

Impurity profile: Significance in Active Pharmaceutical Ingredient

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Abstract

Various regulatory authorities like ICH, USFDA, Canadian Drug and Health Agency are emphasizing on the purity requirements and the identification of impurities in Active Pharmaceutical Ingredient's (API's). Qualification of the impurities is the process of acquiring and evaluating data that establishes biological safety of an individual impurity; thus, revealing 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. There are different methods for detecting and characterizing impurities with TLC, HPLC, HPTLC, AAS *etc.* Conventional Liquid Chromatography, particularly, HPLC has been exploited widely in field of impurity profiling; the wide range of detectors, and stationary phases along with its sensitivity and cost-effective separation have attributed to its varied applications. Among the various Planar Chromatographic Methods; TLC is the most commonly used separation technique, for isolation of impurities; due to its ease of operation and low cost compared to HPLC. An advancement of thin layer chromatography HPTLC, is a well-known technique for the impurity isolation.

Headspace GC is one of the most preferred techniques for identification of residual solvents. The advent of hyphenated techniques has revolutionized impurity profiling, by not only separation but structural identification of impurities as well. Among all hyphenated techniques, the most exploited techniques, for impurity profiling of drugs are LC-MS-MS, LC-NMR, LC-NMR-MS, GC-MS, and LC-MS.

Keywords: Impurity, Analytical method development, Spectrophotometry, Chromatography

1. Introduction

There is an ever increasing interest in impurities present in API's. Recently, not only purity profile but also impurity profile has become essential as per various regulatory requirements. In the pharmaceutical world, an impurity is considered as any other organic material, besides the drug substance, or ingredients, arise out of synthesis or unwanted chemicals that remains with API's. The impurity may be developed either during formulation, or upon aging of both API's and formulated API's in medicines. A good illustration of this definition may be identification of impurity in API's like 1-(1, 2, 3, 5, 6, 7-hexahydro-s-indacen-4-yl)-3,4-bis(1-hydroxy-1-methyl-ethyl)-furan-2-sulphonylurea using Multidisciplinary approach [1]. The presence of these unwanted chemicals, even in small amount, may influence the efficacy and safety of the pharmaceutical products. Impurity profiling (i.e., the identity as well as the quantity of impurity in the pharmaceuticals), is now gaining critical attention from regulatory authorities. The different Pharmacopoeias, such as the British Pharmacopoeia (BP), United States Pharmacopoeia (USP), and Indian Pharmacopoeia (IP) are slowly incorporating limits to allowable levels of impurities present in the API's or formulations.

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has also published guidelines for validation of methods for analysing impurities in new drug substances, products, residual solvents and microbiological impurities [2-5].

A number of articles [8-10] have stated guidelines and designed approaches for isolation and identification of process-related impurities and degradation products, using Mass spectrometry (MS), Nuclear Magnetic Resonance (NMR), High Performance Liquid Chromatography (HPLC), Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS), and Tandem Mass Spectrometry for pharmaceutical substances.

Present article reveals different impurities found in the API's, methods for identifying them and the possible measures to deal with the interferences caused by them.

Impurity profile is the description of identified and unidentified impurities present in new drug substances. Impurities can be described as shown in Table 1.

Impurities have been named differently or classified as per the ICH [11] as follows;

a) Common names

- By-products
- Degradation products

- Interaction products
- Intermediates
- Penultimate intermediates
- Related products
- Transformation products

Table 1. Description of impurity types and their sources

	Impurity type	Impurity source
1	Process-related drug substance	<ul style="list-style-type: none"> - Organic - Starting material - Intermediate - By-product - Impurity in starting material
2	Process-related drug product	<ul style="list-style-type: none"> - Organic or inorganic - Reagents, catalysts, etc
3	Degradation drug substance or drug product	<ul style="list-style-type: none"> - Organic - Degradation products
4	Degradation drug product	<ul style="list-style-type: none"> - Organic - Excipient interaction

b) United State Pharmacopeia

The United States Pharmacopoeia (USP) classifies impurities in various sections;

- Impurities in Official Articles
- Ordinary Impurities
- Organic Volatile Impurities

c) ICH Terminology

According to ICH guidelines, impurities in the drug substance produced by chemical synthesis can broadly be classified into following three categories;

- Organic Impurities (Process and Drug related)
- Inorganic Impurities
- Residual Solvents

Organic impurities may arise during the manufacturing process and or storage of the drug substance may be identified or unidentified, volatile or non-volatile, and may include;

- Starting materials or intermediates
- By-products
- Degradation products

Impurities are found in API's unless, a proper care is taken in every step involved throughout the multi-step synthesis for example; in paracetamol bulk, there is a limit test for p-aminophenol, which could be a starting material for one manufacturer or be an intermediate for the others.

In synthetic organic chemistry, getting a single end product with 100% yield is very rare; there is always a chance of having by-products. In the case of paracetamol bulk, diacetylated paracetamol may be formed as a by-product.

Impurities can also be formed by degradation of the end product during manufacturing of the bulk drugs. The degradation of penicillin and cephalosporins are well-known examples of degradation products. The presence of a β -lactam ring as well as that of an α -amino group in the C6/C7 side chain plays a critical role in their degradation. Another example that may be quoted is, the degradation of ibuprofen (IBP) to 2-(4-formylphenyl) propionic acid (FPPA), 2-(4-isobutylphenyl) propionic acid (IBP), 2-(4-methylphenyl) propionic acid (MPPA), 2-(4-ethylphenyl) propionic acid (EPPA), 4- isobutylacetophenone (4-IBAP), 2-(4-n-propylphenyl) propionic acid (PPPA) and 2-(4-n-butylphenyl) propionic acid (BPPA), which are reported to be well known impurities in IBP [12]. The degradation products of diclofenac-Na and clotrimazole [13], paclitaxel [14] are also reported.

2. ICH limits for impurities

According to ICH guidelines on impurities in new drug products, identification of impurities below 0.1% level, is not considered to be necessary, unless potential impurities are expected to be unusually potent or toxic. According to ICH, the maximum daily dose qualification threshold to be considered is as follows;

$$\leq 2\text{g/day } 0.1 \% \text{ or } 1 \text{ mg per day intake (whichever is lower)} \geq 2\text{g/day } 0.05\%$$

In summary, the new drug substance specifications should include, limits for-

i) Organic Impurities

Each specific identified impurity

- Each specific unidentified impurity at or above 0.1%
- Any unspecific impurity, with limit of not more than 0.1%

- Total impurities

ii) Residual solvents

iii) Inorganic impurities

3. Sources of Impurities

From the preceding discussion, it is clear that impurities can originate from several sources; such as; a) Crystallization-related impurities, b) Stereochemistry-related impurities, c) Residual solvents, d) Synthetic intermediates and by-products, e) Formulation-related impurities, g) Impurities arising during storage, h) Method related impurity, I) Mutual interaction amongst ingredients, h) Functional group-related typical degradation [6].

3.1. Crystallization-related impurities

Based on the realization that the nature of structure adopted by a given compound upon crystallization, could exert a profound effect on the solid-state properties of that system, the pharmaceutical industry is required to take a strong interest in polymorphism and solvatomorphism as per the regulations laid down by the regulatory authorities.

Polymorphism is the term used to indicate crystal system where substances can exist in different crystal packing arrangements, all of which have the same elemental composition. Whereas, when the substance exists in different crystal packing arrangements, with a different elemental composition; the phenomenon is known as *Solvatomorphism* [6].

3.2. Stereochemistry-related impurities

It is of paramount importance to look for stereochemistry related compounds; that is, those compounds that have similar chemical structure but different spatial orientation, these compounds can be considered as impurities in the API's. Chiral molecules are frequently called enantiomers. The single enantiomeric form of chiral drug is now considered as an improved chemical entity that may offer a better pharmacological profile and an increased therapeutic index with a more favourable adverse reaction profile. However, the pharmacokinetic profile of levofloxacin (S-isomeric form) and ofloxacin (R-isomeric form) are comparable, suggesting the lack of advantages of single isomer in this regard [15]. The prominent single isomer drugs, which are being marketed, include levofloxacin (S-ofloxacin), lavalbuterol (R-albuterol), and esomeprazole (S-omeprazole).

3.3. Residual solvents

Residual solvents are organic volatile chemicals used during the manufacturing process or generated during the production. Some solvents that are known to cause toxicity should be avoided in the production of bulk drugs. Depending on the possible risk to human health, residual solvents are divided into three classes [4]. Especially, solvents in Class I, *viz* benzene (2 ppm limit), carbon tetrachloride (4 ppm limit), methylene chloride (600 ppm), methanol (3000 ppm), pyridine (200 ppm), toluene (890 ppm) should be avoided. In Class II, *viz* N, N-dimethylformamide (880 ppm), acetonitrile (410 ppm). Class III solvents, *viz* acetic acid, ethanol, acetone have permitted daily exposure of 50 mg or less per day, as per the ICH guidelines. A selective gas chromatography (GC) method has been developed to determine the purity of acetone, dichloromethane, methanol and toluene. Using this method, the main contaminants of each organic solvent can be quantified. Moreover, the developed method allows the simultaneous determination of ethanol, isopropanol, chloroform, benzene, acetone, dichloromethane, methanol and toluene with propionitrile as the internal standard [16].

3.4. Synthetic intermediates and by-products

Impurities in pharmaceutical compounds or a new chemical entity (NCE) can originate during the synthetic process from raw materials, intermediates and/or by-products. For example, impurity profiling of ecstasy tablets by GC-MS [17], and MDMA samples, produced impurities in intermediates via reductive amination route [18].

3.5. Formulation-related impurities

Many impurities in a drug product can originate from excipients used to formulate a drug substance. In addition, a drug substance is subjected to a variety of conditions in the process of formulation that can cause its degradation or have other undesirable reactions. If the source is from an excipient, variability from lot to lot may make a marginal product, unacceptable for reliability. Solutions and suspensions are inherently prone to degradation due to hydrolysis or solvolysis [19]. Fluocinonide Topical Solution USP, 0.05%, in 60-mL bottles, was recalled in the United States because of degradation/impurities leading to sub-potency [20]. In general, liquid dosage forms are susceptible to both degradation and microbiological contamination. In this

regard, water content, pH of the solution/suspension, compatibility of anions and cations, mutual interactions of ingredients, and the primary container are critical factors.

Microbiological growth resulting from the growth of bacteria, fungi, and yeast in a humid and warm environment may result in unsuitability of an oral liquid product for safe human consumption. Microbial contamination may occur during the shelf life and subsequent consumer-use of a multiple-dose product, either due to inappropriate use of certain preservatives in the preparations, or because of the semi-permeable nature of primary containers [21].

3.6. Impurities arising during storage

A number of impurities can originate during storage or shipment of drug products. It is essential to carry out stability studies to predict, evaluate, and ensure drug product safety [6].

3.7. Method related impurity

A known impurity, 1-(2, 6-dichlorophenyl) indolin-2-one is formed in the production of a parenteral dosage form of diclofenac sodium, if it is terminally sterilized by autoclave [22]. The conditions of the autoclave method (i.e., 123 ± 2 °C) enforce the intramolecular cyclic reaction of diclofenac sodium forming an indolinone derivative and sodium hydroxide. The formation of this impurity has been found to depend on initial pH of the formulation.

3.8. Mutual interaction amongst ingredients

Most vitamins are very labile and on aging they create a problem of instability in different dosage forms, especially in liquid dosage forms. Degradation of vitamins does not give toxic impurities; however, potency of active ingredients drops below Pharmacopoeial specifications.

Because of mutual interaction, the presence of nicotinamide in a formulation containing four vitamins (nicotinamide, pyridoxine, riboflavin, and thiamine) can cause the degradation of thiamine to a sub-standard level within a one year shelf life of vitamin B-complex injections [23]. The marketed samples of vitamin B-complex injections were found to have a pH range of 2.8 - 4.0. A custom-made formulation with simple distilled-water and a typical formulated vehicle including disodium edetate and benzyl alcohol were investigated, and similar mutual interactions causing degradation were observed.

3.9. Functional group-related typical degradation

Ester hydrolysis can be explained with a few drugs *viz* aspirin, benzocaine, cefotaxime, ethyl paraben [23], and cefpodoxime proxetil [25].

Hydrolysis is the common phenomenon for ester type of drugs, especially in liquid dosage forms *viz* benzylpenicillin, oxazepam and lincomycin.

Oxidative degradation of drugs like hydrocortisone, methotrexate, hydroxyl group directly bonded to an aromatic ring (*viz* phenol derivatives such as catecholamines and morphine), conjugated dienes (*viz* vitamin A and unsaturated free fatty acids), heterocyclic aromatic rings, nitroso and nitrite derivatives, and aldehydes (especially flavorings) are all susceptible to oxidative degradation.

In mazipredone, the hydrolytic and oxidative degradation pathway in 0.1 mol L⁻¹ hydrochloric acid and sodium hydroxide at 80°C were studied [26].

Photolytic cleavage includes example of pharmaceutical products that are exposed to light while being manufactured as solid or solution, packaged, or when being stored in pharmacy shops or hospitals for use by consumers.

Ergometrine [27], nifedipine [28], nitroprusside, riboflavin and phenothiazines are very liable to photo-oxidation. In susceptible compounds, photochemical energy creates free radical intermediates, which can perpetuate chain reactions. Most compounds will degrade as solutions when exposed to high-energy UV exposures. Fluroquinolone antibiotics are also found to be susceptible to photolytic cleavage [29].

In ciprofloxacin eye drop preparation (0.3%), sunlight induces photocleavage reaction producing ethylenediamine analog of ciprofloxacin [30].

Decarboxylation of some dissolved carboxylic acids, such as p-aminosalicylic acid; shows the loss of carbon dioxide from the carboxyl group when heated. An example of decarboxylation is the photoreaction of rifloxacin [31].

As seen earlier, impurities in drug products can come from the drug or from excipients or can be brought into the system through an inprocess step by contact with the packaging material.

For most drugs, the reactive species consist of;

- Water- that can hydrolyze some drugs or affect the dosage form performance
- Small electrophiles- like aldehydes and carboxylic acid derivatives

- Peroxides- that can oxidize some drugs
- Metals- which can catalyze oxidation of drugs and the degradation pathway
- Leachable or Extractables- can come from glass, rubber stoppers, and plastic packaging materials. Metal oxides such as NaO₂, SiO₂, CaO, MgO, are the major components leached/extracted from glass [32]. Generally most synthetic materials contain leachable oligomers/monomers, vulcanizing agents, accelerators, plasticizers, and antioxidants [33]. Some examples of leachable/extractables from synthetic materials include styrene from polystyrene, [34] diethylhexylphthalate (DEHP, plasticizer in PVC), [35] dioctyltin isooctylmercaptoacetate (stabilizer for PVC), [36] zinc stearate (stabilizer in PVC and polypropylene), [37] 2-mercaptobenzothiazole (accelerator in rubber stopper), [38] and furfural from rayon [39].

These impurities are needed to be analyzed by using different analytical methods.

4. Analytical method development

New drug development requires meaningful and reliable analytical data to be produced at various stages of the development [40-43].

- a) Sample set selection for analytical method development
- b) Screening of Chromatographic conditions and Phases, typically using the linear-solvent-strength model of gradient elution
- c) Optimization of the method to fine-tune parameters related to ruggedness and robustness

The above three methods are briefly discussed in Table 2.

The impurities can be identified predominantly by following methods;

- Reference standard method
- Spectroscopic method
- Separation method
- Isolation method
- Characterization method

5. Reference standard method

The key objective of this is to provide clarity to the overall life cycle, qualification and governance of reference standards used in development and control of new drugs. Reference

standards serve as the basis of evaluation of both process and product performance and are the benchmarks for assessment of drug safety for patient consumption. These standards are needed, not only for the active ingredients in dosage forms but also for impurities, degradation products, starting materials, process intermediates, and excipients.

6. Spectroscopic methods

The UV, IR, MS, NMR and Raman spectroscopic methods are routinely being used for characterizing impurities [6].

7. Separation methods

The Capillary electrophoresis (CE), Chiral Separations, Gas Chromatography (GC), Supercritical Fluid Chromatography (SFC), TLC, HPTLC, HPLC are regularly being used for separation of impurities and degradation products [6].

Table 2. Achiral method development process

Step: 1 KPSS	Step: 2 Screening	Step: 3 Optimization
Identification of KPSS	LSS (linear solvent strength) model	Gradient methods
- Degradation samples	- Two gradients per condition	- LSS Model
- "Dirty" samples	- Orthogonal conditions	- pH
- PRI'S	Compound specific screening	- Temperature
-Mother liquors	LSS Database	- Slope
- Excipients	LSS predictions	- One- at –a-time approach
- Flavors	Refined optimization	Isocratic methods
-Preservatives	Documentation	- LSS Model
- Fractions		- pH
Impurity isolation		- Ion strength
KPSS Documentation		- Temperature
		- Factorial when LSS model fails

KPSS: (Key Predictive Sample Set)

8. Isolation methods

It is often necessary to isolate impurities. But if the instrumental methods are used, isolation of impurities is avoided as it directly characterizes the impurities.

Generally, chromatographic and non-chromatographic techniques are used for isolation of impurities prior its characterization. The term ‘chromatographic reactor’ refers to the use of an analytical-scale column as both a flow-through reactor, and simultaneously, as separation medium for the reactant(s) and product(s). By using an HPLC, chromatographic reactor approach, the solution-phase hydrolysis kinetics of the Aprepitant (EmendTM) prodrug, fosaprepitant dimeglumine, were investigated [44]. In loratidine, impurity found was ofloratidine [45], other examples include celecoxib [46], and amikacin [47]. A list of methods that can be used for isolation of impurities is given below.

- Solid-phase extraction methods
- Liquid-liquid extraction methods
- Accelerated solvent extraction methods
- Supercritical fluid extraction
- Column chromatography
- Flash chromatography
- TLC
- GC
- HPLC
- HPTLC
- Capillary electrophoresis (CE)
- Supercritical fluid chromatography (SFC)

9. Characterization methods

Highly sophisticated instrumentation, such as MS attached to a GC or HPLC, are inevitable tools in the identification of minor components (drugs, impurities, degradation products, metabolites) in various matrices. For characterization of impurities, different techniques are used; which are as follows;

9.1. NMR

The ability of NMR to provide information regarding the specific bonding structure and stereochemistry of molecules of pharmaceutical interest has made it a powerful analytical instrument for structural elucidation. The ability of NMR- based diffusion coefficient determination to distinguish between monomeric and dimeric substances was validated using a

standard mixture of authentic materials containing both monomers and dimers [48]. Unfortunately, NMR has traditionally been used as a less sensitive method compared to other analytical techniques. Conventional sample requirements for NMR are on the order of 10 mg, as compared with MS, which requires less than 1 mg.

9.2. MS

It has an increasingly significant impact on the pharmaceutical development process over the past several decades. Advances in the design and efficiency of the interfaces, that directly connect separation techniques with Mass Spectrometers have afforded new opportunities for monitoring, characterizing, and quantification of drug-related substances in active pharmaceutical ingredients and pharmaceutical formulations.

If single method fails to provide the necessary selectivity, orthogonal coupling of chromatographic techniques such as HPLC-TLC and HPLC-CE, or coupling of chromatographic separations with information rich spectroscopic methods such as HPLC-MS or HPLC-NMR may need to be contemplated, but hopefully only as a development tool rather than a tool for routine QC use.

Hyphenated Methods:

- LC-MS-MS
- HPLC-DAD-MS
- HPLC-DAD-NMR-MS
- GC-MS
- LC-MS

An example of reverse-phase LC-MS analysis in gradient elution with two distinct soft ionization techniques is the Atmospheric pressure ionization with electrospray source (API-ESI) and the chemical ionization of d-allothrine [49].

The popularity of LC-MS-MS systems for complex mixture analysis of thermally labile and biologically relevant molecules, *viz* mosapride, is largely attributed to the “soft” nature of atmospheric pressure chemical ionization (APCI), and atmospheric pressure ionization (APPI), [50].

HPLC-DAD-MS (HPLC coupled with a diode array UV detector and a mass spectrometer, and such other techniques are almost routinely used.

NMR has now been added to this combination to provide HPLC-DAD-NMR-MS capabilities in a commercial instrument.

In GC-MS of methamphetamine and in LC-MS of risperidone, and cetirizine tablets a number of other chromatographic and spectroscopic configurations are found to be perfectly suitable for initial characterization of the impurities [51-54].

The goal for investigation of impurities is outlined in Table 3. A common goal for investigation of both process and product degradation-related impurities is to determine which of the many potential impurities are, in fact, produced in the manufacturing process and which occur under a given set of storage conditions.

10. Applications

Numerous applications have been sought in the areas of drug designing and in monitoring quality, stability, and safety of pharmaceutical compounds, whether produced synthetically, extracted from natural products or produced by recombinant methods. The applications include alkaloids, amines, amino acids, analgesics, antibacterials, anticonvulsants, antidepressant, tranquilizers, antineoplastic agents, local anesthetics, macromolecules, steroids, miscellaneous [9].

Table 3. Goals of impurity investigations

Process-related impurities	Degradation-related impurities
Identify significant impurities	Identify potential degradation product through stress testing and actual degradation products through stability studies.
Determine origin of impurities and method for elimination or reduction	Understand degradation pathway and methods to minimize degradation.
Establish a control system for impurities involving: <ol style="list-style-type: none"> 1) Processing/manufacturing conditions 2) Suitable analytical methods/ specifications 	Establish a control system for impurities involving: <ol style="list-style-type: none"> 1) Processing/manufacturing conditions 2) Suitable analytical methods/ specifications 3) Long term storage conditions including packaging 4) Formulation.

Following are the few examples of impurities which are reported in the API'S.

Table 4. Various impurities reported in API's

Drug	Impurity	Method	Reference
AmphotericinB	Tetraenes	UV spectroscopy	55
Atropine sulphate	Apo atropine	UV spectroscopy	55
Cloxacillin	N,N dimethyl aniline	GC	55
Dextrose	5 hydroxyl methyl furfural	UV spectroscopy	56
Doxorubicin hydrochloride	Acetone and ethanol	GC	56
Ethambutol hydrochloride	2 amino butanol	TLC	56
Fluorescene sodium	Dimethyl formamide	GC	56
Framycetin sulphate	Neamine	TLC	57
Mercaptopurine	Hypoxanthine	UV spectroscopy	57
Cimetidine	2,5-bis[(N'-cyano-N''-methyl)guinidinoethylthiomethyl]-4-methylimidazole and 1,8-bis[(N' cyano- N''-methyl)guinidino]-3,6-dithiaoctane	HPLC	58
Norgestrel	3,17 α -diethinyl-13-ethyl-3,5-gonadiene-17-ol	TLC, HPLC and UV spectroscopy	59
Celecoxib	[5-(4-methylphenyl)-3-trifluoromethyl-1H-pyrazole], 4-[5-(2'-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]-benzenesulphonamide, and 4-[4-(4'-methylphenyl)-3-(trifluoromethyl)-1H-pyrazole-1-yl]-banzenesulfonamide	HPLC, LC, LC-MS-MS	60
Ethinodiol diacetate	17 α -ethinyl-estr-4-ene-3 β ,17-diol-3-acetate-17-(3'-acetoxy-2'-butenoate), 17 α -ethinyl-estr-4-ene-3 β ,17-diol-3-acetate-17-(3-oxo-butanoate)	HPLC	61

Table 4. (Continued)

Methamphetamine	1,2-dimethyl-3-phenylaziridine, ephedrine, methylephedrine, N-formylmethamphetamine, N-acetylmethamphetamine, N-formylphedrine, N-acetylephedrine, N,O-diacetylephedrine, methamphetamine dimer	GC	62
Repaglinide	4-carboxymethyl-2-ethoxy benzoic acid, 4-cyclohexylaminocarbamoyl-methyl-2-ethoxy-benzoic acid, 1-cyclohexyl-3-[3-methyl-1-2-(piperidin-1-yl-phenyl)-butyl]-urea, 1,3-dicyclohexyl urea	HPLC	63
Morphine	6-monoacetylmorphine	HPLC	63
Morphine sulphate	5-(hydroxymethyl)-2-furfural, 10-hydroxymorphine, 10-oxomorphine	HPLC	64

11. Conclusion

Nowadays, it is mandatory requirements in various pharmacopoeias to know the impurities present in API's.

Isolation and characterization of impurities is required for acquiring and evaluating data that establishes biological safety which reveals the need and scope of impurity profiling of drugs in pharmaceutical research. To isolate and quantify the impurities, various instrumental analytical techniques are routinely been used.

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