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Review

Pharmaceutical impurities and degradation products: Uses and applications of NMR techniques

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ABSTRACT

Current standards and regulations demand the pharmaceutical industry not only to produce highly pure drug substances, but to achieve a thorough understanding of the impurities accompanying their manufactured drug substances and products. These challenges have become important goals of process chemistry and have steadily stimulated the search of impurities after accelerated or forced degradation procedures.

As a result, impurity profiling is one of the most attractive, active and relevant fields of modern pharmaceutical analysis. This activity includes the identification, structural elucidation and quantitative determination of impurities and degradation products in bulk drugs and their pharmaceutical formulations.

Nuclear magnetic resonance (NMR) spectroscopy has evolved into an irreplaceable approach for pharmaceutical quality assessment, currently playing a critical role in unequivocal structure identification as well as structural confirmation (qualitative detection), enabling the understanding of the underlying mechanisms of the formation of process and/or degradation impurities.

NMR is able to provide qualitative information without the need of standards of the unknown compounds and multiple components can be quantified in a complex sample without previous separation. When coupled to separative techniques, the resulting hyphenated methodologies enhance the analytical power of this spectroscopy to previously unknown levels. As a result, and by enabling the implementation of rational decisions regarding the identity and level of impurities, NMR contributes to the goal of making better and safer medicines.

Herein are discussed the applications of NMR spectroscopy and its hyphenated derivate techniques to the study of a wide range pharmaceutical impurities. Details on the advantages and disadvantages of the methodology and well as specific challenges with regards to the different analytical problems are also presented.

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1. Introduction

1.1. Pharmaceutical impurities and approaches to their modern quality control

The quality assurance and quality control of active pharmaceutical ingredients (APIs) and excipients are major issues in pharmaceutical analysis, which intend to prevent damages to the patients. Quality control methods are regulated in the pharmacopeias and other documents, which are continuously revised in order to keep updated drugs, excipients, and also their methods for analysis. Furthermore, internationally agreed recommendations, such as those issued by the International Conference on Harmonization (ICH) are steadily moving pharmaceutical analysis beyond compendial requirements, with the advantage of the most modern analytical technologies [1]. Pharmaceutical impurities are the unwanted chemicals that remain with the API, develop during formulation, or upon degradation of both API and drug products. Their presence, even in small amounts, might influence the efficacy and safety of the pharmaceutical products; therefore, drug purity has always been associated to drug quality.

The evolution of chemical knowledge and the advent of increasingly sensitive and selective analytical methods have been continuously stimulating the interest in the determination of drug purity and the impurities themselves in both, natural and synthetic products. This is in line with the policy of the pharmaceutical industry, which has always demanded that the API should be as pure as possible. The number of recently published books [2], book chapters [3–6] and papers on the subject demonstrate the increasing importance of pharmaceutical impurities [7–11] and impurity profiling [12–16]. These and other [17–19] publications also reflect the continued interest of the regulatory authorities, industry and scientists in these areas.

The ICH Q3A guideline states that impurity profile is “a description of the identified and unidentified impurities, present in a new drug substance” [20]. According to the guide, the impurities are classified into organic, inorganic and organic volatile impurities. The organic impurities can arise from the manufacturing process and/or be formed during storage of the drug substances. They include starting materials, by-products, synthetic intermediates, degradation products, reagents, ligands and catalysts. As specifically indicated, the guide does not cover enantiomeric impurities and polymorphic forms.

The ICH Q3B guideline addresses impurities in new drug products, classified as degradation products of the drug substance or reaction (interaction) products of the drug substance with an excipient and/or immediate container closure system [21].

In addition, in current pharmaceutical development processes, impurities in the API have identification and qualification thresholds of 0.10 and 0.15% respectively, for doses of the API up to 2 g/day [20] and many recent publications reflect interest in compounds ranging from 0.01 to 0.1%.

Reliable assessment of drug purity in accordance with these stringent standards requires the use of state of the art validated analytical methods, increasingly sensitive detections, and mainly an analyst prepared to critically interpret the results.

The available tools for impurity profiling have already been reviewed [22]. Reversed-phase liquid chromatography (RP–LC) is still the primary method for analyte separation toward impurity profiling of drugs. However, an increasing number of examples show the usefulness of both nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) as additional tools for the detection and quantitation of difficult to solve impurity challenges.

When employed as stand-alone techniques, NMR and MS offer a high degree of orthogonality and complementarity [23]. In particular, NMR spectroscopy is relevant because it can simultaneously and selectively detect multiple components in a sample, even without their physical separation.

Mass spectrometric studies can provide structure-rich information of organic compounds, even those found at trace impurity levels, with small amounts of sample. When hyphenated with an LC separation, and when electro-spray (ESI) or atmospheric-pressure chemical ionization (APCI) are used, MS provides very important information about the molecular weights (especially when implemented in high-resolution mode) of the chromatographed compounds. It can also provide the structure (through the fragmentation pattern) of the analytes, especially when some prior information on the molecular skeleton is available and if only minor changes are present in the impurity with respect to the drug substance [24].

Therefore LC–ESI–MS and LC–ESI–MS/MS are currently well-established analytical tools in the rapid identification and characterization of each component in sample mixtures. However, the resulting picture is still often limited with respect to details of molecular structure. Mass spectral methods are rarely capable of de-novo structure determination with full certainty; furthermore, they do not always provide unambiguous structural information and may not be able to distinguish between isomers [25].

In these cases, the next and often final stage is to resort to NMR spectroscopy (usually off-line, but in some cases on-line), in order

to access the required information. Occasionally, single crystal X-ray diffractometry and/or synthesis of the proposed structures have been used as final proofs of the impurities' structure.

Furthermore, NMR is at the center of the new complete molecular confidence concept, which tends to exploit its synergy with LC–MS in order to obtain an estimation of the purity, concentration, and identity of chemical compounds [26].

1.2. NMR spectroscopy

1.2.1. NMR spectroscopy as an analytical tool: Advantages and disadvantages

NMR spectroscopy, an information rich analytical tool, is capable to deliver the most complete molecular structural information, including atomic connectivity, spatial geometry, conformation, and stereochemistry. ^{1}H is the most simple and sensitive, and therefore most frequently detected nucleus. Many more isotopes of elements in the periodic table (^{11}B, ^{13}C, ^{15}N, ^{17}O, ^{19}F, ^{31}P, etc.) are also accessible by NMR. In ^{1}H NMR spectroscopy, ^{1}H chemical shifts and ^{1}H–^{1}H couplings are selectively detected, which relate to the chemical environment of the observed nucleus. The spectral signature of each compound is unique, turning the method highly specific for chemical structures and ideally suited for structural elucidation and confirmation.

Since, the intensity of any given NMR signal depends only on the total amount of the detected nuclei in the sample volume (primary ratio rule), quantitative NMR spectroscopy (qNMR) can be used either as an absolute "standard-free" method or as a relative method [27].

The physical principles of the NMR phenomenon and the relevant spectroscopic parameters, such as chemical shift, signal multiplicity, spin–spin coupling constant (J), and the integral intensity, as well as the wide array of modern NMR experiments [28,29] are detailed in a number of useful books [30–33]. A short list of the most common NMR experiments and the structural information they are able to provide is found in Table 1.

NMR spectroscopy has several advantages; it is a robust and quasi-universal detector, has a non-destructive nature, allows easy sample preparation, and simple method development. Although NMR spectroscopy is used in drug impurity profiling mainly after off-line or on-line separation of the impurities, this technique can be a useful tool even without full analyte separation. Spectra are highly specific, this spectroscopy has the ability to provide global information about the sample in a single analysis and it offers multiple calibration options.

Therefore, ^{1}H NMR can be advantageously used as an alternative to conventional high-performance liquid chromatography (HPLC) when availability of impurity standards is not possible, such as during early stages of drug development [34]. This is documented by a steady increase in the volume of reports that employ NMR spectroscopy to solve this problem, but also by the remarkable interest of drug manufacturers in this technique. Several reviews have been published on the application of NMR in pharmaceutical analysis [35,36].

Since ^{1}H NMR is sensitive to compounds bearing protons, it can be regarded as a quasi-universal detector, especially for low molecular weight organic molecules. Protons are detected with the same sensitivity, regardless of their chemical environment; thus, the requirement of determining response factors is avoided.

Therefore, NMR ranks among the most informative methods, being a key analytical tool for identification, authentication, detailed structural analysis and elucidation of organic compounds (including stereochemistry and even dynamic effects), and quantitation of unknown natural and synthetic compounds in their mixtures in a single run, provided their signals are well separated [37].

On the other side, the two main disadvantages of NMR are its high equipment cost and its comparatively low sensitivity. When related to MS [Limit of detection (LOD) in the picomolar (pM) range], NMR has lower sensitivity, with LOD values in the low mM range. However, the detection sensitivity of NMR spectroscopy may be enhanced by increasing the number of scans, the concentration of the sample solution and by employing higher magnetic field strengths or modern probes. Accordingly, recent improvements in NMR instrumentation and technology, such as shielded and increasingly stronger high-field magnets [38], cryogenic probes [39,40], operation with large sample volumes (several ml), solvent suppression techniques, advanced data processing, versatile pulse sequences and polarization transfer techniques [41], have increased the sensitivity up to the nanomolar range. Other improvements include small OD tubes, Shigemi tubes and microcoil technology.

These characteristics have turned NMR spectroscopy into a powerful tool that can be used in pharmaceutical analysis [42] for the quality control of APIs and excipients for identification, impurity assessment, including adulterations [43,44] and assay purposes. Examination of the most recognized Pharmacopeias and an inspection of the recently summarized major compendial applications of NMR spectroscopy in current pharmaceutical analysis [45], reflect an increasingly important participation of this technique in the quality control monographs.

1.2.2. NMR spectroscopy and qualitative pharmaceutical analysis

The power of NMR for structural elucidation is well established; therefore, one of the major applications of NMR experiments is the 'de novo' structural elucidation of small organic molecules. The later is often considered an almost mechanical process, which can be solved mainly with the aid of NMR and/or MS methodologies, especially when the amount of sample is enough to run the powerful two-dimensional NMR (2D-NMR) experiments.

In practice, however, in the case of impurities this represents a rather intellectually and technically challenging problem, because their amounts are often scarce [46–49] and their structure is not always related directly to the API. Structural elucidation of trace impurities without separation poses additional demands, requiring minimum signal overlapping between structurally useful spectral signals of the impurity and the resonances of the main component.

However, the information provided by NMR is of such magnitude that the technique can be even used to determine the origin (production plant) of a drug based on its spectral profile [44].

1.2.3. NMR spectroscopy and quantitative pharmaceutical analysis: qNMR

The basic principles of quantitative NMR (qNMR) have been discussed elsewhere [50]. The main advantage of NMR spectroscopy is the linear relationship between the integrated intensities of the resonances from separate components in the spectrum and their molar contents in the sample [27]. The quantitative applications (^{1}H qNMR, qHNMR) are based on the nearly equal response of protons, regardless of their chemical shifts or coupling to other nuclei.

Absolute quantification is possible by relating the peak area of interest in the sample to a signal from an appropriate internal standard, without the need for a reference standard of the same chemical structure as the sample [51,52]. When NIST (National Institute of Standards and Technology) standards are used, traceability to the International System of Units (SI) can be established. Relative quantification can be carried out by relating the intensity of the peak of interest to one in the main compound.

In addition, NMR spectroscopy can be considered a relative primary analytical method, because it can be described completely by mathematical equations from which a full uncertainty budget may

Table 1
Summary of the most relevant ^{1}H and ^{13}C NMR experiments for studying small organic molecules.

NMR experiment	Acronym[a]	Information provided
^{1}H NMR	1D-^{1}H (^{1}H)	Number and types of protons.
Nuclear Overhausser effect	1D-NOE (NOE)	Through space H–H vicinity.
Diffusion NMR	DOSY	Molecular size; host–guest interactions.
^{13}C NMR	1D-^{13}C (^{13}C)	Types of carbons.
Special 1D ^{13}C NMR	APT DEPT	Type of carbons (CH, CH_2, CH_3), according to the number of protons attached to them.
2D Homonuclear (^{1}H–^{1}H) correlation	COSY	Through bond H–H correlations (coupled protons).
	TOCSY	Through bond long range correlations (coupled spin network).
	NOESY	Through space H–H correlations.
	ROESY	Long range, through space H–H correlations.
2D Heteronuclear (^{1}H–^{13}C) correlation	HMBC	Short range (^{1}J) H–C correlations.
	HSQC	Long range (usually ^{2}J and ^{3}J) H–C correlations.

[a] For a list with the meaning of the acronyms used in this table and throughout the text, see Appendix A, at the end of the manuscript.

be derived [53,54]. A validation scheme for qNMR methodologies has been proposed [55].

The technique offers a valuable, un-biased and near-universal approach for the analysis of complex mixtures and the simultaneous quantitation of their components, with minimum sample preparation and without the need of a previous separation. Therefore, qHNMR spectroscopy is highly regarded for use in purity assessment of small organic molecules, complementing the identification of potential impurities [56–58]. Currently, sensitivity and accuracy of the quantitative results are suitable for controlling low level impurities and qHNMR measurements are at least as reliable and precise as those obtained by chromatographic techniques [59].

Therefore, qNMR is increasingly gaining interest in pharmaceutical applications and has become an essential tool that may be used for establishing sample composition and components' molar ratio, as well as for assigning content, including the determination of the level of impurities [49]. Furthermore, the capability to perform concurrent identification and quantitation of impurities turns qHNMR into a unique analytical tool. However, the most challenging item is to find proper conditions and separated, pure signals, especially during impurity profiling [60].

An advantage of NMR is that quantitation can be achieved using the main sample component signals thus alleviating the need for time-consuming system suitability and standard runs prior to sample analysis [61]. This strong point was stressed by Deubner and Holzgrabe during their analysis of complex aminoglycoside antibiotics by micellar electrokinetic chromatography, RP–LC and NMR. In that study the authors described the difficulty of evaluating the impurities in gentamicin sulfate which contains no chromophore and has many structurally similar aminoglycoside impurities.

1.2.4. Hyphenated NMR techniques

LC–NMR is one of the most powerful techniques for the characterization of complex mixtures, since it combines a very efficient separation technique and the most important structure elucidation method. However, peak isolation can be also performed by liquid or gas chromatography (GC), as well as by other methods such as capillary electrophoresis (CE). These methods are very different, particularly with respect to sensitivity.

Only recently has LC–NMR coupling become efficient enough and hyphenation allowed the long-expected gain of maximal on-line information about individual constituents of a mixture, with due speed and efficiency. Details of the instrumentation and potential are given in many specific books [62–65]; its general applications in pharmaceutical analysis have also been detailed [66].

Analogous hyphenations [67] such as GC–NMR [68,69], CZE–NMR [70–72], CEC–NMR [73,74] and CE–NMR [75–78] have also been explored. However, in the on-line mode none of them have been used to solve problems in the area of pharmaceutical impurities and currently this application seems still unfeasible, mainly due to sensitivity problems. To date, CE–NMR is not a well-established method; however, development of smaller volume NMR probes and continuous enhancements of NMR sensitivity will probably transform CE–NMR in the near future into a valuable technique for biopharmaceutical analysis. On the other hand, despite its years of evolution and recent advances, GC–NMR is still in its infancy [79]; even more, its sensitivity, interfacing and other instrumental details require strong improvements.

In its different modes (on-flow, stopped flow and loop-storage techniques) HPLC–NMR may allow unambiguous on-line identification of even very complex structures. An integrated LC–NMR and LC–MS approach toward structural elucidation of impurities without their isolation has been informed [80]. However, very often it is learned that subsequent to having spent a considerable time on separation method development, the amount of structural information collected is limited, because it relates to the amount of time the sample remains in the flow cell. Fraction collection is of help, but attempts to isolate an impurity for off-line structure investigation does not always yield pure substances; instead, the resulting mixtures many times are not easily amenable to analysis, or the quantities are so small that they hinder running 2D NMR experiments.

A recent extension capable of providing NMR data of higher quality includes the insertion of a solid-phase extraction (SPE) unit between the HPLC column and the NMR probe [81], which was also employed for detecting adulterants in an herbal medicine [82]. This HPLC–SPE–NMR setup, where lipophilic stationary phases (silica-C18, styrene-divinylbenzene) are commonly utilized as SPE materials, allows the on-line trapping of the impurity peaks while removing the HPLC eluent.

Use of NMR solvents of high elution power (CD_3CN, CD_3OD) allows matching the peak volume to the active volume of the NMR flow cell, when the impurities are automatically back-flushed, submitting them to the NMR probe; this results in an overall increase of the sensitivity of the measurement due to analyte accumulation by multiple SPE collections. The overall process requires careful optimization of the SPE trapping and elution conditions, and it must also take into account that use of lipophilic stationary phases may result in loss of the more polar impurities, that cannot be trapped by the SPE stationary phase.

The problem of isolating the impurity for conventional NMR analysis (~1 mg) can be greatly alleviated by using cryogenically cooled probes, which offer an approximately fourfold increase in S/N, reducing the amount of impurity needing to be purified. Flow and non-flow alternatives are possible, as well as combination with SPE [83]. However, in many cases this does not alleviate the burden of manual preparative-scale isolation of the impurities.

The use of LC–NMR for identification of drug impurities has been impulse during the last 20 years [84]. In this way, NMR and particularly LC–NMR analysis provide complementary information to MS for rapid and unequivocal structural determination. This feature

and the appearance of accessible and more powerful spectrometers designed for routine measurements and by the simplicity of the measurements themselves explain the high and increasing interest in NMR spectroscopy on the part of pharmaceutical analysts [46].

2. NMR-assisted Identification/structure elucidation and quantitation of related impurities

According to the ICH Q3A Guideline [20], a "potential impurity is an impurity that theoretically can arise during manufacture or storage". It has been pointed out that NMR spectrometry is finding increasing application for the identification of unknown organic impurities in pharmaceutical preparations [85]. Impurity profiling is a typical team effort which involves many types of analytical chemists, including UV, IR and NMR spectroscopists, mass spectrometrists, TLC, HPLC and GC chromatographers, as well as experts in hyphenated and several other techniques. When NMR spectroscopy is also necessary for the structure elucidation [86], somewhat larger sample size is necessary. The use of impurity-enriched mother liquors or crude reaction products can overcome problems associated with sensitivity and may be adopted as a general strategy for their identification [87]. These samples are usually obtained by semi-preparative HPLC [88] or by column chromatography.

2.1. Process-related impurities

2.1.1. Impurities resulting from isolation in naturally occurring APIs

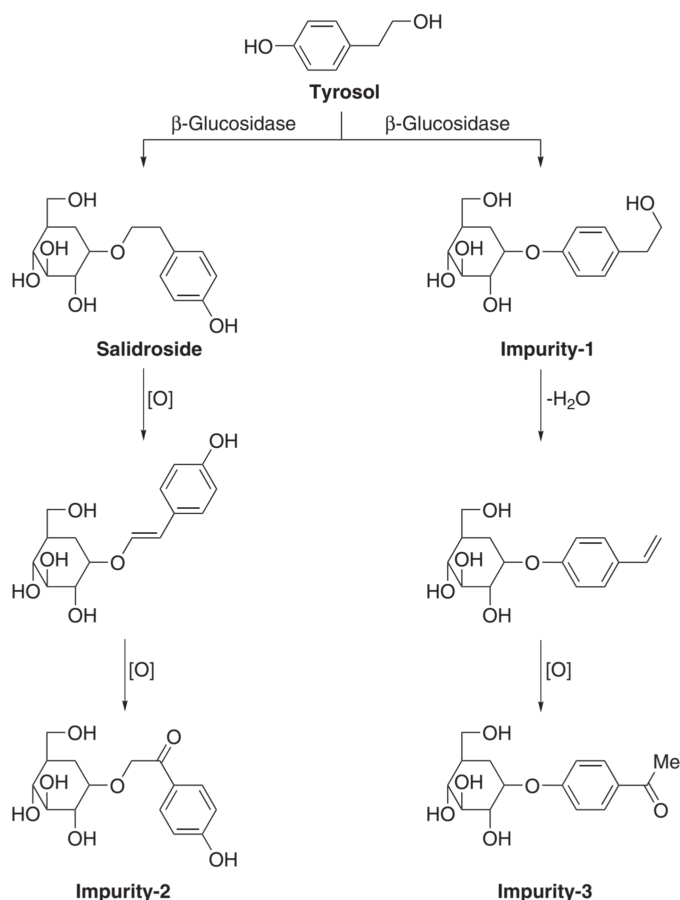
Echinochrome is a quinoid pigment isolated from some sea-occurring invertebrates, which is used in ophthalmology and for the treatment of acute myocardial infarction and ischemic heart disorder. Seven new impurities were isolated from the bulk drug and its formulation and their structures were elucidated by NMR in combination with other spectroscopies. The physicochemical properties of the impurities are close to those of echinochrome. For this reason, they can be present in echinochrome bulk drug after technological operations of isolation and purification. Some of these are also natural products [89].

NMR spectroscopy was employed to identify and quantitate impurities in dihydroquercetin (DHQ) [90]. Quercetin, related flavonoids (naringenin, kaempferol, dihydrokaempferol) and other species, resulting from plant raw material extraction, and impurities resulting from the extraction process, such as acetone, could be identified and quantitated from the proton spectra.

The qualitative and quantitative composition of the impurities is unique for the raw material and the DHQ production method. Therefore, it was proposed that ^{1}H NMR spectra are good fingerprints for determining the origin and preparation method of drugs based on DHQ.

Salidroside has been widely used in Chinese traditional medicine for its antioxidant and antiapoptotic properties and is a drug candidate for the treatment of cardiovascular and cerebrovascular diseases. Three impurities with related chemical structures were found during the analysis of the bulk drug and characterized by various 1D and 2D NMR techniques and other spectroscopies [91]. A pathway for their biosynthesis was also proposed, as shown in Scheme 1.

Sodium tanshinone IIA sulfonate (STS) is a water-soluble derivative of tanshinone IIA, an important lipophilic component isolated from the roots of *Salvia miltiorrhiza*. As an injection, STS is widely and successfully used in China for treating cardiovascular diseases. In the analysis of STS bulk drug, eight related impurities were observed and isolated by column chromatography, which structure was established employing a combined NMR and LC–ESI/MS approach [92]. NMR experiments (^{1}H, ^{13}C, HMBC, HSQC, COSY) were crucial for unequivocally establishing structural features, where MS was not useful, allowing to propose a possible mechanism for the formation of the impurities. Some of the impurities found were known related compounds [93].



Scheme 1. Biosynthetic pathway of salidroside and its impurities.

NMR spectroscopy was also employed for the structural elucidation of previously unknown impurities coming from new isolation processes. The traditional procedures for isolating vincristine and vinblastine from *Catharanthus roseus* are relatively inefficient. Recent efforts have resulted in a more effective manufacturing process at the expense of the appearance of new impurities. These and other known impurities were unambiguously identified by NMR spectroscopy, combined with MS techniques [94,95], after preparative chromatographic isolation of impurities. An 800 MHz NMR spectrometer equipped with a $^{1}H\{^{13}C/^{15}N\}$ Triple Resonance ^{13}C Enhanced Salt Tolerant Cold Probe was employed, where 2D experiments could be run overnight with 10 mg samples. ^{1}H–^{1}H, direct ^{1}H–^{13}C, long-range ^{1}H–^{13}C scalar and dipolar spin–spin connectivities were established from a combination of 1D (^{1}H, ^{13}C) and 2D (gHSQCAD, zTOCSY, gHMBCAD, NOESY and ROESY) NMR experiments. A DBPPSTE DOSY diffusion experiment was also performed.

Collection of the required spectral data was made more difficult by the relatively large size of the molecules, which yielded crowded spectra, turning laborious the extraction of spin–spin connectivities. In addition, these bisindoles exhibit solvent-, field-, and temperature-dependency of the sign and size of the ^{1}H–^{1}H NOEs, serious solvent-dependent line-broadening that affects different signals differently in any given solvent, or line splitting due to slow *N*-formyl rotamerism in vincristine analogs. These difficulties were overcome by running the experiments in two or three different

solvents and using the whole set of data in a complementary fashion in order to extract all the required information.

Applying the mass spectrometric shift technique to HPLC–ESI–MS/MS analysis, four impurities were identified in the unique semi-synthetic vinca alkaloid vinorelbine bitartrate. These were isolated by prep-HPLC and their structures were further confirmed by means of 1D and 2D (HMBC, HMQC, COSY) NMR spectra [96].

Most amino acids lack proper chromophors for UV-detection; therefore, the determination of low levels of impurities is analytically challenging [97,98]. Amino acids are produced by different manufacturing processes, each one conveying to the product a different impurity profile, with fermentation resulting in the most complex array of impurities [99]. NMR was employed as an orthogonal technique to HPLC for quantitative analysis of potential impurities of alanine. Organic acids such as malic, fumaric, aspartic and glutamic, were assessed by ^{1}H NMR spectroscopy with 400 and 600 MHz spectrometers using 128 and 16 scans, respectively [35,98]. In addition, L-Isoleucine and L-leucine present as impurities in L-valine are responsible for some of the process impurities found in lopinavir [100]. The latter were characterized by FT–IR, MS and ^{1}H NMR techniques.

During the 2007–2008 heparin crisis it was detected the presence of oversulfated chondroitin sulfate (OSCS), the adulterant of the API which caused many deaths [101]. NMR spectroscopy has been in use for fractionated medium-sized heparins more than a decade before this case. This event, however, where NMR spectroscopy played a decisive role in the structural determination of the contaminant, spurred intense research activity in this area, which resulted in a series of tests orthogonally aiming to achieve identification, measurement of bioactivity and detection of process impurities or contaminants in these drug products [102]. As a result, the current enoxaparin USP monograph contains a ^{13}C NMR spectroscopy test and the heparin sodium monograph includes a ^{1}H NMR identification test [103].

^{1}H NMR spectroscopy is very sensitive to minor structural variations, allowing easy identification of the repeating pattern of the disaccharide units of heparin. Therefore, not surprisingly NMR spectroscopy proved to be a powerful tool for quali- and quantitative analysis of compounds related to the extraction and purification processes of heparin, including dermatan sulfate, chondroitin sulfate A, galactosamine and 3-*O*-acetylated uronic acid derived impurities [104–107].

In these cases, major advantages of NMR analysis are its unique ability to unambiguously determine sulfation locations and the stereochemistry of the anomeric bonds between the saccharide subunits. The most useful experiments for spectral assignment are COSY and TOCSY (homonuclear correlation), NOESY and ROESY (nuclear Overhauser effect), HSQC and HMQC (heteronuclear single or multiple quantum coherence spectroscopy). Fortunately, some of the characteristic resonances of these contaminants lie outside the heparin regions, easing their detection; chemometrics analysis of the data resulted in more powerful methods [108–111].

Besides the use of NMR spectroscopy for impurity detection and characterization, scattered articles have reported the use of qHNMR as a way to determine the purity of isolated plant metabolites, along with the identity of their impurities [112–114].

2.1.2. *Impurities in synthetic active pharmaceutical ingredients*

APIs are usually prepared through multistep organic synthesis. Process-related impurities can be formed at any step and can ultimately appear in the final bulk drug, especially during scale-up of early-stage drug candidates. These low-level impurities formed by side reactions are always problematic, since they may affect the API through an unwanted pharmacologic activity or just merely from the cosmetic point of view, when colored by-products are formed. Therefore, it is always desirable to investigate the structures of by-products not only in the reactants but also in intermediates and final products. Elucidation of the structures of these process impurities is an important step in refining the process chemistry in order to develop robust routes for manufacturing APIs.

Different techniques, including LC coupled to ion-trap and time of flight mass spectrometry (IT/MS, LC–TOF/MS) and NMR (^{1}H, ^{13}C, DEPT, and HETCOR) allowed characterization of impurities in chloroquine and hydroxy chloroquine bulk drug samples [115]. Process-related impurities were also detected in ezetimibe by LC–MS/MS and elucidated by NMR. NOESY experiments were relevant in this investigation [116,117]. An impurity was found in phenazopyridine hydrochloride; its identification by MS–MS and 2D-NMR spectroscopy allowed to propose a mechanism for its formation [118]. Analogously, a tridesoxy impurity found in didanosine and characterized by 300 MHz NMR (^{1}H and ^{13}C) was proposed to result from deoxygenation during the hydrogenation stage of the preparation of the API [119].

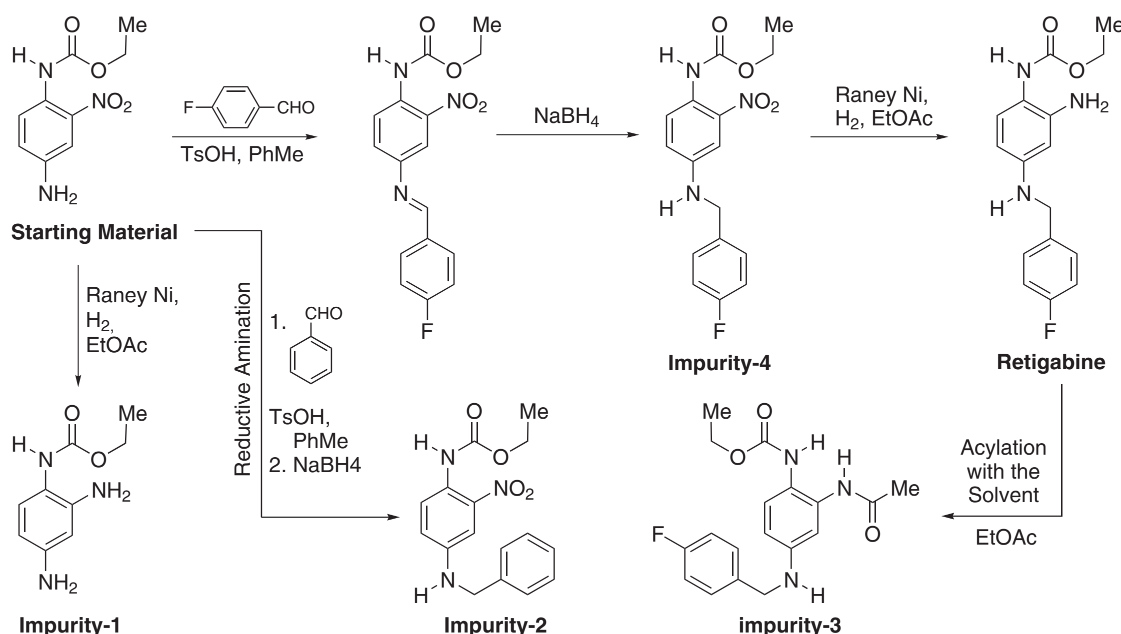
Six process-related impurities were detected in pantoprazole sodium bulk drug substance [120] and another six impurities were found in the structurally related rabeprazole [121]. These impurities differed mainly in the degree of oxidation of both, the sulfur bridge and the pyridinic nitrogen. They were identified, synthesized and characterized using ^{1}H, ^{13}C and DEPT NMR experiments, aided by techniques such as HPLC, LC–MS and IR. Two impurities of tazarotene were characterized by NMR [122].

Process-related impurities found in triclabendazole were identified by 1D and 2D NMR (^{1}H, ^{13}C and DEPT, HSQC, HMBC) spectroscopy, concluding that one of the impurities originates in an impurity of the starting materials [123]. Glutamine as an impurity of glutamic acid generated a process related impurity in leucovorin [124]. Synthesis and spectroscopic characterization (IR, MS, ^{1}H and ^{13}C NMR) of the impurity provided the definitive proof for its postulated structure. The process related impurities in ceftizoxime sodium bulk drug were identified, isolated and characterized by using HPLC (analytical and preparative), LC–MS–MS, IR and NMR (^{1}H, ^{13}C) techniques. Spectra of the impurities and the API were compared [125].

Process-related impurities were also found in telmisartan [126], valsartan [127] and candesartan cilexetil [128], and elucidated/confirmed based on 2D-NMR and MS data. Three impurities were detected in simvastatin substance and tablets By HPLC–DAD. Their proposed structures revealed modifications of the lactone ring of the simvastatin molecule; one of them was synthesized and structurally characterized by NMR [129]. For the development of an HPLC separation, process-related impurities of pridinol mesylate were also synthesized and characterized by ^{1}H and ^{13}C NMR spectroscopy [130].

Classical methods were also employed for the isolation and characterization of process related impurities in eslicarbazepine [131]. A potential process related impurity of phenazopyridine HCl was isolated by preparative HPLC and structurally elucidated by MS–MS and 2D-NMR spectroscopy [118]. Analogously, four process-related impurities were found in the novel antiepileptic drug retigabine [132], which included (Scheme 2) the reduced unreacted starting material (Impurity-1), a defluorinated analog (Impurity-2), the solvent-acylated final product (Impurity-3) and the last intermediate (Impurity-4). Their structures were determined by 1D (^{1}H, ^{13}C, DEPT, ^{19}F), and 2D (^{1}H–^{1}H COSY, HMBC, HSQC) NMR experiments.

Eight related impurities of olanzapine were isolated and characterized, including a new impurity confirmed as 1-(5-methyl-thiophen-2-yl)-1*H*-benzimidazol-2(3*H*)-one by X-ray single diffraction, MS, ^{1}H NMR, ^{13}C NMR and HSQC [133]; a mechanism for its formation was proposed.



Scheme 2. Synthetic sequence of the manufacturing process of retigabine and sources of the impurities.

The structural determination [IR, MS and NMR (^{1}H, ^{13}C, DEPT)] of four impurities in zafirlukast allowed to propose a synthetic scheme to account for their origin [134]; analogously, four impurities were found in montelukast; after their characterization by spectroscopic analysis and the corresponding synthesis, their origin was also proposed [135].

Some examples found in recent literature point out to special precautions required during complex syntheses. The structural analysis (MS, IR, and 200 MHz ^{1}H and ^{13}C NMR) of an impurity isolated from lisinopril revealed it to be a result from incomplete protection of the starting material [136]. An impurity was isolated from benazepril and subjected to spectroscopic analysis using LC–MS/MS, MS, NMR (^{1}H and ^{13}C) and FT-IR. From its structure, it was proposed that it might have been generated due to the presence of small amounts of moisture during a key hydrogenation step [137].

Zolmitriptan-dimer, which was identified as by-product of the last step Fischer indole synthesis, was characterized as an impurity in zolmitriptan by means of LC–MS and NMR studies [138]. Two new dimers (1,2 and 2,2) were found as process impurities in rizatriptan. From the ^{1}H and ^{13}C NMR values it was concluded that *N*,*N*-dimethyl butanal diethyl acetal which is used as reagent in the synthesis of rizatriptan is reacting at indole 2-position of one rizatriptan molecule and at reactive positions of another rizatriptan molecule, forming a dimer with loss of ethanol [139].

Three process impurities were observed in adapalene and characterized by ^{1}H and ^{13}C NMR spectroscopy. Taking into account the synthetic process of the API, one of them is formed during the Friedel–Crafts step employed to prepare the first intermediate, while the others are formed during the Negishi coupling of the latter. Their presence is diagnostic of the involvement of these steps in the corresponding synthetic procedure [140].

After a systematic revision of the synthetic process, the structural elucidation and quantitative determination of seven process impurities (two of them new) of the melatonergic agonist agomelatine was carried out, employing reference standards of the impurities and with the help of NMR and FT-IR techniques [141]. This allowed the development of a new chromatographic method for analysis of the API (Scheme 3).

Relevant impurities were found in citalopram and escitalopram; their structures were elucidated by MS^n (ion trap mass analyzer), accurate mass (Q-TOF mass analyzer), FT-IR and NMR (^{1}H, ^{13}C NMR and DEPT) data [142,143]; this enabled to propose a mechanism for their formation. A process impurity in a crude roflumilast preparation was isolated, characterized by NMR spectroscopy and its identity confirmed by an independent synthesis [144].

Four process and degradation impurities were found in darifenacin. Based on the structures of the impurities, proposed by LC–MS^n, these were independently synthesized and used for further structural confirmation by IR, NMR and MS techniques [145].

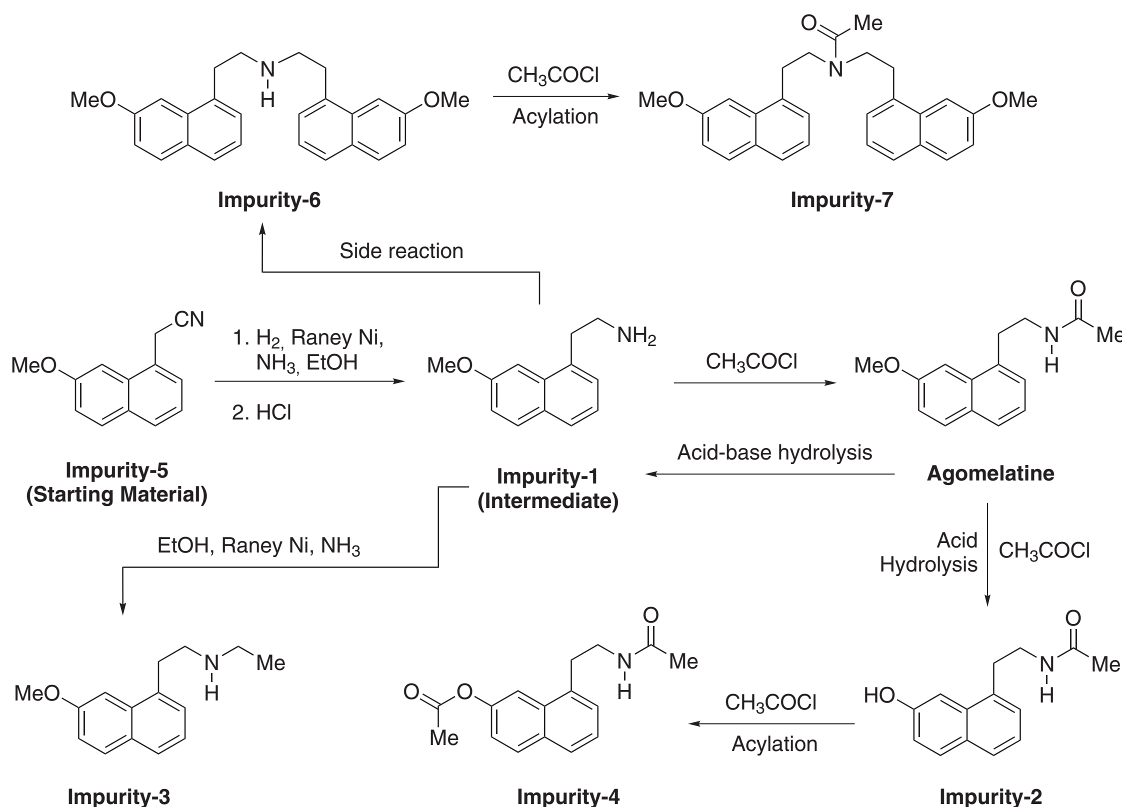
2.1.3. *Process impurities: Geometric and conformational isomers*

The geometric isomer *Z* of endoxifen was confirmed as the active isomer through 2D NMR experiments, including HSQC and COSY [146]. NOE (ROESY) experiments were employed to establish spatial proton–proton correlations between key protons on the ethyl group and the neighbor aromatic rings and confirm the stereochemistry of each isomer (Fig. 1).

The major impurity of the drug substance was found to be the *E*-isomer. The previous identification of the geometric isomers of endoxifen by ^{1}H NMR was empirically based on reported trends for the chemical shifts of the —O—CH_2 protons in the side chain, mentioned to be upfield in the (*Z*)- compared to (*E*)-configuration for similar triarylethylene compounds [147].

The identification and characterization of a geometrical isomeric photo degradation product of eprosartan was reported, using LC–MS and LC–NMR [148].

Five impurities were isolated from a crude reaction mixture of glimepiride. These were spectroscopically characterized by NMR, FT-IR, UV and MS; among them, the *cis* (geometric isomer), as well as the *meta* and *ortho* (positional) isomers were found [149]. On the other hand, the ^{1}H NMR spectrum of enalaprilat exhibits two separate ($\Delta\delta \approx 0.2$ ppm) sets of signals. They correspond to the *cis* and *trans* conformers of the drug [150], which are in equilibrium with a isomer ratio of 71.5:28.5 at 298 °C, and arise from the slow rotation around the amide bond.



Scheme 3. Manufacturing process of agomelatine and origin of the potential impurities originating in side reaction and degradation.

2.1.4. Process impurities: Positional and functional group isomers

A positional isomer of primaquine was detected as an impurity. The proposed compound was synthesized and characterized by NMR spectroscopy [151]. Analogously, four impurities were detected in piperaquine phosphate API by a newly developed gradient RP–HPLC method. They were identified by LC–MS/MS and their structures, which relate to the manufacturing process, were confirmed by NMR and FT-IR spectroscopy, conducted using synthesized authentic compounds. A positional isomeric impurity was identified by 2D NMR spectroscopy (^1H–^1H COSY, HSQC, HMBC). This impurity is formed because of contamination of batches of the 4,7-dichloroquinoline precursor with 4,5-dichloroquinoline [152,153].

LC–MS was employed to detect an isomeric impurity in cefpodoxime proxetil, a third generation cephalosporin, and its hydrolysis product. Detailed structural information was confirmed by LC–NMR [154]. Isomeric impurities in cefdinir were confirmed in an analogous fashion [155].

Ivermectin is produced by hydrogenation of a series of avermectins. Examination of ivermectin by LC–MS data revealed four process impurities. In one of them the fragmentation pattern was identical to avermectin B2a indicating a possible isomer; this suspicion was confirmed by NMR spectroscopy [156].

The related compounds of the novel hypoglycemic drug G004 were identified. Using ^1H, ^{13}C NMR and 2D NMR analysis and MS data, it was found that one of the isolated compounds was an isomer of the drug [157]. Etimicin sulfate is a semi-synthetic aminoglycoside prepared from gentamicin C1a. Ten impurities were detected in the bulk drug by LC–ELSD and LC–ESI–MS^n, many of them followed the similar fragmentation pattern as a result of differing in the position of an ethyl substituent. Six of them were isolated by column chromatography and two impurities proved to be isomers of the drug after ^1H and ^{13}C NMR analysis and comparison with literature data [158].

^1H and ^{13}C NMR spectral analysis helped elucidating a *C*-alkylated isomeric impurity (functional group isomer) in duloxetine hydrochloride [159,160], a *O*-alkylated naphthol (Fig. 2). The

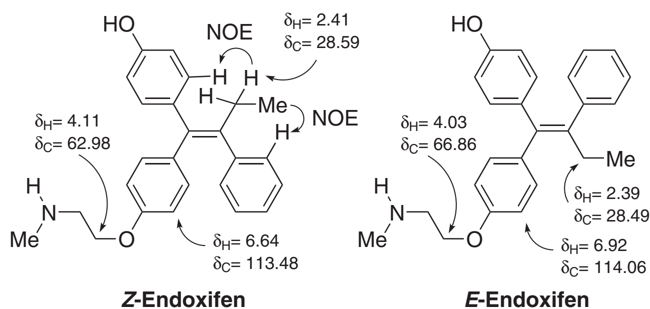


Fig. 1. ^1H and ^{13}C NMR diagnostic signals of *Z*- and *E*-endoxifen (ppm values).

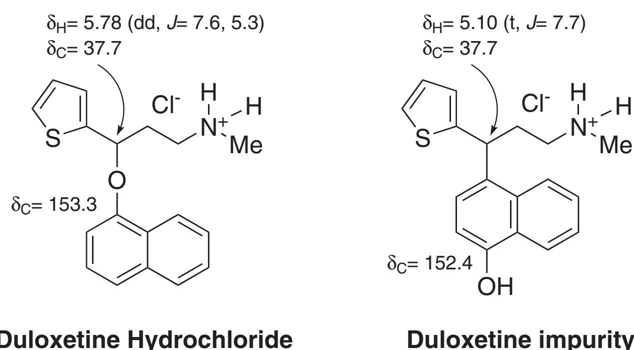


Fig. 2. Chemical structures and diagnostic NMR resonances (in ppm) in duloxetine and its impurity.

formation of this unusual reaction product was attributed to the ambidentate nature of the nucleophile naphthol.

2.1.5. Process impurities: Configurational isomers. Epimers and enantiomers; NMR determination of enantiomeric purity

Molecules having stereogenic centers are highly important as pharmaceutical products; some of them are natural products, but semi-synthetic and fully synthetic small organic molecules constitute an increasing proportion of these APIs in the current pharmaceutical arsenal, many of which are sold as a sole optically active enantiomer. The ICH Q6A guideline states that "where a new drug substance is predominantly one enantiomer, the opposite enantiomer is excluded from the qualification and identification thresholds given in the ICH Guidelines on Impurities in New Drug Substances and Impurities in New Drug Products because of practical difficulties in quantifying it at those levels. However, that impurity in the chiral new drug substance and the resulting new drug product(s) should otherwise be treated according to the principles established in those Guidelines" [161].

Accordingly, the quality control of chiral reagents and products requires methods capable of discriminating between enantiomers and for determining their enantiomeric purity, to resolve questions such as whether racemization has occurred during the synthesis of a chiral molecule or distinguish the enantiomeric impurity among other impurities in a sample. Typically, a 98% pure catalyst, ligand or auxiliary reagent will intrinsically afford at least 2% of the wrong enantiomer, lowering the enantiomeric excess of the final product. Therefore, since 1998 the control of impurities in chiral reagents [162–164], involves NMR among other methodologies, is a routine task.

Currently, the method of choice for limiting enantiomeric impurities is chromatography (GC, HPLC) with chiral columns. Among the other alternatives, NMR spectroscopy is the only non-separative technique useful for the determination of the stereochemical purity of materials of pharmaceutical interest, employing for that purpose chiral derivatizing agents, lanthanide shift reagents and chiral solvating agents (CSA). CSA-based chiral NMR methods can be developed when only a single enantiomer of the analyte is available [165], provided both enantiomers of the CSA are at hand. This and other developments have converted ^{1}H NMR into a straightforward and more time-efficient approach toward detection and quantification of enantiomers [166,167].

NMR spectroscopy is also well known to be one of the most powerful tools for the elucidation of the chiral recognition mechanism on a molecular level [168]. NMR signal separation in chiral environments has been studied and the corresponding mechanisms were related to those operating in separative techniques such as HPLC and CE [169]. The chiral recognition can also be observed by ^{13}C NMR spectroscopy [170]; however, since this is a less sensitive alternative, the method is not used as frequently as ^{1}H NMR.

The enantiomeric purity of acyl-L-carnitine was determined by 500 MHz ^{1}H NMR employing fast diastereomeric interaction with chiral shift reagents such as chiral lanthanide-camphorato {[Eu(hfc)$_3$], [Pr(hfc)$_3$]} or chiral samarium-pdta shift reagents. The LOD of the determination was 0.5% for the D-enantiomer [171].

NMR is also a powerful tool for distinguish between diastereomers. Seven related impurities were detected in asperosaponin VI bulk drug and their structures were elucidated by TOF-MS, IR and NMR techniques. One of the impurities was found to be a mixture of two epimers (α- and β-glucosyl anomer derivatives) [172]. Four impurities were detected in the semisynthetic drug docetaxel. Two of them are oxazolidinones resulting from side reactions during the introduction of a Boc protecting group and acid deprotection, while the remaining pair are epimers of the API, formed by isomerization of two different hydroxyl groups [173,174].

Three process impurities were recently detected in the fluorine-containing steroid clocortolone pivalate by HPLC. They were isolated by semi-preparative LC and identified by MS and NMR spectroscopy. One of them was the 6β-fluor epimer of the API. Their formation was also proposed [175]. Table 2 summarizes the role of NMR spectroscopy in providing improved knowledge on process impurities of pharmaceutically relevant active ingredients.

2.1.6. Genotoxic, toxic and mutagenic impurities

Genotoxic impurities (GTIs) are chemical compounds that may be mutagenic and could potentially damage DNA with an accompanying risk of cancer.

The analysis of GTIs has many special characteristics and difficulties. First of all, it can be very challenging because usually they must be controlled at levels significantly lower than 0.1% (1000 ppm). According to the current guidance, the threshold for toxicological concern in commercial products is 1.5 μg/day. Current regulations expect to control GTIs to levels in the ppm range [176]. Therefore, the analytical procedure should ideally allow detection limits in the range of 1–5 ppm.

Another special issue is solubility, since high sample concentrations are required (100 mg/ml) to enable analyses of the low-level GTIs. This poses the risk of sample precipitation, especially if the sample solution needs to be cooled in order to control degradation.

In addition, there is no single analytical procedure for analysis of all GTIs, because of their wide structural diversity. Furthermore, their reactive and often volatile nature poses difficulties on sample preparation in order to ensure the integrity of the analytes.

Therefore, the analyst should always decide on which is the correct analytical technique for each particular GTI determination. Among the five major alternatives (GC, LC, GC–MS, LC–MS and NMR), the GC-based techniques are preferred for the most volatile compounds, and the MS-based alternatives should be used for the cases requiring the highest sensitivity.

The chromatographic techniques are very complementary with each other. However, NMR has areas of application which can overlap with all of them, being most suitable when the compounds are not too volatile and their abundance is not too low [178].

The advantages, disadvantages and potentials for the use of NMR in GTI analysis have been recently discussed in depth [179]. Therefore, with the modern digitizers, the LOD and LOQ in qNMR are dependent on the number of scans, the solubility of the API and the strength of the magnetic field.

Although not many examples are currently available to illustrate the potential of NMR spectroscopy in this area, it is clear that this is one of the methods that will increase in importance in the coming years for detecting and quantitating these impurities.

A potential genotoxic impurity from the synthesis of an API was used to challenge the LOD/LOQ on a 400 MHz spectrometer with a limit test. Samples were spiked with the impurity up to 100 ppm and spectra were acquired (4096 scans, D1 = 2 s) with the inverse-gated ^{13}C decoupling sequence to prevent overlapping satellites with the impurity. Unequivocal detection of the spiked genotoxic impurity was demonstrated at a level of 25 ppm [180]. In addition, qNMR was used for quantitation of methanosulfonic acid throughout the production process of an API and a practical quantification limit of 100 ppm was consistently attained [181].

Hydrazines, hydrazides and hydrazones must also be controlled in APIs [182]. Aryl hydrazone isomerization, resulting in the formation of residual geometrical *E*-isomer impurities, were determined by HPLC with DAD and MS–MS detection, followed by confirmatory NMR data [183].

Combined application of LC and NMR spectroscopy allowed characterization of mutagenic impurities in vestipitant. The starting material for the synthesis was identified as the root cause of impurities [184].

Table 2
Process impurities found in some active pharmaceutical ingredients.

Drug	Field (MHz)	NMR experiments	Remarks	Ref.
Agomelatine	500	^{1}H, ^{13}C, DEPT, COSY, HMBC, HSQC	Imp-1: First intermediate. Imp-2 and Imp-3: Side reaction products of Imp-1. Imp-4: Acetylated derivative of Imp-2. Imp-5: Starting material. Imp-6: Dimer of Imp-1. Imp-7: Acetylated derivative of Imp-6.	[141]
Chloroquine (CQ) Hydroxychloro-quine (HCQ)	400	^{1}H, ^{13}C, DEPT, COSY, HETCOR (^{1}H–^{13}C)	Imp-1 and Imp-2 (CQ-I and HCQ-I): Formed by deethylation of intermediates 5-(*N*,*N*-diethyl-ethylamino)-2-pentyl amine and 5-(*N*-ethyl-*N*-2-hydroxyethylamino)-2-pentyl amine, respectively. Imp-3 and Imp-4 (CQ-II and HCQ-II): Quaternary ammonium products formed by chloromethylation of the amino side chain of the APIs in basic medium, with dichloromethane.	[115]
Citalopram	400	^{1}H, ^{13}C, DEPT-135	Imp-1: The Grignard reagent performs a double addition in the first step of the synthesis. The product undergoes all the following reactions toward the API. Identified as 1-(1,1-bis(4-fluorophenyl)-1,3-dihydro isobenzofuran-5-yl)-4-(dimethylamino) butan-1-one HBr.	[142]
Clindamycin palmitate hydrochloride	300	^{1}H, ^{13}C, DEPT	Imp-1 (Clindamycin palmitate sulphoxides α/β-isomers): Formed by oxidation at the sulfur atom. Imp-2, Imp-4, Imp-7 Imp-9 and Imp-10 (laurate, myristate, pentadecanoate, heptadecanoate and stearate ester of Clindamicin respectively): From impurities in the starting palmitoyl chloride. Imp-3 (Lincomycin palmitate): Lincomycin is the starting material for the synthesis of Clindamycin. Imp-5 (7-epiclindamycin palmitate): Formed by C-7 epimerization. Imp-6 (Clindamycin palmitate 3-isomer): Formed by esterification on C-3. Imp-8 (Clindamycin B-palmitate): Clindamycin B present as an impurity in the starting Clindamycin. Imp-11 and Imp-12: Starting palmitic acid and Clindamycin.	[177]
Clocortolone pivalate	200, 300, 600	^{1}H, ^{13}C, NOESY	Imp-1: 6β-Fluor epimer. Imp-2: 4-Fluor isomer. Imp-3: 11-Pivaloylated $\Delta^{60,07}$ derivative.	[176]
Escitalopram	400	^{1}H, ^{13}C, DEPT, HMBC, HSQC, DQF-COSY	Imp-1 (ESC-I): Uncyclized precursor of the API. Imp-2 (ESC-II): Formed by chloromethylation of the amino side chain of ESC with CH_2Cl_2 (solvent). Imp-3 (ESC-III): Formed by chloromethylation of the amino side chain of ESC-I with CH_2Cl_2.	[143]
Eslicarbazepine acetate	400	^{1}H, ^{13}C, COSY	Imp-1: Due to the presence of propionic anhydride in acetic anhydride, used in the last step of the synthesis.	[131]
Ezetimibe	500	^{1}H, ^{13}C, DEPT, COSY, HSQC, HMBC	Imp-1: Formed during hydrogenation of the last intermediate (benzyl ezetimibe), by deoxygenation of the benzyl alcohol with cleavage of the β-lactam C-N bond. Imp-2: Formed by deoxygenation of the benzyl alcohol moiety.	[117]
G004	500	^{1}H, ^{13}C, HMBC	Imp-1: 1-(4-(2-(2-bromobenzenesulphonamino) ethyl) phenylsuphonyl)-3-(trans-4-methyl cyclohexyl) urea is the 2-bromo analog. Isolated using LC.	[157]
Montelukast sodium	500	^{1}H, ^{13}C, DEPT, 2D (unspecified experiments)	Imp-1: Result of incomplete transformation of the cyano group into a carboxate during the basic hydrolysis stage. Imp-2: Formed during the Montelukast work up stage by protonation and subsequent dehydration of the tertiary hydroxyl moiety. Imp-3 and Imp-4: Saturated and deschloro analog of the starting material, which undergo all the sequence of reactions leading to the API.	[135]
Naproxen	400	^{1}H, DQFCOSY, gHSQC, gHMBC	Imp-1: Identified as 2-(6-methoxynaphthalen-2-yl) acrylic acid, the acrylic acid of naproxen.	[33]
Phenazopyridine.HCl	400	^{1}H, COSY, NOESY	Imp-1: Formed by addition of phenyl radical to phenazopyridine HCl during last step of the synthesis. Identified as 3-phenyl-5-phenylazo-pyridine-2,6-diamine.	[118]
Retigabine	400	^{1}H, ^{13}C, ^{19}F, COSY, HMBC, HSQC	Imp-1: Produced by reduction of ethyl 2-nitro-4-aminophenylcarbamate as a vestigial intermediate. Imp-2: Resulting from benzaldehyde being an impurity in 4-fluoro benzaldehyde. Imp-3: Acetylation product of retigabine. Imp-4: Vestigial intermediate of retigabine.	[132]
Rizatriptan Benzoate	300	^{1}H, ^{13}C	Imp-1: Formed during the Fischer indole reaction, rizatriptan further undergoes a dimerization reaction through electrophilic substitution, yielding 1,2 and 2,2 rizatriptan dimers.	[139]
Sodium Tanshinone IIA Sulfonate	300, 500	^{1}H, ^{13}C, COSY, HMQC, HMBC	Imp-1 and Imp-5: Hydroxylated derivatives of the API. Imp-2: Formed by removal of the methyl group at C-4 of Imp-4. Imp-3 and Imp-6: Side products attributed to oxidation of C-19. Imp-4: Formed by dehydration of Imp-1. Imp-7: Formed by hydration of the furan ring of Imp-1. Imp-8: Produced by oxidation and further esterification at C-19 of Imp-4.	[92]

Table 2 (*Continued*)

Drug	Field (MHz)	NMR experiments	Remarks	Ref.
Tazarotene	300	^{1}H, ^{13}C	Imp-5: Side reaction of a PCl_3-mediated Pummerer rearrangement. Imp-6: Side product of a Sonogashira coupling reaction.	[122]
Telmisartan	300	^{1}H, ^{13}C, DEPT, COSY, HSQC, HMBC	Imp-1: Formed during radical bromination of the starting material 4-methyl biphenyl-2-carboxylate.	[126]
Valsartan	400	^{1}H, ^{13}C, DEPT	Imp-1 and Imp-2: Synthetic intermediates. Imp-3 and Imp-4: Products resulting from free radical desulfurization. Imp-5: Result from reaction of 5-phenylthiovaleric acid with Imp-1 and subsequent alkaline hydrolysis.	[127]
Vincristine (VCR) Vinblastine (VLB)	800	^{1}H, ^{13}C, ^{15}N, COSY, HSQC, NOESY, ROESY, HMBC, gHMBCAD, ^{1}H–^{15}N gHSQCAD, zTOCSY	Three new impurities. Imp-2: Cyclo-VCR. Imp-4: [VCR]-C(16)-COOEt, from oxidization of Imp-5. [VLB]-C(16)-COOEt: This impurity is a new natural product.	[94,95]
Zafirlukast (ZLK)	200, 400	^{1}H, ^{13}C, DEPT	Imp-1: Formed by trans-esterification of the cyclopentyl group of ZLK with methanol under basic conditions. Imp-2: Formed from Imp-1 by condensation with o-toluene sulfonamide. Imp-3 and Imp-4: Due to the presence of *m*/*p*-toluene sulfonamide impurities in *o*-toluene sulfonamide. Imp-5: Synthetic intermediate.	[134]

Finally, a toxic impurity was isolated from the experimental anti-neoplastic drug XP315. Characterization by combination of NMR (400 MHz, including gCOSY, gHMBC, gHSQC) and mass spectral methods revealed that it resulted from the dimerization of an intermediate by ring fusion, during the synthetic process [185].

2.1.7. Organic volatile impurities (residual solvents) and other accompanying impurities

The organic volatile impurities (OVIs) are a potential toxic risk for pharmaceutical products and have been a concern of manufacturers for many years. The ICH addresses this topic in its Q3C guideline [186]. The most widely used method for analysis of OVIs is GC [187]; however, there has been a slow but increasing use of NMR in this area. When the first coupled continuous-flow capillary GC–solenoidal microcoil ^{1}H NMR spectroscopy (400 MHz) experiments were disclosed, the separation of a mixture of diethyl ether, dichloromethane and tetrahydrofuran, at an oven temperature of 60 °C was informed [69]. Acetone, resulting from the extraction process was identified and quantitated by NMR spectroscopy in dihydroquercetin, isolated from natural sources [90].

Preparation by-products such as ethanol and acetone are among the most common impurities identified in heparin preparations by ^{1}H and ^{13}C NMR spectroscopy [104,188,189]. Being a process impurity, for the development of a chemometric-NMR approach to heparin analysis, the presence of EtOH was considered a potential interference for the determination of the drug or other impurities [106]. Other residual solvents like methanol, formic acid [104], and butanol (δ = 0.91, 1.35, 1.53, and 3.61 ppm) were also found in heparin samples [190], as well as sodium acetate and the chelating agent ethylenediaminetetraacetic acid (EDTA) ($\delta \sim 3.5$ ppm) [191].

It has been reported [192] that when initial studies concluded that direct injection or headspace GC could not be employed to determine the levels of *N*-methyl pyrrolidinone (NMP) in a process intermediate, a ^{1}H NMR assay was developed and validated to directly quantitate the solvent at a 140 ppm level in the sample matrix.

Aldehydes and silicon derivatives have also been observed by NMR spectroscopy. Bortezomib is a modified dipeptidyl boronic acid [193]. Recent comparison between two brands of the drug revealed the presence of *tert*-butanol, a compounding solvent. In addition, this API proved to have silicon-containing impurities such as silanes and siloxanes and isovaleraldehyde as an impurity of unknown origin [194].

The related aldehydes, acetaldehyde and propionaldehyde, were quantitatively determined as impurities in poloxamer 188 by ^{1}H NMR [195]. On the other hand, the NMR detection limit of residual silicone oil in poly(lactic acid) and poly(lactic acid–co-glycolic acid) microspheres, was determined to be 100 ppm, which compared favorably with IR determinations (5000 ppm) [196].

NMR spectroscopy was employed to determine the levels of residual benzene, toluene, acetone, and ethyl ether in cocaine [197]; ethyl acetate and methylene chloride, which were previously not detected in cocaine samples, were also found. An excellent correlation was obtained between the results of Schöniger flask combustion analysis for chlorine as an appraisal of chlorinated solvent levels and NMR-based estimates of chloroform. This demonstrated the suitability of NMR spectroscopy for the quantitation of chlorinated solvents [198].

2.2. Degradation products

2.2.1. Impurities resulting from degradation of synthetic APIs

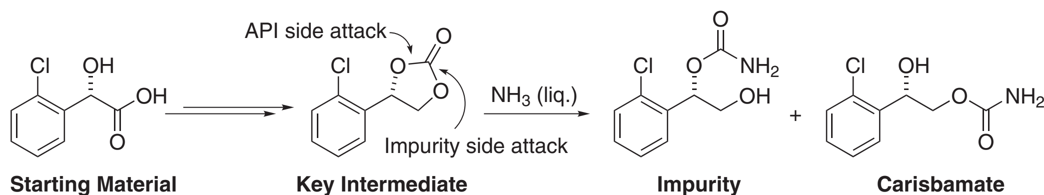
Forced degradation is a useful tool for impurity profiling [199,200]. Due to its multiple advantages, NMR spectroscopy is widely used in pharmaceutical analysis in order to understand the course of the degradation and also identify and structurally elucidate its embedded impurities.

Stress tests of eprinomectin, a synthetic acetylated amino derivative of abamectin, revealed two impurities. Given their isomeric nature (2-epimer and $\Delta^{2,3}$-isomer) their structures became fully elucidated only after analysis of ^{1}H, ^{13}C, NOESY and COSY experiments [201].

The novel antiepileptic drug carisbamate undergoes significant degradation when subjected to stress conditions. The degradation products were isolated and characterized. MS/MS and 2D-NMR (COSY and HSQC) studies revealed that one of the degradation products resulted from positional isomerization of the carbamate moiety of the API [202], being prepared as shown in Scheme 4.

Twelve impurities of clindamycin palmitate hydrochloride were isolated by preparative HPLC and characterized by a combination of LC–MS, FT-IR and simple 1D NMR (^{1}H, ^{13}C and DEPT) techniques [176]. Thorough NMR analysis was used for the characterization of an oxidation impurity of clopidogrel [203] and impurities of pridinol mesylate [204].

An impurity of citalopram was isolated by semi-preparative HPLC and its structure was established by MS, NMR and IR spectroscopy [142]. Analogously, chloroquine-*N*-oxide was identified as the major oxidative degradation product of chloroquine [205].



Scheme 4. Synthesis of carisbamate and origin of its isomeric impurity.

An *N*-oxidation product of cetirizine was isolated and characterized by 400 MHz NMR spectroscopy along with LC–MS/MS. The product is also a metabolite of the drug [206].

Two isomeric monoacylated diacerein impurities (MW = 326) were characterized by NMR spectroscopy, which confirmed their structures and revealed the exact position of the acetyl group in each case [207]. Unknown impurities of the antidepressant drug escitalopram were elucidated by ^{1}H and ^{13}C NMR and their structures were confirmed by synthesis [143]. Preparative HPLC, LC–MS/MS, UPLC–TOF-MS, NMR and FT-IR spectroscopy were employed to study the forced degradation of dipyridamole product, resulting in two new impurities (one of them an interaction product) which were characterized by NMR spectroscopy [208]. LC–NMR and LC–MS allowed to elucidate a new impurity in the antifungal drug icofungipen [209].

A novel impurity in eprosartan bulk drug was characterized by ESI/MS^n and NMR [210], and five impurities were found in candesartan cilexetil subjected to stability and forced degradation studies. They were structurally elucidated employing NMR techniques [211]. Low-level unknown impurities in GW876008, a novel corticotrophin-release factor 1 antagonist were isolated and characterized employing LC–NMR and HR-NMR in conjunction with mass spectrometric experiments [212]. Similarly, degradation products of anastrozole [213] and etimicin sulfate were determined by LC–MS/MS and NMR spectroscopy and LC–ESI–MS^n and NMR, respectively [158]. LC–NMR was employed for structural characterization of the oxidative degradation products of SCH 56592, an antifungal agent [214].

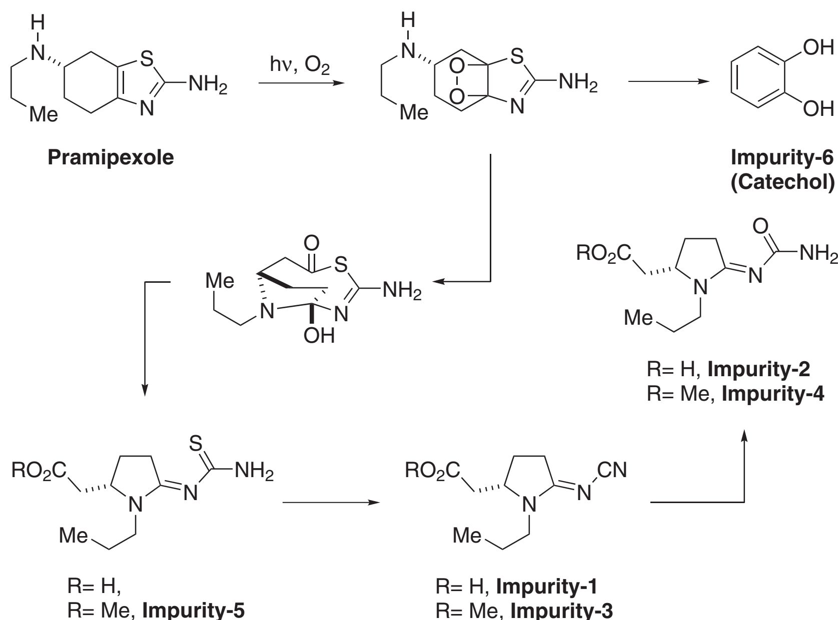
Two unknown impurities of moxidectin were isolated by flash chromatography during stress testing of the drug, and fully characterized by 1D (^{1}H, ^{13}C) and 2D (NOESY, COSY, HMBC and HSQC) NMR experiments [215].

The photodegradation of fleroxacin was investigated in different injections and solutions. The three major photodegradation products were detected and isolated by HPLC, and elucidated employing FT-IR, MS^n and TOF-MS, as well as ^{1}H, ^{13}C, DEPT, and 2D NMR. Significant differences in the photodegradation process and photostability between the concentrated injection and the dilute injection have been demonstrated, and photo-degradation pathways were proposed [216].

Analogously, photo-irradiation of tablets containing pramipexole afforded pyrrolidine carboxylic acids (Impurities 1 and 2) and catechol (Impurity-6). The drug was also smoothly degraded in the methanolic solution yielding carboxylic esters; a possible degradation mechanism was proposed in which the reaction starts by a [2+2] cycloaddition of $^{1}O_2$ to the thiazole ring (Scheme 5). Cleavage of the C—C bond of the thiazole, followed by attack of the secondary amine attack to the carbonyl group affords a pyrrolidinic structure, which is solvolyzed to furnish carboxylic acids and methyl esters depending on the reaction medium [217].

A photolytic degradation product of telmisartan and degradation products of irbesartan were detected and characterized using LC–MS/TOF, LC–MS^n, LC–NMR and on-line H/D exchange mass studies [218,219].

The structure of the major degradation product of ezetimibe was initially proposed by NMR spectroscopy (^{1}H and ^{13}C) and MS studies [220]. Shortly after, and without providing a structure, it was pointed out that the previously proposed structure was inconsistent with the reported spectroscopic data [221]. Finally, a third group of scientists [222] effected detailed analysis of ^{1}H, ^{13}C, COSY,



Scheme 5. Proposed photo-degradation mechanism of pramipexole.

Table 3
Degradation and process impurities detected in some active pharmaceutical ingredients.

Drug	Field (MHz)	NMR Experiments	Remarks	Ref.
Asperosaponin VI	500	^{1}H, ^{13}C, HSQC, HMBC, COSY, ROESY, TOCSY	Imp-1, Imp-2, Imp-5 and Imp-6: Natural impurities. Imp-4: Resulting from acid hydrolysis. Formed by loss of a unit of arabinose. Imp-7: Resulting from acid and basic hydrolysis. Formed by loss of two units of glucose. Imp-3: Mixture of two epimers that are isomers of Asperosaponin VI.	[172]
Bicalutamide	200	^{1}H	Imp-1 (I) and Imp-3 (III): Formed by basic degradation. Imp-2 (II) and Imp-4 (IV): Side products. Imp-5 (V): Starting material. Imp-6 (VI) and Imp-7 (VIII): Process intermediates.	[226]
Carisbamate	300	^{1}H, ^{13}C, COSY, HSQC	Imp-1 – Imp-4: Process intermediates. Imp-5 and Imp-6: Degradation products.	[202]
Doxofylline	300	^{1}H, ^{13}C	Imp-1: Starting material. Imp-2–6: Process intermediates. Imp-7 (DP-I): Resulting from acid degradation. Imp-8 (DP-II): Produced by basic and acid degradation.	[227]
Lopinavir	300	^{1}H	Imp-1 (RS1): Process intermediate. Imp-2 (RS2): Starting material. Imp-3 (RS3): Resulting from acid hydrolysis. Imp-4 (RS4) and Imp-5 (RS5): Side products and thermal degradation products, formed on extended storage at higher temperature. Imp-6 (RS6) and Imp-8(RS8): Impurities derived from the starting material. Imp-9 (RS9) and Imp-10 (RS10): Side products.	[100]

HSQC, HMBC and NOESY experiments, proposing a structure which agreed with a previously reported compound; ^{19}F NMR was also employed in this research [223,224].

The thermal and alkaline degradation products of meropenem were identified by NMR and MS analysis. The in vitro cytotoxicity assay against mononuclear cells demonstrated their cytotoxicity, rising safety concerns and suggesting that special care must be taken to avoid exposure of the drug to the degradation conditions during the handling and storage of the pharmaceutical preparation [225].

Table 3 summarizes the degradation and process impurities found in some active pharmaceutical ingredients, while Table 4 contains a brief list of degradation products found by submission of different pharmaceutical ingredients to stress or forcing degradation conditions.

2.2.2. *Impurities resulting from degradation of natural product APIs*

Artesunate is the hemisuccinic ester of dihydroartemisinin, a product resulting from the reduction of artemisinin. When held under ICH zone IV storage conditions during 6 months (40 °C and 75% RH), artesunate is known to yield several products above the threshold of identification [228].

Artesunate-amodiaquine bilayer tablets were subjected to heat treatment (70 °C, 14–30 days, 75% RH), furnishing a new and degradation product (Impurity B). This was purified by flash chromatography followed by semipreparative HPLC and unequivocally identified by 500 MHz NMR experiments (especially COSY, TOCSY and HMBC) as the tetrahydrofuranyl acetate-rearranged derivative of anhydrodihydroartemisinin (Fig. 3); MS analysis completed the identification.

Further, an authentic standard of the impurity was synthesized. An analogous rearrangement has been described to occur for artemisinin congeners during a biotransformation process [234]. The product has been shown to be a surrogate secondary marker for the Fe(II)-catalyzed radical reaction to rearranged tetrahydrofuranyl acetate products [235,236].

An isomeric 2-deoxy-4α-hydroxy-anhydrodihydroartemisinin derivative was also isolated. This product, a result of intrinsic thermal unstability of artesunate itself, is produced by the homolytic radical opening of the endoperoxide bond, a transformation already recorded for other artemisinin derivatives [228].

Tacrolimus (FK506) is an immunosuppressive drug used to avoid organ rejection in patients that have undergone organ transplantation. This is a potent drug which has a narrow therapeutic index. Following clinical reports suggesting that use of generic brands resulted in a significant reduction in the tacrolimus concentration/dose ratio in the plasma of liver and kidney recipients, a group of analytical methods were employed to assess the level of impurities and potency of products found in the US marketplace [237]. Ascomycin has immunosuppressant properties similar to tacrolimus, which fundaments its determination. In addition, it has been reported that the drug tautomerizes, yielding related rearrangement products [238,239], and the analysis can be further complicated by the presence of mixtures of interconverting rotamers [240].

After preparing standards of tacrolimus, ascomycin and the excipients commonly found in the pharmaceutical formulations and performing the ^{1}H NMR spectral comparisons, no ascomycin was detected above the LOD (0.7 w/w%) nor other known impurities were observed in any of the products. This clearly indicated that the clinically observed difference does not originate from quality (purity and potency) differences between the generic and innovator tacrolimus.

Even established APIs still hide secrets that await to be uncovered. The structure of a labile estradiol-related degradant, isolated from a pharmaceutical formulation containing estradiol and norethisterone acetate was elucidated by off-line and on-line NMR [241] as well as MS techniques. The product, which proved to be unstable, contains a 9,10-epoxy function. Its secondary and tertiary decomposition products were also identified, and a new oxidative degradation mechanism was proposed, which provides an improved explanation for the formation of some oxidative secosteroid degradation products [231]. The study also cleared some literature ambiguities.

Erythromycin is a complex macrolide antibiotic mainly composed of erythromycin A and also of erythromycins B-F. Several related substances may be formed by fermentation along with the aforementioned compounds. Unknown compounds found in an erythromycin formulation were investigated by LC coupled to MS and NMR. The degradation products were structurally elucidated, especially employing an array of off-line NMR techniques (1D ^{1}H and ^{13}C, and 2D TOCSY, NOESY, COSY, HSQC and HMBC); two of the degradation products were new [242].

Table 4
Degradation products observed after exposing APIs or their products to stress conditions.

Drug	Field (MHz)	Experiments	Remarks	Ref.
Anastrozole	300	^{1}H, ^{13}C	Imp-1: Formed by basic hydrolysis of both nitrile groups of the API (diacid). Imp-2: Formed by basic hydrolysis of one nitrile group of the API (monoacid).	[213]
Artesunate-Amodiaquine	500	^{1}H, ^{13}C, COSY, HSQC, HMBC, TOCSY, NOESY	Imp-1: Rearranged derivative of anhydro dihydroartemisinin.	[229]
Biapenem	500	^{1}H, ^{13}C, COSY, HSQC, HMBC	Imp-1 – Imp-3: Products of hydrolysis. Imp-2: Dimer A Imp-3: Dimer B	[230]
Chloroquine	500	^{1}H, ^{13}C, HSQC	Imp-1 (Chloroquine *N*-oxide): Resulting from oxidative degradation.	[205]
Clopidogrel	400	^{1}H, ^{13}C, DQF-COSY, HSQC	Imp-1: Oxidation impurity. Imp-2: Clopidogrel related compound A. Imp-3 (Related compound B1) and Imp-4 (Related compound B2): Positional stereoisomers of the API. Imp-5 (Related compound C): Optical isomer.	[203]
Dipyridamole	400	^{1}H, gDQCOSY, gHSQC, gHMBC	Imp-1 (DPI): Formed by hydrolytic loss of a piperidine moiety resulting under acidic and oxidative (peroxide) conditions. Imp-2 (DPII): Interaction product with tartaric acid formed during stress treatment under acidic, oxidative (peroxide) and thermal degradation conditions.	[208]
Eprinomectin (EPM)	400	^{1}H, ^{13}C, NOESY, COSY	Imp-1 (Unknown 1): Alkaline degradation product. Identified as the 2-epimer of EPM. Imp-2 (Unknown 2): Alkaline degradation product of Imp-2. Identified as $\Delta^{20,03}$-EPM.	[202]
Estradiol	500	^{1}H, ^{13}C	Imp-1: Oxidative product of estradiol.	[231]
Ezetimibe	400, 500	^{1}H, ^{13}C, gCOSY, gHSQC, gHMBC, 1D NOESY	The impurity (2R,3R,6S)-N,6-bis(4-fluorophe nyl)-2-(4-hydroxy phenyl)-3,4,5,6-tetrahydro-2H-pyran-3-carboxamide was mis-identified in previous publications.	[222]
Irbesartan	500	LC–NMR was used. ^{1}H, COSY (with solvent suppression)	Imp-1 (DP-I): Produced by acid degradation. Imp-2 (DP-II): Basic degradation product. Imp-3 (DP-III): Resulting from photo-acid degradation.	[219]
Meropenem	500	^{1}H, ^{13}C, COSY, HSQC	Imp-1 (DP1): Thermal degradation product. Imp-2 (DP2): Alkaline degradation product.	[227]
Olmesartan medoxomil	600	^{1}H, ^{13}C, using a ^{1}H–^{13}C/^{15}N triple resonance inverse probe in stopped-flow LC–NMR.	Imp-1 (DP-1): Esterified dimer of Olmesartan.	[232]
Pramipexole hydrochloride hydrate	400	^{1}H, ^{13}C, 2D (unspecified experiments)	Imp-1: Photo-degradation product.	[217]
Pridinol mesylate	300	^{1}H, ^{13}C	Imp-1 (ELI): Dehydration product formed under acidic conditions. Imp-2 (NOX): Formed under oxidative degradation conditions.	[204]
Thiocolchicoside	300	^{1}H, ^{13}C	Imp-1 (D1SO) and Imp-2 (D1SO2): Formed by oxidative degradation with m-CPBA. Imp-3 – Imp-5 (D2, D3, D4): Acid degradation products.	[233]

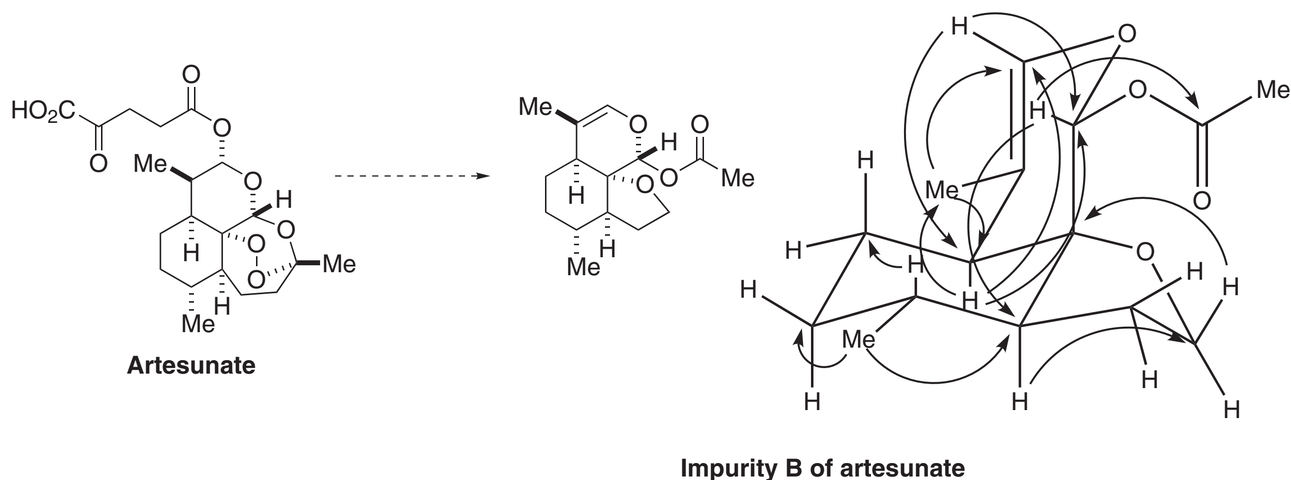


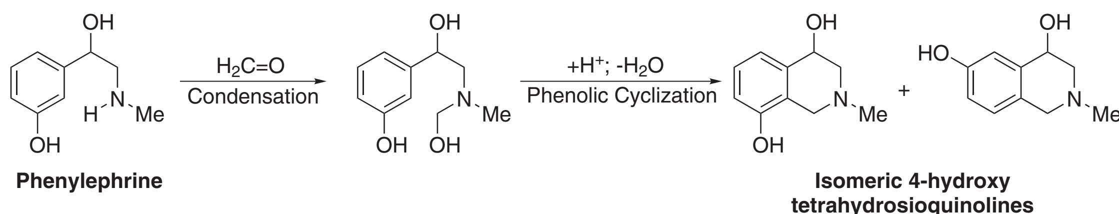
Fig. 3. Degradation of artesunate and three-bond H → C correlations observed in the HMBC spectrum of Impurity B.

2.3. Miscellaneous impurities

2.3.1. Impurities and degradation products related to excipients

Examples are scarce, and include degradation products of the excipients themselves and a case where an excipient promoted degradation of an API. Interaction products involving excipients are detailed separately. An unknown degradation product found in non-MS compatible HPLC analysis of a drug formulation was studied using a multidisciplinary approach. NMR analysis revealed it to be 5-hydroxymethyl furfural, resulting from degradation of lactose [243].

In addition, ^{1}H NMR approaches to the determination of free fatty acids [244,245] and peroxidation products [246] in pharmaceutical lipids were recently reported. The first method was



Scheme 6. Formation of interaction products between phenylephrine and sugar-derived formaldehyde.

demonstrated in different fatty oils, fat samples, waxes and oleyloleat. The lipids are a group of widely used pharmaceutical excipients, many of which also have therapeutic properties [247,248].

A constituent of the artificial flavoring of a losartan potassium extemporaneous suspension formulation was held responsible for the photosensitized degradation of the API in the drug product [249]. The structures of the degradation products were determined using a combination of preparative HPLC, LC–MS and NMR (^{1}H, ^{13}C and 2D) experiments.

2.3.2. Leaching impurities

Low levels of extractables have been observed in vials of interferon-α2b finished product, following periods of filling line down time. Their structures were elucidated as chlorinated biphenyl derivatives by MS and NMR spectroscopic methods and their origin was found in the Rehau RAU-SIK silicone tubing used on the Bosch filling line [250]. The structures of these impurities revealed that they are formed during the polymerization process used to make the raw material for the tubes.

2.3.3. Interaction products

During a stability study of an enema formulation containing 5-aminosalycylic acid (5-ASA) and citric acid, the formation of three new peaks was observed by HPLC–MS and further investigated by HPLC–SPE–NMR. These proved to be citric acid amides of 5-ASA; therefore, a recommendation was issued for avoiding the use of citric acid in liquid 5-ASA formulations [251]. Impurities in 5-ASA have also been elucidated by LC–NMR [252].

Analogously, one of the impurities found after submission of a dipyridamole drug product preparation to stress conditions was a tartrate ester, resulting from esterification with tartaric acid, employed as an excipient [208].

Different pharmaceutical preparations against the common cold containing phenylephrine (PHE) and saccharose were studied and new tetrahydroisoquinoline-type impurities were discovered, arising from a phenolic cyclization between the API with sugar-derived formaldehyde (Scheme 6). These were characterized by LC–MS and NMR techniques [253].

An amide interaction product between phenylephrine and maleic acid in a combined cold formulation containing phenylephrine hydrochloride and chlorpheniramine maleate, has been reported NMR analysis confirmed the structure of the correct isomer formed, the α-hydroxyamide [254].

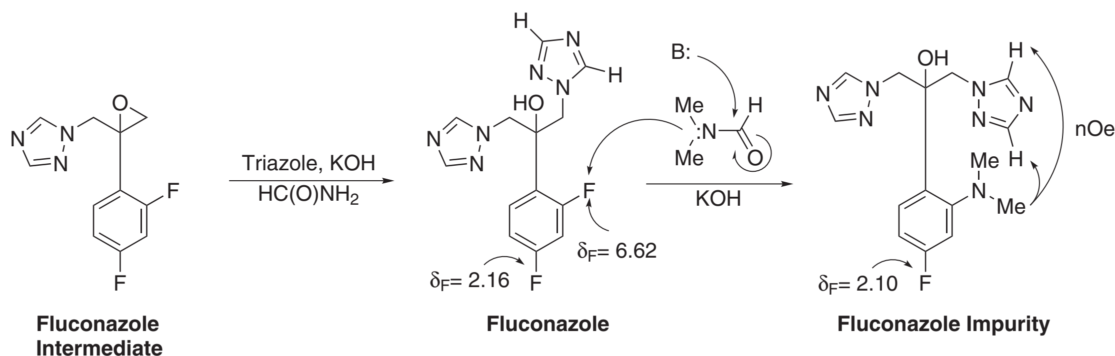
In addition, a hydroxylamine-type degradation compound of carvedilol drug product was characterized by NMR; its generation was attributed to an interaction of the drug with polyvinyl pyrrolidone (PVP) in the presence of moisture [255].

3. Other NMR active nuclei

Fluorine (^{19}F) NMR spectra are invaluable in the analysis of fluorine containing compounds [256].When the impurity profile of 16 commercial formulations of ciprofloxacin was studied, and characterized using ^{19}F and ^{1}H NMR spectroscopy, it was found that four to twelve fluorinated impurities, among them fluoride ion and two already known compounds, were detected and quantified in the formulations analyzed by ^{19}F NMR [257].

The ^{19}F NMR analysis of five pharmaceutical preparations of fluoxetine and fluvoxamine revealed that one of the samples contained an unidentified fluorinated impurity [258]. NMR spectroscopy (^{1}H, ^{13}C, ^{19}F, ^{1}H–^{1}H, ^{1}H–^{13}C, HMBC) and especially a nOe experiment helped to elucidate the place where substitution of a fluorine by a dimethylamino group took place in fluconazole (Scheme 7), resulting in a new, monofluorinated impurity of the latter [259]. No changes were observed by NMR when the drug was subjected to ionizing radiation (20–200 kGy) [260]. The degradation product of a fluorinated thiazole-containing compound was identified by extensive application of NMR spectroscopy, including ^{19}F and ^{15}N techniques [261].

^{19}F Solid state NMR spectroscopy was associated with chemometrics in order to study the solid phases of atorvastatin. The proposed method allows recognizing the mixtures of the prepared forms of the API with a LOD of the crystalline phase impurities in the amorphous phase < 1% [262]. In addition, the distinction between monomeric and dimeric fluorinated impurities was carried out by ^{19}F NMR spectroscopy [263]. The complete assignment of a mixture of isomers of 1,3-perfluorodimethylcyclohexane as well as



Scheme 7. Last step of the synthesis of fluconazole and generation of the monofluorinated impurity.

some impurities were identified using a combination of ^{19}F-COSY, ^{19}F-TOCSY and ^{19}F-NOESY experiments [264].

^{15}N NMR spectroscopy has very low sensitivity; however, it has been employed for confirming the identity of impurities and drug degradation products [260]. A cationic impurity was identified in preladenant employing a series of 1D and 2D NMR experiments, including the gHMBCAD experiment for accessing ^{1}H–^{15}N long-range correlation information [265] and the structure of an impurity in valsartan was confirmed with the aid of ^{15}N NMR experiments [266].

4. Conclusions and perspectives

The presence of minor amounts of unwanted chemicals might influence the efficacy and safety of the pharmaceutical products. Therefore, their structures should be elucidated when present at level higher than 0.1%. These impurities are also synthesized based on the suggested structures, as standards for selective analytical methods for their quantitation in drug substance and products.

NMR spectroscopy is finding increasing application for the identification of unknown organic impurities embedded in pharmaceutical preparations. In addition, this is the most suitable technique for the identification and the qualitative analysis of mixtures, where some of the compounds are unknown, and almost unsubstitutable for structural analysis of epimers, rotamers and even some cases of enantiomers. NMR is highly informative and it allows to draw conclusions on the structure of the components in a mixture of related compounds because the NMR spectrum is sensitive to even small modifications in their molecular geometry or bonding.

NMR spectroscopy is usually employed to obtain structural information. In some cases, it is used to confirm structural proposals made as a result of MS experiments, since the combination of NMR and MS is one of the most powerful approaches to acquire complete information on the structure of a studied molecule or mixture of compounds.

The quantitative determination of the level of impurities is one of the major applications of qNMR in this area. This can be done along with acquisition of their structural information. The determination of related impurities in a mixture, as well as content of residual solvent, isomeric composition, molar ratio and even the course of a degradation can be also achieved.

It has been shown that LC–NMR ranks among the most powerful hyphenated techniques for the separation and structure elucidation of complex organic molecules, also providing quantitative information. Due to the success of LC–NMR hyphenation, it can be foreseen that this approach will be extended to other multidimensional separation schemes in the near future. Despite the number of years of their evolution, coupled NMR techniques are still in their infancy and will require much more time to become established. Particularly, GC–NMR could be highly useful for volatiles. It is also expected that instrumental improvements will make more feasible the coupling of fractionation with ^{13}C NMR detection.

The relatively low sensitivity, sometimes poor reproducibility and high instrumental costs are current substantial limitations of this spectroscopy. State-of-the-art NMR equipment, including higher magnetic fields and cryo-probes exhibit enhanced sensitivity. However, despite the latest advances, it should be stressed that current sensitivity still makes NMR unsuitable for quantitative analysis of most of the genotoxic impurities, even employing the most sensitive ^{1}H NMR techniques.

Conquering all these technological frontiers required for trace and impurity analysis will open a completely new area of exploration and research in pharmaceutical analysis.

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Appendix A. Acronyms, abbreviations and their definitions, regarding NMR experiments

Acronym[a]/abbreviation	Meaning
1D, 1D-NMR	Mono-dimensional NMR experiment
^{1}H qNMR, qHNMR	Quantitative proton NMR experiment
2D, 2D-NMR	Two-dimensional NMR experiment
APT	Attached Proton Test
COSY	Homonuclear Correlation Spectroscopy
DBPPSTE DOSY	**DOSY** Bipolar Pulse Pair Stimulated Echo
DEPT	Distortionless Enhancement by Polarization Transfer
DOSY	Diffusion Ordered Spectroscopy
DQF-COSY	Double Quantum Filtered COSY
HETCOR	Heteronuclear Shift Correlation
HMBC	Heteronuclear Multiple-Bond Correlation
HMQC	Heteronuclear Multiple-Quantum Correlation
HSQC	Heteronuclear Single Quantum Correlation
NOE	Nuclear Overhauser Effect
NOESY	Nuclear Overhauser Effect Spectroscopy
qNMR	Quantitative NMR Spectroscopy
ROESY	Rotating-Frame NOE Spectroscopy
TOCSY	Total Correlation Spectroscopy
zTOCSY	z-Filtered Total Correlation Spectroscopy

[a] Prefix and suffix letters are added to more elaborate experiments. Letter 'g' is employed to denote the field gradient version of the experiment. The suffix 'AD' indicates the use of adiabatic pulses.

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