-MS and APPI-MS are not capable of detecting the ionic sample<sup>[66-72]</sup>. Accordingly, it can be also aid to differentiate between the ionic or non-ionic unknown impurity.

The sensitivity of the charge solution decreases in the following order ESI-MS $\geq$ TSI-MS $\geq$ APPI-MS $\geq$ APCI-MS. For detection of the solution which is not changed and for less polar compounds, APCI is mostly employed. ESI-MS is mostly employed due to its sensitivity and softness. V Dora *et al.* reported a comparison study between three methods such as RP-HPLC, Capillary electrophoresis and also included CE-ESI-MS/MS for impurity profiling in galantamine hydrochloride in a stressed condition. Important information obtained regarding the impurity by comparison between three methods, two impurities were detected at a low level. It is a combination of separation and identification technique, since requiring a small volume of the sample

as well as provide high resolution and sensitivity<sup>[73-77]</sup>.

#### Limitations of CZE-MS:

Capillary electrophoresis is a very complicated technique since it requires the optimization of all the parameters that have an impact on the separation mechanism. The less sensitive technique, therefore cannot detect the trace quantity of impurity.

#### CONCLUSION

According to the regulatory guideline, the analytical monitoring of impurity in a new drug substance is a mandatory requirement for market authorization. Thus, pharmaceutical products should be analytically monitored for any impurity present in trace amounts. As APIs are not 100 % pure those trace amounts of impurity may affect the safety and efficacy of the final formulation and patient consuming those products. Even standard assay procedures are not sufficient to describe impurities both qualitatively and quantitatively. All the techniques specify for impurity profiling may contribute effectively to the identification and structural elucidation of the unknown impurity. The application of this technique will significantly depend on the nature of impurity and the type and origin of impurity present in the drug substance. For the separation of the impurities mainly liquid chromatography techniques are widely employed, but the isolated fraction should be enough to carry out further structural analysis in off-line mode. In the current era, many advancements have brought huge changes in the analysis technique and also faster the process of analysis and have reduced the efforts. The on-line mode of analysis has not only saves our time but also provide qualitative and quantitative data for the unknown impurity. The hyphenated technique has been extensively used for impurity profiling. For the monitoring of the impurity either one of the methods can be utilized or else a combination of the technique can be employed based upon the requirement.

#### Conflict of interest:

The authors report no declarations of interest.

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