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Original Article

Impurities in Pharmaceuticals- A Review.

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Abstract

Impurity is defined as any substance coexisting with the original drug, such as starting material or intermediates or that is formed due to any side reactions. The impurity may be developed either during formulation, or upon aging of both API's and formulated API's in medicines. The presence of these unwanted chemicals, even in small amount, may influence the efficacy and safety of the pharmaceutical products. The control of impurities is currently a critical issue to the pharmaceutical industry. Impurity profiling includes identification, structure elucidation and quantitative determination of impurities and degradation products in bulk drug materials and pharmaceutical formulations. Identification of impurities is done by variety of Chromatographic and Spectroscopic techniques, either alone or in combination with other techniques. The advent of hyphenated techniques has revolutionized impurity profiling, by not only separation but structural identification of impurities. This review highlights the different types of impurities and various methods for isolation, separation and characterization of impurities.

Keywords: Isolation, Separation, Characterization of impurities.

1. Introduction

Chemically a compound is impure if it contains undesirable foreign matter i.e. impurities. An impurity in a drug product is any component of the drug product that is not the chemical entity defined as the drug substance or excipients in the drug product.[1] Impurities in pharmaceuticals are the unwanted chemicals that can develop during synthesis, formulation or with aging of active pharmaceutical ingredient (API). Presence of impurity even in small quantity may influence the efficacy and safety of pharmaceutical products[2]. Now a day's majority of the drugs used are of synthetic origins, which are further formulated into different finished dosage forms. These formulations deliver the drug substances in a stable, non-toxic and acceptable form, ensuring its bio-availability and therapeutic activity[3].

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The major challenge for both bulk drug industries and pharmaceutical industries is to produce quality products. To meet this, vigorous quality control tests are carried out to maintain quality, purity, safety and efficacy of pharmaceuticals. The pharmacopoeias specify not only purity but also puts limits which can be very stringent on levels of various impurities[4]. An impurity as defined by the ICH guidelines is "Any component of the medicinal product which is not the chemical entity defined as the active substance or an excipient in the product[5]. The efficacy and safety of pharmaceutical product is affected by presence of unwanted traces of impurities. Impurity profiling is deals with detection, identification/structure elucidation and quantitative determination of organic and inorganic impurities as well as residual solvents in bulk drugs and pharmaceutical formulations. Biological safety of impurity is established by qualification of impurities present[6]. Qualification is the process of acquiring and evaluating data that establish

the biological safety of an individual impurity or a given impurity profile at the level(s) being considered[7]. Impurities present in excess of 0.1% should be identified and quantified by selective methods.[8] The different Pharmacopoeias, such as the British Pharmacopoeia (BP), United States Pharmacopeia (USP), and Indian Pharmacopoeia (IP) are slowly incorporating limits to allowable levels of impurities present in the API's or formulations[9]. The aim is to minimize the adverse effects of drug materials and the preparations made thereof. After establishing the pharmacological-toxicological profile of a drug substance, pharmacologists, clinicians and drug-registration authorities consider its beneficial and adverse effects to the human organism and, on the basis of the benefit / risk ratio thus obtained, make the decision with respect to the possibility of introducing it into therapy.[10] ICH Q3A and Q3B cover drug substance and drug products respectively. Identification of impurities is done by variety of chromatographic and spectroscopic techniques, either alone or in combination with other techniques. The advent of hyphenated techniques has revolutionized impurity profiling, by not only separation but structural identification of impurities. According to ICH guidelines, impurities in drug substance can be classified into the following categories,

1) Organic impurities (process- and drug-related)
2) Inorganic impurities
3) Residual solvents
4) Others

According to United States Pharmacopoeia (USP) impurities can be classified as,

1) Impurities in Official Articles
2) Ordinary Impurities
3) Organic Volatile Impurities

1. Organic Impurities

These impurities arise during the manufacturing process and/or storage of the drug substance. These impurities includes,

Starting Materials or Intermediates

These are most common impurities found in API in multistep synthesis. Although end products are always washed with solvents there is always chance to remain as residual starting material or intermediate in final product unless proper care is taken[1]. For example, In the synthesis of amlodipine besylate traces of 4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-2-[(2-phthalimidoethoxy)methyl]*p*-1-4-dihydroxy pyridine is synthesis related impurity[11].

Degradation products

Degradation products arise from synthetic process, storage formulation of dosage form and aging. For example, penicillin's and cephalosporin's are classic examples of impurities from degradation products. In the synthesis of hydrochlorthiazide it is degraded to disulfonamide[11].

By-Products

In synthetic organic chemistry, getting a single end product with 100% yield is very rare; there is always a chance of formation of by-products[11]. By products can be formed due to variety of side reactions like incomplete reaction, over reaction, dimerization, isomerization, or due to unwanted reactions between starting materials or intermediates with catalysts or chemical reagents. For example, in the case of Paracetamol bulk, diacetylated paracetamol may form as a by-product[12].

2. Inorganic impurities

Inorganic impurities may also arrive from manufacturing processes used for bulk drugs. They are normally known and identified and include reagents, ligands, catalysts, heavy metals (From water used in the processes and the reactors, e.g., stainless steel reactors, where acidification or acid hydrolysis takes place) and other materials (filter aids, charcoal)[1].

3. Residual solvents

Residual solvents are organic or inorganic volatile liquids used during the manufacturing process or generated during the production. Some solvents that are known to cause toxicity should be avoided in the manufacturing of bulk drugs[9]. Residual solvents either modify the properties of certain compounds or may be hazardous to human

health. The residual solvents also affect physicochemical properties of the bulk drug substances such as crystallinity of bulk drug, which in turn may affect the dissolution properties, odour and colour changes in finished products[11]. It is very difficult to remove these solvents completely by the workup process. To remove them, various manufacturing processes or techniques (usually under increased temperature or/and decreased pressure) are in use.[12] Even after such processes, some solvents still remain, yet in small quantities. Residual solvents are divided into 4 classes,

Class-I

Class-I residual solvents should not be employed in the manufacture of drug substances, excipients, and drug products because of the unacceptable toxicities or deleterious environmental effects of these residual solvents. However, if their use in order to produce a medicinal product is unavoidable, their levels should be restricted. These solvents include benzene (2ppm), carbon tetrachloride (4ppm), methylene chloride (600ppm), methanol (300ppm), pyridine (200ppm), toluene (850ppm).

Class-II

Class-II residual solvents should be limited in drug substances, excipients, and drug products because of the inherent toxicities of the residual solvents. These solvents include N, N-dimethyl formamide (880ppm), acetonitrile (410ppm).

Class-III

Class III residual solvents may be regarded as less toxic and of lower risk to human health than Class I and Class II residual solvents. Class III includes no solvent known as a human health hazard at levels normally accepted in pharmaceuticals. However, there are no long-term toxicity or carcinogenicity studies for many of the residual solvents in Class III. Available data indicate that they are less toxic in acute or short-term studies and negative in genotoxicity studies.[13] These include, Acetic acid, Ethanol, Acetone, permitted daily exposure of 50mg or less as per ICH guidelines.

Class-IV

For these solvents, adequate toxicity data is not available. The manufacturers should justify the residual levels for these solvents in pharmaceutical products. The solvents are 1, 1-diethoxypropane, 1, 1-dimethoxypropane, petroleum ether etc.

3. Others

Other types of impurities includes

a. Formulation-Related Impurities

These impurities arise during formulation of different dosage forms such as tablet, syrup, capsule, semisolids etc. These are,

Method related impurities

E.g. Formation of impurity 1-(2,6-dichlorophenyl)-indolin-2-one on autoclaving of Diclofenac sodium. These impurities are due to exposure to heat, light, change of pH, solvents etc.

Environment related impurities

Environment related impurities arise by exposures to adverse temperatures (e.g. vitamins are very sensitive to heat), light (e.g. sunlight having about 8000 foot-candles can destruct nearly 34% of vitamin–B in 24hrs), Humidity (important factor in case of hygroscopic compounds such as aspirin and ranitidine). [11]

b. Dosage form factors related impurities

In case of liquid dosage forms impurities are significantly noticeable because they are very much susceptible to both degradation and microbiological contamination. For example fluocinonide topical solution USP, 0.05% in 60-mL bottles, recalled in the United States because of degradation/impurities leading to sub- potency.

c. Functional group related impurities

Different functional groups are also responsible for different impurities in pharmaceuticals; examples are given in table 1.

Table 1. Functional group related impurities.

Functional Group Reaction	Example
Ester hydrolysis	Formation of Salicylic acid impurity from aspirin
Hydrolysis	Bezylpenicillin, Chlordiazepoxide.
Oxidative degradation	Hydrocortisone, Methotrexate
Photolytic cleavage	Photolytic cleavage of Ciprofloxacin in eye preparation
Decarboxylation	Photoreaction of Rufloxacin

d. Packaging Material

Impurities may also results from containers and closures. For most reactive species impurities consists of water (hydrolysis of active ingredients), small electrophiles (aldehydes and carboxylic acid derivatives), peroxides(oxidize some drugs), metals (catalyse oxidation of drugs and their degradation pathway), extractables and leachables (emerge from glass, rubber stopper and plastic materials, in oxides like NO_2, SiO_2, CaO, MgO are major components leached from glass).[12]

Isolation and Identification of Impurities in Active Pharmaceutical Ingredients

Number of methods can be used for separation, isolation and characterization of impurities. But the application of any method depends on the nature of impurity (i.e.) its structure, physicochemical properties and availability.

Flash Chromatography

In column chromatography, if the solvent is forced down the column by positive air pressure, it is called flash chromatography. Flash chromatography is basically an air pressure driven hybrid of medium pressure and shorter column chromatography which has been optimized for particularly rapid separation.[10] Flash chromatography is a technique used to separate mixtures of molecules into their individual constituents, frequently used in the drug discovery process. Flash chromatography utilizes a plastic column filled with some form of solid support, usually silica gel, with the sample to be separated placed on top of this support. The rest of the column is filled with an isocratic or gradient solvent which, with the help of pressure, enables the sample to run through the column and become separated.[16] Satinsky D and co-workers developed flash chromatographic method for simultaneous determination of paracetamol, caffeine and acetylsalicylic acid using benzoic acid as an internal standard. A Chromolith Flash RP-18e, 25-4.6mm column (Merck, Germany) and a FIAlab 3000 system (USA) with an 8-port selection valve and a 5 mL syringe were used for injection. The mobile phase used was acetonitrile-(0.01 M) phosphate buffer (10:90, v/v) pH 4.05, flow rate 0.6 mL min^{-1}. UV detection was at 210 and 230 nm.[17]

High Performance Liquid Chromatography (HPLC)

HPLC is the method of choice for impurity testing of the final products. In the majority of cases the use of traditional reversed phase (RP) HPLC conditions and UV detection mostly employed for separation. Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. With these stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily.[16] In some cases normal phase HPLC also used. Today HPLC is a basic tool for analysis of pharmaceuticals.

Nageswara Rao and V. Nagaraju separated and determined synthetic impurities from difloxacin by RP HPLC. The separation was achieved on a reversed-phase C_{18} column using methanol–water–acetic acid (78:21.9:0.1, v/v/v) as a mobile solvent at a flow rate of 1.0 ml/min at 28^0C using UV detection at 230 nm. Difloxacin synthesized by condensation of 7-chloro-6-fluoro- 1-(4-fluorophenyl)-4-oxo-1,4-dihydro-3-quinoline carboxylic acid (CFQ) with *N*-methyl piperazine. During its synthesis not only the unreacted CFQ, but also its related analogues: (i) methyl 2-(2,4-dichloro-5- fluorobenzoyl)-3-(4-fluoroanilino)-(E)-2-propenoate (MFP), (ii) methyl 2-(2,4-dichloro-5-fluorobenzoyl)-3-(2,4-

difluoroanilino)-(E)-2-propenoate (MDF) and (iii) 7-chloro-1- (2,4-difluorophenyl)-6-fluoro-4-oxo-1,4-dihydro-3-quinoline carboxylic acid (CDF) are usually carried over in small quantities in to the bulk of difloxacin.[18] Joseph Sunder Raj et al were studied degradation products of dicloxacillin sodium by HPLC and three impurities were detected.[19] Isolation and characterisation of impurity in Phenazopyridine HCl bulk drug was done by analytical HPLC and the impurity was identified to be 3-phenyl-5-phenylazo-pyridine-2,6-diamine.[20] Estimation of related substances of Naproxen in pharmaceutical dosage form was performed on a YMC-ODS A Pack (250mm × 4.6mm, 5µ) column using mobile phase containing acetonitrile and 10 mM ammonium acetate buffer pH 3.8 in ratio 550:450 v/v (pH 3.8 adjusted with acetic acid) at the flow rate 0.8 ml/min and detection was performed at 254 nm.[21] Anuradha Gajjar and Vishal Shah, performed forced degradation study of ezetimibe which was subjected to thermolytic, photolytic, hydrolytic (acidic and alkaline) and oxidative stress conditions. Extensive degradation of ezetimibe occurred only in alkaline hydrolytic conditions. Major degradation product of alkali hydrolysis of Ezetimibe was found at RRT of 0.80. This degradant was isolated by preparative HPLC. On the basis of spectral data, the structure of the degradant was confirmed as 5-(4-fluorophenyl)-2-[(4-fluorophenyl amino)-(4 hydroxyphenyl)methyl]-pent-4-enoic acid.[22] S. G. Hiriyanna and coworkers detect one unknown impurity in azoxystrobin bulk material by a gradient reverse phase HPLC. This impurity was isolated from a crude sample of azoxystrobin using reverse phase preparative LC and characterized by NMR, MS. The impurity was characterized as methyl 2- (2- (6-(2 cyanophenoxy)-2-((4-(2-cyanophenoxy)-6-(2- (1, 3-dimethoxy-3- oxoprop-l-en-2-yl)phenoxy) pyrimidin-5-yl) methyl)pyrimidin-4-yloxy)phenyl)- 3-methoxyacrylate.[23] Two unknown impurities in linezolid bulk drug viz (S)-N-[[-(3-(3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]methyl] acetate and (S)-N-[[-(3-(3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]methyl] chloride, were detected by a simple isocratic reverse phase high performance liquid chromatography by Krishna Reddy and co-workers.[24] Impurities in clarithromycin were studied by using High-performance liquid chromatography . The sample is chromatographed on a YMC C_{18} column using an eluent of acetonitrile-0.033 M KH_2PO_4 (48:52) at an apparent pH of 5.4 and ultraviolet detection at 205 nm.[25] Palavai Reddy and co-workers developed impurity profiling method and degradation studies for Sumatriptan Succinate and Naproxen Sodium tablets by HPLC using Waters Spherisorb ODS-1 column (250mm X 4.6mm, 5µm) by the gradient program using 0.05 M Phosphate buffer (ph 3.0), Acetonitrile and methanol at a flow rate of 1.0 ml min^{-1} with detection wavelength at 225 nm.[26] Finasteride contains four impurities namely Imp -A, Imp-B, Imp-C and Imp-D which are result of oxidative degradation determined by simple high performance liquid chromatographic method by using Symmetry C_{18} column and mobile phase is mixture of water and Acetonitrile (64:34, v/v) as mobile phase.[27] Two unknown impurities were detected in verapamil hydrochloride bulk drug using isocratic reversed-phase high performance liquid chromatography (HPLC) which are characterized as 2-(3,4-dimethoxyphenyl)-3-methylbut-2-enenitrile and 2-(3,4-dimethoxyphenyl)-2-isopropyl-3-methylbutanenitrile.[28] Impurities from rivastigmine isolated by preparative HPLC and analysed by HPLC. These impurities were characterized as N,N-dimethyl-3-[1-dimethylaminoethyl]phenylcarboxylate (dimethyl-rivastigmine) and N,N-diethyl-3-[1-dimethylaminoethyl]phenylcarboxamide (diethyl-rivastigmine). [29] Similarly an unknown impurity in mitoxantrone hydrochloride bulk drug was detected by HPLC at levels around 0.5%. This impurity was isolated from a sample of crude mitoxantrone using preparative HPLC and was characterized as 1, 4-dihydroxy-5-2-2-hydroxyethyl-amino-ethyl-amino-8-2-bis 2-hydroxyethyl-amino-ethyl-amino-9,10-anthracenedione based on its spectral data (NMR, IR and MS).[30]

Thin-Layer Chromatography (TLC)

Thin-Layer Chromatography (TLC) is a good technique to use when normal phase solvents provide optimum separation. Typical thin-layer

separations are performed on glass plates that are coated with a thin layer of stationary phase. TLC plays an essential role in the early stage of drug development when knowledge about the impurities and degradants in drug substance and drug product is limited. It is an ideal technique for the isolation of compounds because of its simplicity. However, in order for TLC to be successful, the impurity and/or degradant should be at or above the 1% level. Anything below this level is very difficult to isolate on a TLC plate due to higher detection limits.[15] Thin layer chromatography is a widely used method in the pharmaceutical analysis both in its classical semi quantitative form, and equipped with sophisticated analytical instruments like special chamber-types, densitometers, or coupled with different interfaces with Mass spectrometers for identification and quantitative analysis of impurities. The synthetic pathway of an API usually spans over several manufacturing steps which are discrete or continuous. In such case intermediates are isolated, characterized and analyzed individually by TLC. In continuous manufacturing processes the intermediates remaining in the reaction mixture are not isolated and controlled individually, however their presence is checked only in the final step of the synthesis. Nevertheless, in both cases in-process control have to be performed to track the progress of the syntheses of the intermediates. As for all in-process control tests there is a need to be performed rapidly and to deliver appropriate information to decide whether the reaction could be stopped or not. Usually this is a simple chromatographic task: starting material and the reaction product should be separated sufficiently to track reaction progress. A simple TLC test also used for monitoring fermentation process. For example formation and degradation of a carbohydrate from starch to a monosaccharide on the same chromatographic plate.[31] An unknown autoxidation product in an aerated cholesterol sol was isolated by preparative thin layer chromatography. This compound was identified as cholesterol-5β, 6β-oxide by gas liquid chromatography along with infrared and mass spectrometry.[32] A. Mohammad and co-workers developed thin layer chromatographic method for on-plate identification of ketoprofen from pure, formulated and spiked urine samples. The proposed method involves use of amino acid impregnated silica gel layers as stationary phase with mixed micelles (0.5% aqueous solutions of sodium dodecyl sulphate plus Triton X-100 and acetone (8:5:1.5, v/v) as mobile phase.[33]

Capillary electrophoresis (CE)

Capillary Electrophoresis (CE) is a separation technique based on the differential transportation velocities of charged species in an electric field through a conductive medium. Primary candidates for CE separation are ions. The basic instrumental set-up consists of a high voltage power supply (0 to 30 kV), a fused silica (SiO_2) capillary, two buffer reservoirs, two electrodes, and an on-column detector.[16] Oversulfated chondroitin sulfate (OSCS), an impurity found in some porcine intestinal heparin samples was separated from intact heparin by capillary electrophoresis (CE) using a 600mM phosphate buffer, pH 3.5 as the background electrolyte in a 56cm x 25microm i.d. capillary.[34] Tonon M.A. developed capillary electrophoretic enantioselective method with UV detection for the simultaneous quantification of zopiclone enantiomers and its impurities, zopiclone-N-oxide enantiomers, and 2-amino-5-chloropyridine, in tablets. The analytes were extracted from the tablets using ACN and were separated in an uncoated fused-silica capillary (50 µm, 42 cm effective length, 50 cm total length) using 80 mM sodium phosphate buffer pH 2.5 and 5 mM carboxymethyl-β-cyclodextrin as running buffer.[35] A capillary electrophoresis (CE) method for testing the stability of a novel oral anticancer metallodrug, tris(8-quinolinolato)gallium is proposed by Lidia S. Foteeva and co-workers.[36] Marta Zalewska studied Capillary Electrophoresis for analysis of the anti-cancer drugs impurities for example Cisplatin, Carboplatin, Lobaplatin, Methotrexate, Tamoxifen, Paclitaxel and their derivatives.[37]

Gas Chromatography (GC)

It is very useful for isolation and characterization of volatile and semivolatile organic compounds in complex mixtures or

those components that can be made volatile by derivatization technique and the detector used should be non destructive.[6] Headspace GC analysis is the most widely used technique for residual solvent determination in pharmaceuticals. In this technique only volatile substances and dissolution medium can be injected onto the column. Also HS systems are fully automated, in addition, a sample preparation is easy, and the sensitivity of analysis is sufficient for the majority of solvents mentioned in ICH guidelines.[13]In gas chromatography, the components of vaporized sample are separated as a consequence of being partitioned between a mobile gaseous phase and a liquid or solid stationary phase held in column. In performing a gas chromatographic separation, sample is vaporised and injected onto the head of column. Elution is brought about by the flow of inert gaseous mobile phase.[38]

Supercritical Fluid Chromatography (SFC)

SFC is a technique in which mobile phase is supercritical fluid (supercritical fluid is formed whenever a substance is heated above its critical temperature). SFC is a hybrid of gas and liquid chromatography that combines some of best features of each.[37] Supercritical fluid chromatography is based on the principle of density of supercritical fluid which corresponds to solvating power. As pressure in the system is increased, the supercritical fluid density increases and correspondingly its solvating power increases. Thus as the components retained in the column get eluted.[39] Supercritical fluid chromatography (SFC) has developed rapidly in recent years, particularly in the area of enantioseparations.[40]

High Performance Thin Layer Chromatography (HPTLC)

The separation depends on the relative affinity of compounds towards stationary and mobile phase. The compounds under the influence of mobile phase (driven by capillary action) travel over the surface of stationary phase. During this movement the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Once separation occurs individual components are visualized as spots at respective level of travel on the plate. Their nature or character are identified by means of suitable detection techniques.[16] High performance thin layer chromatography (HPTLC) is an enhanced form of thin layer chromatography (TLC). A number of enhancements can be made to the basic method of thin layer chromatography to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative measurements. Automation is useful to overcome the uncertainty in droplet size and position when the sample is applied to the TLC plate by hand. Nowadays, HPTLC has become a routine analytical technique due to its advantages of reliability in quantitation of analytes at micro and even in nanogram levels and cost effectiveness. Recently an HPLC and HPTLC method has been reported for simultaneous estimation of levocetirizine dihydrochloride and Montelukast sodium in pharmaceutical dosage forms which are either tedious or expensive methods.[41]

Solid Phase Extraction (SPE)

Solid-phase extraction (SPE) is an extraction method that uses a solid phase and a liquid phase to isolate the impurity of interest from a solution.[16] It is usually used to clean up a sample before using a chromatographic or other analytical method to quantify the amount of analyte(s) in the sample. The general procedure is to load a solution onto the SPE phase, wash away undesired components, and then wash off the desired analytes with another solvent into a collection tube. Solid-phase extractions use the same type of stationary phases as are used in liquid chromatography columns. The versatility of SPE allows use of this technique for many purposes, such as purification, trace enrichment, desalting, derivatisation and class fractionation. The principle of SPE is involving a partitioning of solutes between two phases. SPE involves partitioning between a liquid (sample matrix or solvent with analytes) and a solid (sorbent) phase. The most common retention mechanisms in SPE are based on van der Waals forces (“non-polar interactions”), hydrogen bonding, dipole-dipole forces (“polar” interactions) and cation-anion interactions (“ionic” interactions). [42]

Supercritical fluid extraction (SFE)

Supercritical Fluid Extraction (SFE) is the process of separating one component (the extractant) from another (the matrix), using supercritical fluids as the extracting solvent. A pure supercritical fluid (SCF) is any compound at a temperature and pressure above the critical values (above critical point).Carbon dioxide (CO_2) is the most used supercritical fluid, sometimes modified by co-solvents such as ethanol or methanol. Extraction conditions for supercritical CO_2 are above the critical temperature of 31°C and critical pressure of 72 bar.[43]

Hyphenated Techniques

Hyphenated techniques are those techniques, where two or more analytical techniques are combined. The two most commonly used hyphenated techniques for impurity profiling are LC-MS and LC-MS-NMR. In these techniques chromatographic techniques are coupled with a spectroscopic detector. Thus impurity structure determination can be performed in real time during chromatographic separation and both isolation and characterization is performed in one single step. The use of hyphenated techniques for impurity determination is on rise due to easy availability of bench-top instrumentation and their distinct advantages like versatility, sensitivity, possibility of profiling sub structural analysis and rapid selective quantitative determination of targeted compound even in mixtures. The various hyphenated techniques used for impurity characterization are-

LC-MS

Mass spectrometry coupled with modern high performance liquid chromatography (HPLC) allows trace components in complex mixtures to be studied directly with no prior preparative purification or fractionation to enrich the impurities.[44] LC-MS has become the primary approach for the identification of low-level impurities in samples resulting from synthesis or from degradation of APIs. Full scan and product ion scan analysis, providing molecular weight information and fragmentation data, respectively, offer rich structural information on candidate structures.[45] Liquid chromatography -Mass spectroscopy (LC-MS) is an analytical technique that couples high resolution chromatographic separation with sensitive and specific mass spectroscopic detection. This is one of the hyphenated techniques which revolutionized impurity profiling and degradation products formed during the formulation and production procedure. The technique is still fast developing, with high resolution and high sensitivity, particularly in mass spectrometry area.[46] During stress degradation studies of pioglitazone hydrochloride, one major unknown oxidative degradation impurity and two major unknown base degradation impurities were identified by LC-MS. The oxidative degradation impurity, base degradation impurity-1 and base degradation impurity-2 were characterized as pioglitazone N-oxide, 3-(4-(2-(5-ethylpyridine-2yl) ethoxy) phenyl)-2-mercaptopropanoic acid and 2-(1-carboxy-2-{4-[2-(5-ethylpyridine-2yl)-ethoxy] phenyl}-ethyl disulfanyl)-3-{4-[2-(5-ethylpyridine-2yl)-ethoxy] phenyl propanoic acid, respectively.[47] LC-MS was performed to identify and characterize impurities present in Raloxifene , these impurities were characterized as Raloxifene-N-Oxide [Imp-1]; EP impurity A [Imp- 2]; EP impurity B [Imp-3]; Raloxifene Dimer [Imp-4]; (6-Acetoxy-2-[4-hydroxyphenyl]-1-benzothiophene or 6-Hydroxy-2- [4-acetoxyphenyl] -1-benzothiophene)[Imp-5]; (Methyl[4-[2-(piperidin-1-yl) ethoxy]]benzoate)[Imp-6];(1-[6-hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-3-yl] ethanone) [Imp-7]; (7-Acetyl-[6-hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-3-yl][4-[2-(piperidin-1-yl)ethoxy]phenyl methanone)[Imp-8].[48] Sonal Desai, et al, used LC-MS for the characterization of impurities in 8-chlorotheophylline these impurities were characterized as 3,7-dihydro-1,3-dimethyl-1H-purine-2,6-dione (impurity I), 3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione (impurity II) and isomer of 8-chloro-1,3-dimethyl-2,6(3H,1H)-purinedione (impurity III).[49]Unknown impurity in bulk drug eprosartan was detected by liquid chromatography tandem multi-stage mass spectrometry it was finally elucidated as 4,4-(5,5-(1E,1E)-3,3-(4,4-methylenebis(thiophene-4,2-diyl))bis(2-carboxyprop-1-ene-3,1-diyl)bis(2-butyl-1H-imidazole-5,1-

diyl))bis(methylene) dibenzoic acid by Cuirong Sun, et al.[50] A gradient elution LC method was developed to separate methoxsalen from three of its known impurities: isopimpinellin, bergapten, and ammidin. The method employs a methanol-6%THF (aq) mobile phase, phenyl column, and detection at 254 nm. Identification of the impurity as isopimpinellin was accomplished by a combination of analytical and preparative LC, LC/MS, and NMR.[51] Gulshan Bansal et al conducted Forced degradation studies on glipizide The drug is shown to degrade in acidic conditions to two products: 5-methyl-*N*-[2-(4 sulphamoylphenyl) ethyl] pyrazine-2-carboxamideand methyl *N*-[4-[2-{(5-methyl-2-pyrazinoyl)amino}ethyl] phenyl] sulfonyl carbamate). The degradation products are characterized through through LC–mass spectrometry (MS) fragmentation pattern study. Three unknown impurities in rosiglitazone maleate bulk drug atlevel below 0.1% (ranging from 0.05 to 0.1%) were detected by simple reverse phase high performance liquid chromatography and preliminarily identified with LC-MS.[52] For identification by LC-MS a column (Inertsil ODS 3V 250 X 4.6 X 5.0 µ) with a mobile phase consisting of 0.01M ammonium acetate (pH=6.0) adjusted with dilute acetic acid and acetonitrile in the ratio of 65:35, with a flow rate of 1.0 mL/min, UV detection at 280 nm was used.[53]

GC-MS

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. In the case of GC-MS, GC coupled to a Mass spectrometer through an interface that enriches the concentration of the sample in the carrier gas by taking advantage of the higher diffusivity of the carrier gas. Scanning times are rapid so that several MS can be obtained during the elution of a single peak from the GC unit. For example GC-MS technique is used in impurity profiling of synthetic pesticide d-allethrin.[16] GC-MS allowed the highly specific and sensitive quantification of thermo-stable molecules below a molecular weight of about 500 and became a key method in the toxicology field. With the respect to standardization and quality assurance of small molecule analytical routine methods the introduction of GC-MS as a reference method was an essential progress, in particular for endocrinology.[54]It is very useful for the determination of molecular weights and (sometimes) the elemental compositions of unknown organic compounds in complex mixtures.GC-MS is widely used for the quantitation of pollutants in drinking and wastewater. To use GC-MS, the organic compounds must be in solution for injection into the gas chromatograph. The solvent must be volatile and organic (for example, hexane or dichloromethane). Depending on the ionization method, analytical sensitivities of 1 to 100 pg per component are routine. GC-MS can also be used to measure the concentration of one or more analytes in a complex mixture. Quantitation can be based on peak areas from mass chromatograms or from selected ion monitoring.[55]

HPLC-DAD-MS

In this technique HPLC coupled with a diode array UV detector and a mass spectrometer, used in characterization of impurities in pharmaceuticals. For example analysis of doxycycline and its related impurities like metacycline and 6-epidoxycycline.[16] HPLC-DAD is used for the detection of a wide polarity range of compounds present in water. With this technique, the spectra of all eluting (UV absorbing) organic compounds are acquired. In this study, the combination of both HPLC-DAD and HPLC-Q-TOF MS techniques was used for the detection and identification of an unknown micro-contaminant in water samples.[56]

LC-MS-MS

LCMS/ MS technique is proposed as a modern alternative for the characterization of pharmaceuticals. First, the parent drug is analyzed with LC-MS. The retention time and molecular weight information are obtained. Using LC-MS/ MS, the product-ion analysis of the parent drug is obtained, and specific product ions and neutral losses are assigned to the substructures of the molecule.[45] Triple stage quadrapole and ion trap mass spectrometers are presently used for this technique, because of their sensitivity and

selectivity.[54] For the detection of a wide range of polar water soluble compounds present in water, HPLC-MS-MS is a powerful technique.[56] A liquid chromatographic-tandem mass spectrometric method using an Xterra MS C_{18} chromatographic column (100mm × 2.1mmi.d. 3.5m) that allows complete separation of oxytetracycline (OTC) and the impurities: 4-epi-oxytetracycline (EOTC), tetracycline (TC), 4-epi-tetracycline (ETC), 2-acetyl-2-decarboxamido-oxytetracycline (ADOTC), apo oxytetracycline (AOTC) and apo-oxytetracycline (AOTC) was developed by Anne Kruse Lykkeberg.[57]

LC-NMR

LC-NMR is an innovative technique that connects NMR with HPLC online and can offer not only 1-D but also 2-D NMR spectra for the components separated by HPLC. LC-NMR has come into wide use because of improved sensitivity due to higher magnetic fields of superconductive magnet and advanced techniques, especially the solvent suppression method. For example, LCNMR has been applied for the analysis of medicinal metabolites, impurities in medicinal specialties, and metabolites of natural products. NMR provides information about conformational geometry and is thus a powerful tool for structural analysis.[58] Vestipitant is a novel NK1 antagonist currently under investigation for the treatment of CNS disorders and emesis. The first synthetic step comprised a Grignard synthesis. An impurity was identified and initially expected to be a symmetric biphenyl. LC-NMR, is successful for impurities identification in Vestipitant. The HPLC method was initiated with 70% D_2O containing 0.1% TFA–30% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA), followed by a gradient to 100% ACN containing 0.1% TFA over 9min (total run time 15min), with a flow rate of 1ml/min and UV detection at 254 nm.[59]

Conclusion

Impurity profiling is an important aspect regarding quality, safety and efficacy of pharmaceuticals. To minimise toxicity in API (Active pharmaceutical ingredient) and finished dosage form different regulatory agencies reveals the need and scope of impurity profiling of drugs in pharmaceutical research. Identification of impurities is done by variety of Chromatographic and Spectroscopic techniques, either alone or in combination with other techniques. Chromatographic techniques such as HPLC, TLC, GC, HPTLC, Flash chromatography, Supercritical fluid chromatography etc. are routinely employed for isolation and characterisation of impurities. Some extraction methods also used in isolation of impurities like Solid Phase extraction, Supercritical fluid extraction etc. Today advanced hyphenated techniques are used for impurity profiling, by not only separation but structural identification of impurities. Among all hyphenated techniques, the most exploited techniques, for impurity profiling of drugs are GC-MS, LC-MS, LC-MS-MS and LC-NMR.

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