U. Holzgrabe, I. Wawer, B. Diehl

NMR Spectroscopy in Drug Development and Analysis

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Prof. Dr. Ulrike Holzgrabe
University of Würzburg
Department of Pharmaceutical
Chemistry
Pharmaceutical Institute
Am Hubland
D-97074 Würzburg
Germany

Prof. Dr. Iwona Wawer
Medical University of Warsaw
Department of Physical
Chemistry
Faculty of Pharmacy
Banacha 1
PL-02097 Warsaw
Poland

Dr. Bernd W. K. Diehl
Spectral Service
Vogelsanger Str. 250
D-50825 Köln
Germany

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Preface

Only few fields of scientific techniques had such an astonishing and wide spread development into different areas as nuclear magnetic resonance. Starting more than fifty years ago as a purely physical technique it soon entered the field of organic and inorganic chemistry, passed over to structural biology and is now probably the most important imaging technique in medicine, since it can create images from the inner of the human body without any invasive means.

The possibilities of NMR even in a more limited field such as in pharmaceutical and drug research are countless, as will be demonstrated in this volume which was collected and edited by U. Holzgrabe, I. Wawer and one of my first Ph.D. students B. K. Diehl, which made a great effort to present here a number of timely and well researched articles which demonstrate the state of the art of nuclear magnetic resonance applications in this area.

The volume starts with a report on the contributions offered by NMR in the field of drug admission and control (S. K. Branch) and describes how NMR has already invaded the international regulations as documented in the Pharmacopoeias (Diehl and Holzgrabe). We see a review on pH dependent NMR measurements (Haegele and Holzgrabe) which demonstrates the fundus of information which can be gained from NMR titration curves. Similar, articles how NMR is used to observe complexation and stereochemical information are provided (Diehl and Holzgrabe). The rapid development of on line coupling between chromatographic techniques and NMR as the possibly best chromatographic detector is covered by one of the major contributors in this field (Albert). It is this area where we may expect further significant technical breakthroughs in the next decade. The important results of these techniques are covered in a review of NMR of Body Fluids (Holzgrabe) which shows the exciting possibilities NMR can offer to medical research.

In every volume of collected reviews one finds one article which best meets ones own personal interest. In this volume for me it is the outstanding article by Gemmecker which outlines the current possibilities of SAR by NMR and the use of ^{15}N labelled proteins to demonstrate their interaction with drugs.

There can be no volume in modern NMR without considering the tremendous advantages obtained by the solid state methods and this is covered by Waver shedding light on galenic questions which can be solved by solid state NMR. Finally the volume ends with an review of the current state of imaging and localized spectroscopy with respect to pharmaceutical questions.

In summary, the collection of articles presented in this volume seems to be well chosen in topics covering the most pertinent questions of current pharmaceutical applications. It is a volume from which much can be learned and many stimulating ideas may emerge. It demonstrates the liveliness of NMR and its continuing importance in all fields of structural and analytical problems.

S. Berger, Leipzig

Contributors

Prof. Dr. Klaus Albert	Universität Tübingen, Institut für Organische Chemie, Auf der Morgenstelle 18, 72076 Tübingen, Germany
Prof. Dr. Gottfried Blaschke	University of Münster Institut für Pharmazeutische Chemie, Hittorfstr. 56, 48149 Münster, Germany
Dr. Sarah K. Branch	Medicines Control Agency Market Towers 1 Nine Elms Lane SW8 5NQ London, England
Prof. Dr. Bezhan Chankvetadze	Tbilisi State University, Department of Chemistry, Chavchavadze Ave 1, 380028 Tbilisi, Georgia
Dr. Bernd W.K. Diehl	Spectral Service, Vogelsanger Str. 250, 50825 Köln, Germany
PD Dr. Gerd Gemmecker	TU München, Insti. für Org. Chemie und Biochemie II, Lichtenbergstr. 4, 85747 Garching, Germany
Prof. Dr. Ulrike Holzgrabe	University of Würzburg, Lehrstuhl f. Pharmazeutische Chemie, Pharmazeutisches Institut, Am Hubland, 97074 Würzburg, Germany

Prof. Dr. Giorgio Pintore	University of Sassari Dipartimento Farmaco Chimico Tossicoligio Faculty of Pharmacy Sassari, Italy
Prof. Dr. J. K. Seydel	Forschungszentrum Borstel, Zentrum für Medizin und Biowissenschaften, Parkallee 1-40, 23845 Borstel, Germany
Prof. Dr. Iwona Wawer	Medical University of Warsaw, Department of Physical Chemistry, Faculty of Pharmacy, Banacha 1, 02097 Warsaw, Poland

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Chapter 1

B.W.K. Diehl

1 Introduction

Since the development of the high-resolution NMR spectrometer in the 1950s, NMR spectra have been a major tool for the study of both newly synthesized and natural products isolated from plants, bacteria etc. In the 1980s a second revolution occurred. The introduction of reliable superconducting magnets combined with newly developed, highly sophisticated pulse techniques and the associated Fourier transformation provided the chemist with a method suitable for determining the 3-dimensional structure of very large molecules, e.g. biomacromolecules such as oligopeptides, in solution. An interesting development of NMR spectroscopy is demonstrated in Figure 1-1. It shows a nearly linear increase of the used magnetic field strength of superconducting magnets with time, this relation seems to be valid at least until the end of this millennium.

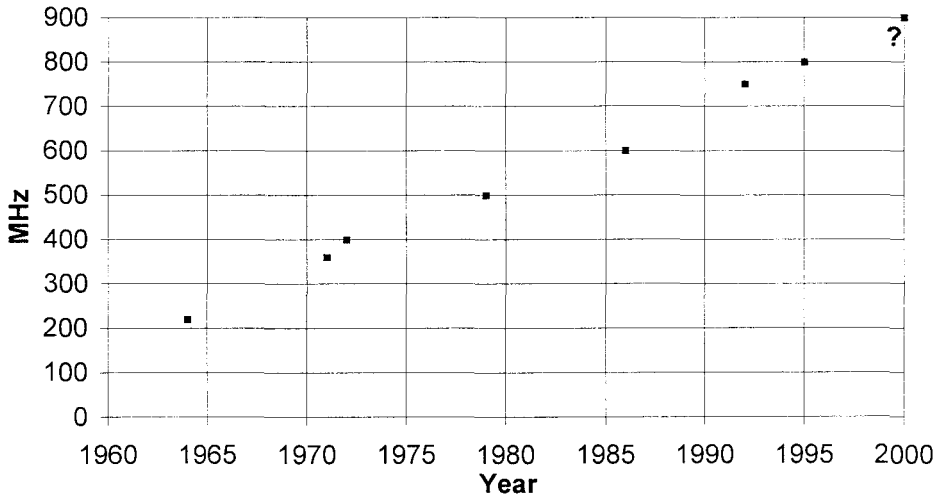

Figure 1-1: Evolution of the magnetic-field strength of superconducting magnets.

Since drugs in clinical use are mostly synthetic or natural products, NMR spectroscopy has been mainly used for the elucidation and confirmation of structures. For the last decade, NMR methods have been introduced to quantitative analysis in order to determine the impurity profile of a drug, to characterize the composition of drug products, and to investigate metabolites of drugs in body fluids. For pharmaceutical technologists, solid-state measurements can provide information about polymorphism of drug powders, conformation of drugs in tablets etc. Micro-imaging can be used to study the dissolution of tablets, and whole-body imaging is a powerful tool in clinical diagnostics. Taken together,

this review will cover applications of NMR spectroscopy in drug analysis, in particular methods of international pharmacopeias, pharmaceutics and pharmacokinetics. The authors have repeated many of the methods described here in their own laboratories.

1.1 The Instrument

Organic compounds are composed basically of the elements hydrogen, carbon, phosphorus, nitrogen and oxygen. Additionally, there are the halogens fluorine, chlorine, bromine and iodine and sometimes metal atoms. Each of these elements has an isotopic nucleus which can be detected by the NMR experiment. The low natural abundance of ^{15}N and ^{17}O in nature prevents NMR being routinely applied to these elements without the use of labeled substances, but ^{1}H, ^{13}C, ^{19}F and ^{31}P NMR spectroscopy are daily routine work. Many instruments are equipped with a so-called QNP (quattro nuclei probe) for sequential NMR analysis of ^{1}H, ^{13}C, ^{31}P and ^{19}F, without the hardware having to be changed. Modern NMR spectrometers are available up to field strengths of 18.8 Tesla or a proton resonance frequency of 800 MHz. Routine analysis is made at proton frequencies between 300 and 500 MHz.

Depending on the kind of experiments, the high-field instruments allow analysis of concentrations down to some µg/ml, but the "normal" case is a concentration of 1–100 mg/ml. The data are recorded using 32-bit ADCs (analog-digital converters). This results in a high spectral dynamic range.

1.2 Principles

The NMR experiment makes the direct observation of atoms possible. The integral of an NMR signal is strictly linearly proportional to the amount of atoms in the probe volume. The signals are a measure of molar ratios of molecules, independently of the molecular weight. There are no response factors such as those in UV-detection caused by varying extinctions dependent on molecular structures; non-linear calibration curves such as those found with light-scattering detectors are unknown to NMR spectroscopy.

1.2.1 Spectra

The frequency at which an NMR signal appears depends mainly on the magnetic field strength. For example, protons have a resonance frequency of 300 MHz at 7.05 Tesla. The chemical environment of an active nucleus leads to a small shift in the resonance frequency, the "chemical shift". Functional groups find their expression in the chemical shift. The result is an intensity/frequency diagram, the NMR spectrum. This collection of more or less separated NMR signals is analogous to intensity/time diagrams in chromatography, in which one component is represented by one signal. In ^{1}H NMR spectroscopy, each H atom leads to at least one signal. Since most molecules of analytical interest contain more than one H atom, spectra are more complex than chromatograms. What is crucial to the information taken from NMR spectra is the spectral dispersion, which is a linear function of the magnetic field strength. The homonuclear spin coupling of protons leads to a low dispersion of ^{1}H NMR spectroscopy. In ^{1}H NMR spectra of complex mixtures, it is often not possible to detect single components, but the sum of

functional groups in the mixture can be determined. In ^{13}C NMR spectra, the dispersion is much higher. The low natural abundance of the NMR-active ^{13}C isotope has a detrimental effect on the sensitivity, but signals are singlets after heteronuclear decoupling. The high spectral dispersion makes parts of the ^{13}C NMR spectra directly comparable to chromatograms. An example is the carbonyl region in a ^{13}C NMR spectrum of a lipid mixture, where each fatty acid is represented by a specific signal.
^{31}P NMR spectroscopy is the method of choice for phospholipids or any other phosphorus-containing compound. Most phospholipids contain only one phosphorus atom, so the ^{31}P NMR spectrum of lecithin reads like an HPLC chromatogram. There are some advantages in comparison with HPLC: specific detection of the phosphorus nucleus, high dispersion and high dynamics. The role of ^{31}P NMR spectroscopy will be discussed later in detail.

1.2.2 Response

The area of an NMR signal is directly proportional to the molar amount of the detected isotope. The ratio between two different signals of one molecule should be 1:1, the number of represented atoms being taken into account. In practice, there are differences caused by different relaxation times. This is the time an excited atom needs to fall to the ground state. In case of heteronuclear decoupling, the nuclear Overhauser effect can cause response factors as well. These response problems are influenced by the measuring parameters, they disappear or minimize with the correct (problem-oriented) choice. Within a family of atoms in similar chemical surroundings, e.g. all end-positioned methyl groups in the ^{13}C NMR spectra of fatty acid-containing material, these effects may be neglected. The same applies to corresponding carbonyl groups, but it is incorrect to compare areas of carbonyl and methyl signals without an appropriate experimental design or experimentally determined response factors. The response factors change from ±10% in ^{1}H NMR spectra up to ±50% in ^{13}C NMR spectra, and this is in fact advantageous in comparison to HPLC/UV detection.

1.2.3 Reproducibility

During an NMR experiment, there is no contamination of sample and probe head. The electronic stability of NMR spectrometers is very good. The spectra of a stable sample stored in a sealed tube are reproducible over many years with a variation of less than 1%. These facts allow minima expenditure on validation measurements when using NMR methods.

1.2.4 Calibration

Like all classical quantitative analysis methods, NMR spectroscopy needs calibration, calibration standards and a validation procedure. The standard techniques are used for calibration: external calibration, the standard addition method and the internal standard method. A fourth is a special NMR calibration method, the tube-in-tube technique. A small glass tube (capillary) containing a defined amount of standard is put into the normal, larger NMR tube filled with the sample for analysis. In most cases, there are slight differences in the chemical shift of corresponding signals of the same molecule in the inner

and outer tube. The spectrum shows two signals at different frequencies, and evaluation of the signal ratio allows quantification.

1.3 Experimental

Methods marked with "SSL" were developed by Spectral Service. To illustrate the published NMR methods, some spectra shown in the Figures were recorded by Spectral Service and replace those of the original studies. The 300-MHz NMR spectra were measured at Spectral Service GmbH, Cologne (Germany) on an NMR Spectrometer AC-P 300, at 7.05 Tesla (BRUKER, Karlsruhe, Germany) equipped with automated sample changer and QNP head for nuclei ^{1}H, ^{13}C, ^{19}F and ^{31}P. The data processing was performed using BRUKER WIN NMR 5.0 software under Microsoft Windows 95.

1.4 Acknowledgment

Thanks to Dr. Werner Ockels and the Spectral Service team, for their support during the preparation of this book.

Chapter 2

S. K. Branch

2 NMR Spectroscopy in the European Regulatory Dossier

The development of NMR spectroscopy since its inception in the 1950s has been remarkable in demonstrating its power and versatility in the fields of chemistry, biochemistry, biology and medicine, as exemplified in other chapters of this book. The pharmaceutical industry has employed various aspects of the technique during development of drug substances and new medicinal products and thus NMR spectroscopy has an established role in the regulation of medicinal products which is described in this chapter.

2.1 Directives, Rules and Guidelines

There are various routes by which the sale of a medicinal product can be authorised in the European Union (EU). Some applications are made to the European Medicines Evaluation Agency (EMEA) and are dealt with by the Committee for Proprietary Medicinal Products (CPMP) through the 'centralised procedure', whilst others are made directly to the national or 'competent' authorities of the EU member states. The original legal basis of applications for marketing authorisations in the EU was set out in Council Directive 65/65/EEC together with a brief description of the documents and particulars which should accompany such applications in order to establish the quality, safety and efficacy of the product. Subsequent directives have extended the legal system for authorising medicines, introducing new procedures and expanding on existing legislation. The basic requirements for the contents of the dossier of information accompanying the application are the same whatever the route and are laid out in detail in Directive 75/318/EEC and its subsequent amendments. Provisions are made in Directive 65/65/EEC for the omission of data in certain circumstances where information is available to the regulatory authorities from other sources, for example, in the case of applications for line extensions to existing products or for generic drugs. Applications which do not include a full dossier of information are referred to as 'abridged applications'. The legal basis for the different types of applications for product licences will not be considered further since this chapter will concentrate on the technical aspects of the dossier. Further information may be found in *The rules governing medicinal products in the European Union*, published by the European Commission [1] The relevant legislation is presented in Vol. 1 while guidance on submission of applications is given in Vol. 2, the *Notice to applicants for marketing authorisations for medicinal products for human use in the European Union.*

Directive 75/318/EEC indicates that the dossier should be presented in four parts, Part I taking the form of a summary of the information presented. Part II relates to the quality of the product and gives details of its chemical, pharmaceutical and biological testing. In cases where the active ingredient is made by a manufacturer other than the applicant or product manufacturer, some of the information required in Part II may be presented in a

separate file, a Drug Master File (DMF), to maintain the confidential nature of the synthetic process. Part III describes the toxicological and pharmacological tests conducted with the drug (pre-clinical tests) and the clinical documentation is presented in Part IV. Vol. 2B of the *Rules governing medicinal products* gives a detailed breakdown of the structure of a European regulatory dossier.

Vol. 3 of the *Rules* comprises *Guidelines on the Quality, Safety and Efficacy of Medicinal Products for Human Use* and is a compilation of the notes for guidance produced by the CPMP through its Working Parties or its membership of the International Conference on Harmonisation (ICH). The ICH is a tripartite body which is committed to harmonising the technical requirements for registration of pharmaceuticals in the EU, USA and Japan. The aim is to avoid unnecessary experimental duplication and to streamline the process of drug development world-wide. None of these guidelines are legally binding but are intended to be sufficiently flexible so as not to impede scientific progress in drug development. However, where an applicant chooses not to apply a guideline, the decision must be explained and justified in the dossier. The notes for guidance are continually being updated and added to, and applicants need to be aware of the current versions when preparing their dossiers. In the EU, the current guidelines and draft versions which have been released for consultation are available from EuroDirect [2] or the EMEA website [3].

The following discussion will focus on the information required in Part II to establish the quality of a medicinal product containing a chemical active substance. Additional regulations apply to radiochemical and biological medicinal products. Sections of the dossier other than Part II may also feature NMR spectroscopy, for example, reports of clinical studies using *in vivo* NMR imaging as a diagnostic tool or where the product is an NMR imaging agent itself. NMR spectroscopy is also used to identify drug metabolites isolated during animal and human pharmacokinetic studies, an area where hyphenated techniques are increasingly being used (see Chapter 8 on the NMR of biofluids).

2.2 Information Required to Establish Quality

Table 2-1 lists the headings under which information should be provided in Part II, according to the *Notice for Applicants*. NMR methods are most likely to appear in Parts IIC, IIE and IIF which describe respectively the control of starting materials, control of finished product and stability of active ingredient and finished product. As a general rule, any analytical methods should be described in sufficient detail to enable the procedures to be repeated if necessary, for example, by an official laboratory.

It should be noted that one of the ICH topics (M4) currently under discussion is a common technical document suitable for registration of medicinal products in the EC, USA and Japan. The draft requirements are at an early stage, and any harmonisation of requirements is likely to be an involved procedure given the currently differing regulatory practices in the three participating regions.
A selection of guidelines issued by the CPMP relevant to Part II of the dossier are listed in Table 2-2. The following sections will describe in more detail some of the information expected in Part II of the dossier, highlighting areas where NMR spectroscopy makes a

A selection of guidelines issued by the CPMP relevant to Part II of the dossier are listed in Table 2-2. The following sections will describe in more detail some of the information expected in Part II of the dossier, highlighting areas where NMR spectroscopy makes a contribution. The requirements of the different Directives and relevant guidelines will be drawn together under headings selected from those presented in the *Notice to Applicants.*

Table 2-1: Requirements for the product quality aspects of the European regulatory dossier

CHEMICAL, PHARMACEUTICAL AND BIOLOGICAL TESTING OF MEDICINAL PRODUCTS	
A.	Qualitative and quantitative particulars of the constituents
	1. Composition of the medicinal product
	2. Brief description of container
	3. Clinical trial formulae
	4. Development pharmaceutics
B.	Description of the method of preparation
	1. Manufacturing formula
	2. Manufacturing process
	3. Validation of the process
C.	Control of starting materials
	1. Active substance
	2. Excipients
	3. Immediate packaging material
D.	Control tests on intermediate products
E.	Control tests on the finished product
	1. Specifications and routine tests
	2. Scientific data
F.	Stability tests
	1. Stability tests on the active substance
	2. Stability tests on the finished product

2.3 Control of Starting Materials

2.3.1 Specifications and Routine Tests

Starting materials, whether the active ingredient itself or an excipient used in the manufacture of the finished product, are controlled by a specification comprising a list of tests and associated limits. The substance must comply with these limits before a batch can be deemed of suitable quality for use in the manufacture of the proposed medicinal product. The information required differs according to whether or not a substance appears in a pharmacopoeia.

2.3.1.1 Starting Materials Listed in a Pharmacopoeia

Compliance with monographs of the European Pharmacopoeia (Ph Eur) applies to all substances appearing in it and, for other substances, national pharmacopoeias may be enforced. Thus, where a pharmacopoeial monograph for an active substance or pharmaceutical excipient employs NMR spectroscopy in a test method, the substance must comply with this test. However, a test other than the pharmacopoeial test may be used if proof is supplied that the starting material meets the quality requirements of the relevant pharmacopoeia. If a pharmacopoeial monograph is applicable to a substance, then there is no need for the applicant to provide full details of the analytical tests or their validation, as reference to the pharmacopoeia in question is deemed sufficient.

However, where a starting material has been prepared by a method liable to leave impurities not controlled by the monograph, these impurities and their maximum tolerance limits must be declared and a suitable test procedure described. For example, changing a route of synthesis might lead to different solvent impurities, catalysts or related substances. NMR techniques are, of course, particularly useful for identifying new organic impurities if they can be isolated in sufficient quantities.

A competent authority is also at liberty to request more appropriate specifications if it considers that the monograph is insufficient to assure adequate quality of the substance. Further to the examples given above which result from differences in the synthesis route, additional tests may be required for particle size, polymorphic form, microbial contamination and sterility as necessary to ensure the correct performance of the starting material in the finished medicinal product. Limits which are tighter than the pharmacopoeial specification may be imposed if appropriate for the particular product in question. In addition, the competent authority will require stability data for active substances on which to base the storage conditions for the drug substance and its re-test period (the period of time for which it is expected to remain within specification and after which it must be re-tested for compliance and used immediately).

These requirements also apply to drug substances for which a Certificate of Suitability has been issued by the European Pharmacopoeia. This scheme was introduced in 1994 and certifies that the Ph Eur monograph for a substance is suitable for the control of that substance using *a particular method of manufacture*. Presentation of Certificates of Suitability are the preferred way for applicants to satisfy the guideline for such active substances (Table 2-2). A Certificate assures that the pharmacopoeial tests are adequate to

control the drug substance manufactured by a particular synthetic route, even though that route may be different to the one used when the monograph was originally devised.

Table 2-2: Some guidelines relevant to Part II of the regulatory dossier.

Title	Reference number	Relevant section
Development pharmaceutics	CPMP/QWP/155/96	Part IIA
Manufacture of the finished dosage form	CPMP/QWP/486/95	Part IIB
Chemistry of new active substances	CPMP/QWP/130/96*	Part IIC
Summary of requirements for active substances	CPMP/QWP/297/97	Part IIC
Impurities in new drug substances	CPMP/ICH/142/95	Part IIC
Investigation of chiral active substances	CPMP/III/3501/91	Part IIC and Part IIF (also Parts III and IV)
Validation of analytical procedures: Definition and terminology	CPMP/III/5626/93	Part II: all sections
Validation of analytical procedures: Methodology	CPMP/ICH/281/95	Part II: all sections
Excipients in the dossier for application for marketing authorisation of a medicinal product	CPMP/III/3196/91	Part IIC
Specifications and control tests on the finished product	CPMP/III/3324/89	Part IIE
Impurities in new medicinal products	CPMP/ICH/282/95	Part IIE
Stability testing of new active substances and medicinal products	CPMP/ICH/380/95	Part IIF
Stability testing of existing active substances and related finished products	CPMP/QWP/556/96	Part IIF

*Draft revised guideline

NMR is most frequently used as an identity test in pharmacopoeial monographs, the spectrum of the sample being compared to that of a reference standard. Examples where active ingredients are subject to such pharmacopoeial NMR tests are licensed products containing tobramycin or one of the low-molecular-weight heparins (classified as biological medicinal products). Monographs for tobramycin use ^{1}H NMR spectroscopy, while ^{13}C NMR spectroscopy is used for heparins. Poloxamer is an example of an excipient with a monograph containing an NMR test: in this case ^{1}H NMR spectroscopy is used to determine the percentage of oxyethylene in the starting material. Chapter 3 gives further details of these particular tests and other examples of pharmacopoeial NMR methods, including some under discussion for inclusion in future editions.

2.3.1.2 Starting Materials not Listed in a Pharmacopoeia

Constituents not listed in a pharmacopoeia should be described in the same form as a monograph. The note for guidance on *Chemistry of new active substances* (Table 2-2) provides current recommendations for developing drug substance specifications, but the draft ICH guideline on *Specifications: test procedures and acceptance criteria for new drug substances and new drug products: chemical substances* should also be noted [4]. The latter seeks to provide guidance on the setting and justification of acceptance criteria and the selection of test procedures for new drug substances of synthetic chemical origin with the intention of establishing a single set of global specifications.

The most frequent use of NMR spectroscopy in drug substance specifications is as an identity test: ^{1}H, ^{13}C or multinuclear spectroscopy may be used as appropriate. A possible advantage of multinuclear NMR is the increased specificity afforded by the wider spectral width and the ability to distinguish the active ingredient from compounds not containing the observed heteroatom. NMR may offer greater structural specificity than other spectroscopic techniques, and it has therefore been used in identity tests for more complex molecules such as peptides and proteins as well as heparins. Another use of NMR in specifications has been to confirm that the drug substance is present in the correct polymorphic form (see below).

2.3.2 Scientific Data

A variety of chemical data are required under this heading, including information on the nomenclature of the drug substance, its description, its manufacture and quality control during manufacture, development chemistry, details of impurities and results of batch analyses. Some of this information may also be necessary for excipients which have not been previously used in a medicinal product.

Quality control of the drug substance manufacturing process requires the application of specifications to starting materials, intermediates, solvents and reagents. Any of these may include NMR tests, usually to provide identification of the substance in question. NMR spectroscopy comes into its own in the section on development chemistry, which should include evidence of chemical structure, discussion of potential isomerism, physico-

chemical characterisation, characterisation of reference materials and analytical validation data.

2.3.2.1 Evidence of Structure

Evidence of structure provided for new active substances should be related to the actual material to be used in the marketed product, particularly when complex molecules are involved. Where the data provided in this section relates to substance produced by a different route of synthesis, the structural identity of the different materials in question should be confirmed, and this principle should also be applied to existing active ingredients. It is expected that NMR (^{1}H and ^{13}C), together with other spectroscopic techniques and elemental analysis, should be used as a matter of routine to confirm the structure of the drug substance, where applicable. Care should be taken to ensure that reproductions of spectra are completely legible (a common problem in dossiers), and full assignments should be given where possible. The route of synthesis can also be used as supporting evidence for proof of structure, and NMR can help confirm that the reactions involved have led to the expected structures by establishing the identity of intermediate products.

A wide range of proton and carbon NMR techniques, almost exclusively using Fourier Transform (FT) spectroscopy, are presented in applications to support proposed chemical structures, with multinuclear NMR being used where appropriate. Polarisation transfer experiments (e.g. DEPT) are used to indicate carbon multiplicity and the use of two-dimensional techniques, which facilitate signal assignment, is seen more frequently in applications. Typical techniques include ^{1}H-^{1}H correlation (COSY and related phase-sensitive experiments) ^{1}H-^{13}C proton-carbon heteronuclear correlation (including inverse spectroscopy) to identify short- and long-range couplings, and experiments to identify intramolecular Nuclear Overhauser Enhancement (NOESY) and its rotating frame equivalent (ROESY).

2.3.2.2 Potential Isomerism

The potential isomerism of the active ingredient should be discussed by the applicant, and NMR spectroscopy is of value in establishing the stereochemistry of the molecule. An example here is the use of difference NOE spectroscopy to identify *cis-trans* isomerism. The absolute configuration of molecules containing chiral centres is best achieved by single-crystal X-ray diffraction studies, but NMR methods such as those employing chiral shift reagents may also be of value in establishing the presence or absence of optical isomers (see Chapter 6 for examples).

Conformational data may be required for macromolecules, e.g. proteins, particularly where the correct conformation is essential for activity. As reported in the literature, NMR has been of substantial value in this area through the analysis of coupling constants, use of NOE and relaxation measurements amongst other techniques, though this type of data has not yet been seen in regulatory dossiers (in any case, demonstration of appropriate biological activity would be necessary).

2.3.2.3 Physico-chemical Characterization

Physico-chemical data are needed (whether or not the active ingredient is listed in a pharmacopoeia) if the bio-availability of the product depends on them. The information to be presented includes the crystalline form and solubility of the drug substance, its particle size (after pulverisation if necessary), state of solvation, partition coefficient, pH and pK_a, even if these tests are not included in the final specification. The existence of different polymorphic forms of a drug substance can be established by examination of the spectroscopic and thermal characteristics of material re-crystallised in a variety of solvents and conditions. Control of polymorphs is particularly important for those active ingredients of low solubility where dissolution of the drug may have a significant effect on its bio-availability from the dosage form. Solid-state NMR, particularly the ^{13}C-MAS technique, is being used more frequently to characterise the polymorphic forms of drug substances, and in some cases has been the routine test method chosen for the specification where its specificity and sensitivity have proved superior to alternative methods such as IR spectroscopy or X-ray powder diffraction (see Chapter 12 for further discussion of solid state NMR in drug analysis).

2.3.2.4 Analytical Validation

Where an NMR method is used in the specification for a drug substance, it should be validated according to the ICH guidelines on analytical validation (Table 2-2), in the same way as other analytical methods. Revalidation may be necessary following changes in the synthesis of the drug substance or in the analytical method. The specificity of a method used for an identity test needs to be established to ensure lack of interference from related substances or other impurities. The power of NMR in distinguishing between even closely related structures is well recognised, and thus specificity is less likely to be a problem in an NMR test compared to other spectroscopic or chromatographic techniques. Indeed, the specificity of NMR has been used to advantage in the validation of test methods for other techniques, for example to ensure peak purity in chromatographic methods. Hyphenated methods such as HPLC-NMR (discussed in Chapters 7 and 8) may be used for this purpose.

With NMR methods, it is particularly important to ensure that the magnetic field is reproducible or that any fluctuations are compensated for by the use of appropriate standards. Full qualitative and, where necessary, quantitative characterisation of any reference standard is required. NMR spectroscopy has traditionally been limited in quantitative application by its relative lack of sensitivity compared to other methods, however, advances in technology, such as the introduction of high-field super-conducting magnets and FT spectroscopy, have overcome this problem to some extent. NMR has thus become a feasible option for quantitative methods in some cases (see Chapter 3), although the cost and availability of the instrumentation may limit its wider application to testing drug substances. The advantage of using NMR for determining related substances is the opportunity for simultaneous measurement and identification of the compounds present in the sample.

2.3.2.5 Impurities

The guideline on *Chemistry of new active substances* (Table 2-2) requires the applicant to discuss potential impurities and give details of any which have been synthesised and analytical methods used to detect them. Impurities may arise from the raw materials and solvents or reagents used in the manufacture of the active ingredient, from intermediates or by-products of the synthesis, and from degradation of the substance. According to the note for guidance on *Impurities in new drug substances* (Table 2-2), structural characterization is required for organic impurities at or above an apparent level of 0.1% (assuming the same analytical response factor as the drug substance), or lower if they are expected to be particularly toxic. Identification of related substances in these circumstances normally includes NMR methods in conjunction with other spectroscopic techniques and confirmation by independent synthesis.

The setting of impurity limits in the specification should take into account both the *quality* of the drug substance, i.e. the actual levels of related substances found in batch analysis, and also the *safety* of an individual impurity or given impurity profile at the specified levels. The acquisition and evaluation of data which establishes this safety is referred to as *qualification.* An impurity or impurity profile is considered qualified if it has been adequately tested in pre-clinical or clinical studies and such studies are needed for impurities present above the dose-dependent qualification thresholds prescribed in the note for guidance.

2.3.2.6 Batch Analysis

The information provided in this section should illustrate the actual results, including those from any NMR tests, which have been obtained from routine quality control of the active ingredient to establish compliance with the proposed specification. Explanations should be included in circumstances where earlier batches were tested against slightly different specifications, e.g. with wider assay or higher impurity limits. Data for batches used in toxicity tests and clinical trials should be reported, including the actual levels of impurities found, to facilitate assessment of the qualification process.

2.4 Control of the Finished Product

The dosage form administered to a patient is controlled by a finished product specification with which a batch of product must comply before it can be released for marketing. There may be justification for some tests not being applied on a routine basis, but the frequency of such skip or periodic testing must be stated. The general monographs for pharmaceutical forms (tablets, capsules, creams etc.) of the European or national pharmacopoeias are applicable to the finished product. In some member states, for example the UK and Germany, the national pharmacopoeias have monographs for specific preparations. Products are required to comply with these monographs, where applicable. Tests other than those in the pharmacopoeia may be used, but it must be shown that the product would comply with the monograph if tested. It should be noted that pharmacopoeial monographs apply to the preparation throughout its shelf-life, but competent authorities require release specifications to be stated as well, and these may be

tighter than the compendial limits. As with the drug substance, the national authority may prescribe additional tests to those in the pharmacopoeia if they are considered necessary to assure the quality of the product. Full details of analytical tests are required if a pharmacopoeial method is not available or applicable.

In addition to the pharmacopoeial monographs, the guideline on *Specifications and control of finished products* (Table 2-2) provides details of requirements. Reference should also be made to the ICH draft note for guidance on *Specifications* [4].The latter details the tests which might be expected for the control of different types of dosage forms.

Validation of analytical methods used in the finished product specification is required in the same way as for drug substance. Revalidation of these methods may also be necessary if the composition of the product has been changed during development, e.g. to reassess specificity.

As for drug substances, stressed stability studies (see below) are used to establish likely degradation products. NMR spectroscopy has been used to assist characterisation of any compounds which can be isolated. Decomposition may result from the usual mechanisms of chemical breakdown or may be due to specific interactions with excipients. The process may occur during manufacture of the formulation (e.g. induced by heating), thus requiring control in the release specification, or on storage of the product, in which case limits would be needed in the shelf-life specification. (Impurities which are limited in the drug substance specification and arise only from the synthetic process do not require further control in the finished product.) Degradation products should be identified and qualified as indicated in the guideline on *Impurities in new drug substances and new drug products* (Table 2-2), in the same way as discussed for the drug substance itself. The reporting, identification and qualification thresholds appropriate for different maximum daily doses of drug substances are presented in the note for guidance.

NMR, including multinuclear spectroscopy, has mainly been used as an identity test in the specifications for licensed products either for the active ingredient or an excipient. It has also been used to control the composition of polymeric excipients in the dosage form. Paramagnetic agents allow the use of NMR relaxation measurements in control of the quality of finished products.

2.5 Stability Studies

Stability studies fall into two categories. Firstly, 'stressed' studies referred to above are conducted in which the active substance or the finished product are subjected to extreme heat, light, acidic, basic and oxidative conditions with the intention of forcing degradation. Such experiments, when coupled with the isolation and identification of decomposition products (with the aid of NMR spectroscopy where appropriate) may allow the elucidation of degradation pathways in the drug substance and dosage form. In addition, these degraded samples may be used to test the specificity of the analytical methods applied to the drug substance and product.

The second type of stability studies are used to establish the re-test period for the active ingredient and the shelf-life of the finished product. The ICH guideline on stability testing (Table 2-2) stipulates standard conditions for storage (25°C/60%RH long-term and 40°C/75%RH accelerated) and gives recommendations on the number of batches, testing frequency and evaluation of results. The tests used should be the same as those in the drug substance and finished product specifications, including any based on NMR techniques, but additional stability-indicating methods may be included in the protocol. A relevant NMR example of the latter might be the inclusion of a solid-state method for identifying polymorphs in the stability trials for an active ingredient, even though the results eventually indicate it to be unnecessary in the final specification.

Full information on the batches tested and their packaging, the test methods (description and validation) and results and proposals for re-test period and shelf-life must be included in the dossier, together with details of any on-going studies.

2.6 Concluding Remarks

Regulatory dossiers, particularly for new active substances, tend to reflect state-of-the-art analytical techniques from a few years prior to the application because the drug development takes place over a relatively long time-scale. The unique power of NMR spectroscopy in structure elucidation is already well recognised, and its versatility is gradually becoming more widely utilised in other areas. Regulators can expect to see a wider range of techniques described in the future as advances are made in NMR technology and methodology. It is anticipated that the more sophisticated experiments for structure elucidation will be applied to a wider range of drug substances and will thus appear more frequently in descriptions of development chemistry. It is interesting to speculate on the use of imaging techniques, such as those described in Chapter 13, for examining tablets and excipients, and whether these techniques will be able to address problems encountered in pharmaceutical development. NMR techniques may become more common as routine analytical methods for control of drug substance or finished product as the sensitivity of the method increases and instruments become more accessible.

References

1. Eudralex: The rules governing medicinal products in the European Union, Vol.s 1-9, Office for Official Publications of the European Communities, Luxembourg, 1998.
2. Eurodirect Publication Service, Room 1207, Market Towers, 1 Nine Elms Lane, London, SW8 5NQ.
3. http://www.eudra.org/emea.html
4. ICH Topic Q6A *Specifications: test procedures and acceptance criteria for new drug substances and new drug products: chemical substances*, Step 3, draft, 16 July 1997 (CPMP/ICH/367/96).

Chapter 3

B. W. K. Diehl and U. Holzgrabe

3 Analysis of Drugs

3.1 NMR Spectroscopy in International Pharmacopoeias and Related Applications

Even though the reproducibility of NMR spectroscopic methods in terms of qualitative and quantitative analysis is proved to be very high, the European pharmacopoeias use NMR spectroscopy mostly for the identification of drugs and reagents, for instance, in the cases of tobramycin (Pharmacopoeia Europaea, Ph. Eur.) and hydrocortisone sodium phosphate (BP 93). Due to heavy signal overlapping, the spectra of these compounds are very complicated (see Figure 3-1 and Figure 3-2) and could be assigned only by means of 2D experiments. Thus, the ^{1}H NMR spectra are used in the same manner as IR spectra, which can be described as a sort of pattern recognition. In addition, an increasing number of reagents, e.g. adenine, adenosine, aesculin, butoxycaine, chamazulene, guaiazulene, nitrilotriacetic acid etc., are identified by ^{1}H and ^{13}C NMR spectra in European pharmacopoeias.

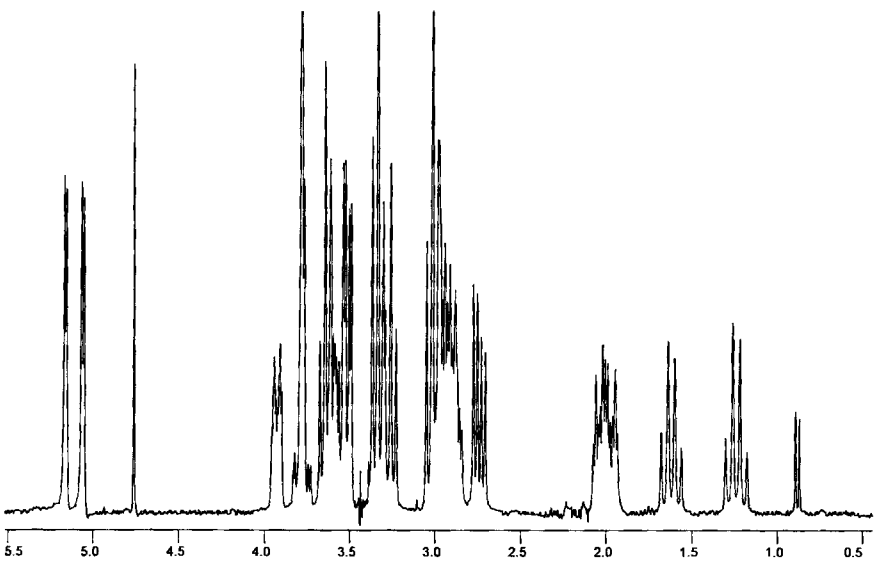

Figure 3-1: Expansion of the ^{1}H NMR spectrum of tobramycin, 300 MHz, solvent D_2O, "SSL".

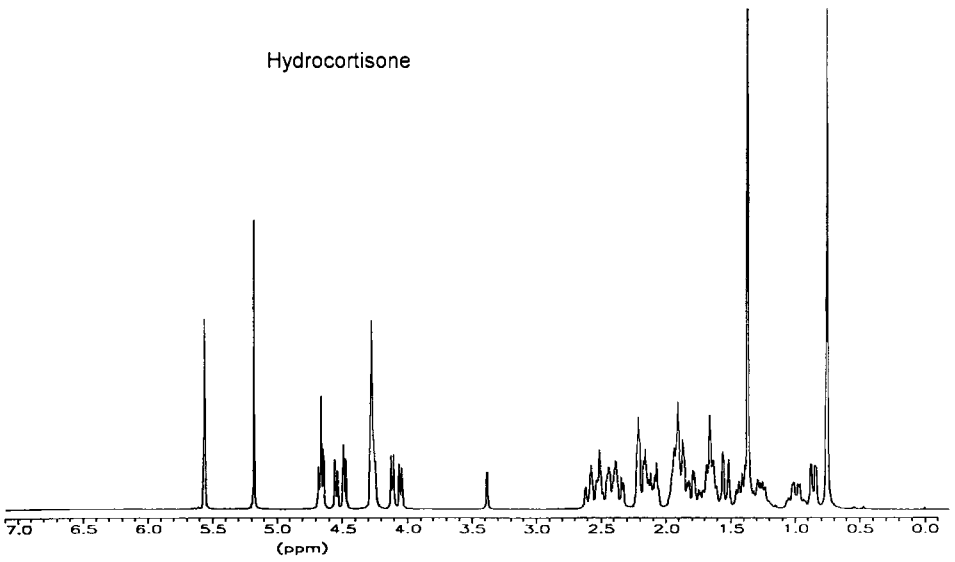

Figure 3-2: ^{1}H NMR spectrum of hydrocortisone sodium phosphate, 300 MHz, solvent DMSO-d_6, "SSL"

Whereas the European pharmacopoeias describe the method of NMR spectroscopy only in principle, the United States Pharmacopoeia 23 gives detailed information about the procedures of qualitative and quantitative applications. In the section on qualitative application, the correlation between chemical shifts and coupling constants on the one hand and the structure of a molecule on the other hand is stressed. For quantitative applications, an absolute method, utilizing an internal standard, and a relative method are given. Consequently, NMR spectroscopy is used in the USP for identification of drugs and their impurities (see test section) and for quantification (see assay section of a monograph).

For example, amyl nitrite, a mixture consisting chiefly of *iso*-amyl nitrite [$(CH_3)_2CH$–CH_2-CH_2–O–N=O] as well as other isomers, is identified by an ^{1}H NMR spectrum which is characterized among other peaks by a doublet centered at about 1 ppm and a multiplet centered at about 4.8 ppm representing the methyl hydrogens and methylene protons in α-position to the nitrite group. In the assay the substance is subjected to the absolute method, using benzyl benzoate as an internal standard. The quantity of amyl nitrite is calculated from the signal area of the α-methylene group of the drug (at 4.8 ppm) and the signal area of the methylene hydrogens of benzyl benzoate at 5.3 ppm.

The relative method of quantification is used in the orphenadrine citrate monograph in order to determine the content of *meta*- and *para*-methylphenyl isomer in the *ortho*-methylphenyl substituted drug (see Figure 3-3).