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Analytical Survey

NMR spectroscopy: analytical applications from chemistry to the clinic

J. C. LINDON

Department of Physical Chemistry, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, UK

Abstract: The impact of new technology in NMR instrumentation is described with reference to a range of problem areas in the pharmaceutical and biomedical fields. In particular, the contribution of very high field instruments based on superconducting magnets is considered, together with Fourier transform and related software developments. Specific application areas discussed include quantitative analysis, structure elucidation, NMR detection in high-performance liquid chromatography, analysis of body fluids, metabolic studies in single cells, analysis of intact tissue *in vitro* and *in vivo*, and NMR imaging.

Keywords: *Nuclear magnetic resonance spectroscopy; Fourier transfer NMR; biomedical applications; quantitation; NMR imaging.*

Introduction

The commercial introduction of Fourier transform (FT) nuclear magnetic resonance (NMR) spectrometers in about 1969 opened up the subject to whole new areas of application. Prior to this, NMR was used in both academic and industrial laboratories to study simple molecules mainly in response to a need to identify their functional groups; in general, large biologically interesting species were considered too complex for the technique. However, from this period two major technological developments have enhanced the capabilities of NMR many-fold. These are: the development of ever higher magnetic fields through the use of superconducting solenoid magnets; and computer-aided advances in digital electronics allowing very complex software packages to control experiments and to process acquired data. With the additional developments in two-dimensional NMR occurring since about 1975, NMR techniques are now widely used in chemistry, biochemistry, physiology and medicine. This article aims to highlight a few areas of application to biomedical and related analytical problems.

It is assumed that the reader is familiar with the basic details of NMR spectroscopy, in that the technique depends on the detection of radiowave emissions from certain atomic nuclei subjected to a strong static magnetic field and a pulsed radiofrequency field.

Almost all elements have an isotope which is, in principle, observable by NMR spectroscopy. The most frequently studied and most sensitive of the stable isotopes is the proton, ^{1}H, although ^{13}C, ^{31}P, ^{15}N, ^{17}O, ^{19}F, ^{29}Si, ^{27}Al and others are often used. The background to the NMR techniques and many of the experiments discussed herein are well described in the recent book by Harris [1]. Many practical and technical points are covered in the comprehensive volume by Martin *et al.* [2].

One of the most useful aspects of NMR spectroscopy for the analyst is the fact that, if the experimental conditions are properly chosen, the various peaks in the spectrum assignable to chemically different functionalities have areas that are quantitatively proportional to the amounts present. These areas or integrals form the basis for the use of NMR spectroscopy for quantitative analytical purposes, whilst the peak positions and their fine structure (chemical shifts and coupling constants respectively) are used for identification of the species.

Instrumental Developments

The sensitivity of the NMR experiment is generally regarded as being rather low relative to other techniques such as mass spectrometry. The main effort to increase sensitivity has resulted in the use of superconducting solenoid magnets, which have increased the available magnetic field from 2.1–2.3 Tesla (T) in about 1970, to commercially available 11.7 T magnets and with further increases on the horizon. This corresponds to a change in the original ^{1}H radiofrequency from 90–100 MHz to 500 MHz. This field effect combined with greatly improved probe detector design has meant an increase in ^{1}H detection sensitivity of about 50-fold.

Apart from simple 60 MHz instruments used for undergraduate teaching experiments and for routine fast analysis of reaction products, NMR spectroscopy has departed from the principle of using a swept magnetic field or radiofrequency (r.f.) signal in order to observe the signals, the so-called CW or continuous wave approach. All contemporary spectrometers exploit the application of pulsed radiofrequency power to excite the nuclei, followed by the observation of the emission of r.f. as the nuclei undergo relaxation processes. This decaying voltage can be signal-averaged to improve sensitivity even further and the usual NMR spectrum is obtained by Fourier transformation, using the spectrometer's own controlling computer system.

On the data analysis front, the computing hardware has advanced considerably in power; multi-tasking virtual memory processors coupled to array processors can perform Fourier transforms in a few milliseconds on 32K words of data. Because of the multi-tasking capabilities, acquisition of data can be performed concurrently with mathematical processing and/or plotting of other results; moreover, the coupling of the instrumental computers into laboratory networks is also possible. Software is now often written in a high-level language such as PASCAL, making modification of programs that much simpler than when written in assembler language; and many routines are usually included in manufacturers' packages to facilitate manipulation of the data to permit operations such as resolution enhancement and spectrum baseline correction [3].

The software on research machines allows the design and execution of complex sequences of r.f. pulses with various phases. These play spin-physics tricks on the nuclei and have the effect of perturbing or modulating the resultant NMR spectrum to give only the information required. Examples could include the editing of a ^{13}C NMR spectrum into four subspectra, each consisting of one type of carbon, i.e. quaternary, methine,

methylene or methyl carbons. Some of these methods imply that one can no longer consider a compound's NMR spectrum to be immutable, it being characteristic only of a particular NMR experiment; examples are given below.

One area where NMR promises to be fruitful in pharmaceutical analysis is through the use of a fully automated spectrometer operating unattended outside normal working hours. A number of commercial instrument manufacturers have collaborated with individual laboratories in developing technological solutions for this problem. Thus under computer control 50–60 samples can be presented consecutively to the spectrometer. For each sample data are then acquired and stored on computer disk before being processed and plotted automatically, ready for the analyst or chemist to examine the next day.

Essentially NMR spectroscopy was a technique for studying materials in solution until the development of methods for obtaining high resolution ^{13}C spectra of solids. These innovations include very high power transmitters for removing large ^{1}H–^{13}C interactions found only in solids, and high speed spinning (250,000 rpm) at the so-called "magic angle", i.e. 54.7° relative to the static magnetic field. More details can be found in a book published by the Royal Society following a discussion meeting there [4]. The method is useful for rare nuclei, e.g. ^{13}C (1.1% abundant) or ^{29}Si (4.7% abundant), in solids and has found much use in the analysis of synthetic polymers (^{13}C) and zeolites (^{29}Si and ^{27}Al). For some years the method has promised to be useful for distinguishing polymorphic forms of drugs, information which may be useful in bioavailability studies. However, this promise has yet to be fulfilled, bearing in mind the ease of infrared spectroscopy and thermal analysis of solids.

As the applications of NMR have extended into physiology and medicine, new techniques have been developed to allow the examination of tissues, and even whole animals and human beings, into specially adapted NMR magnets. This has paralleled the development of surface coils placed upon the limb or part of the body to be studied. In general, the high resolution spectrum obtained is that due to ^{31}P because of the problem of differentiating all the protons from the many molecules in any tissue. Again, some applications of these techniques are discussed below. A condensed but very readable summary of the history of NMR has recently been given by Derbyshire [5].

Specific Applications of NMR to Pharmaceutical Analysis

Quantitative analysis

NMR spectroscopy, usually of ^{1}H, has found wide application for the analysis both of pure drugs and formulated materials, although the aims and difficulties of the two applications are quite different. In the former, the detection and quantitation of very small amounts of impurities are necessary and here a suitable internal intensity standard may be the ^{13}C satellite peaks of one of the main component peaks, the intensities of each being 0.56% of the parent. This application has to take into account errors introduced by the dynamic range problem of detecting both large and small signals simultaneously [3]. In analysing formulated materials the proportions of interest are not usually so diverse, but cognisance must still be taken of other features in the spectrum such as spinning sidebands. In the book by Martin *et al.* [2] a whole chapter is devoted to the problems associated with obtaining accurate integrals both in CW and FT modes. These include a discussion on internal/external standards, suppression of Overhauser intensity distortions and the use of relaxation reagents. In addition, a review by Shoolery

[6] addresses the problem of quantitative ^{13}C NMR and shows applications to the analysis of mixtures of animal and vegetable oils. The problems are magnified even more for ^{15}N NMR spectroscopy, where nuclear Overhauser effects (nOe) are negative, causing a diminution in peak intensities; furthermore, relaxation times are often very long and the low natural abundance necessitates long acquisition periods. Levy *et al.* [7] have provided some methods of surmounting the problems using ^{1}H decoupled spectra with nOe suppression and the addition of paramagnetic relaxation agents. However, it is fair to say that in general NMR is not used in quantitative analysis unless cheaper methods are inadequate or inappropriate.

Structural elucidation

The elucidation of the chemical structures of small molecules of pharmaceutical interest and for biological macromolecules has advanced tremendously with the increased armoury of pulsed NMR experiments. Increased field strengths have led to higher sensitivity and greater chemical shift resolution, so that smaller amounts (e.g. drug metabolites) and larger molecules (e.g. proteins, polysaccharides) can both be studied. This field of NMR is too vast to consider in detail in this review, so a few highlights will be presented. NMR spectroscopy is making headway in the structural elucidation of microgram quantities of drug metabolites using ^{1}H NMR, thus nicely complementing the established mass spectrometric approaches. One innovation has been the development of tritium ^{3}H NMR spectroscopy, attractive because this nuclide has an even higher sensitivity than ^{1}H and resonates at the highest frequency of all nuclei. A team from the University of Surrey has pioneered this approach and shown that 10 μCi of ^{3}H per site would be sufficient to obtain a ^{3}H spectrum overnight on a spectrometer operating at 400 MHz for ^{1}H. Although ^{3}H is radioactive, only soft β-radiation is emitted, making it relatively safe to use given proper facilities. A recent review by Elvidge describes the operating requirements for ^{3}H NMR and demonstrates a wide range of applications including structural elucidation, stereochemistry of catalysis and biosynthesis [8].

One of the major advances in structural NMR studies has been the development of two-dimensional NMR experiments, largely by the groups of Ernst and Freeman. In normal FT NMR the emission decay which is a function of time is Fourier-transformed to present intensity as a function of frequency. In a two-dimensional experiment, two time intervals are built into a multiple pulse sequence, one being the usual acquisition time, the other being some incrementable delay. After the acquisition of data as a function of both time intervals, two Fourier transforms are performed orthogonally on the two-dimensional data matrix to provide intensity as a function of two frequency parameters. The theory and background to the whole range of experiments has been summarized by Bax [9].

The experiments fall into two classes, described as "resolved" or "correlated". "Resolved" experiments have the property of having one NMR parameter projected on to one axis, while a second parameter, which normally also appears in the spectrum, is rotated 90° into an orthogonal plane, thus avoiding any overlap. An example would be the ^{1}H spectrum of a fairly complex small molecule with all the coupling constant multiplets rotated through 90° allowing each to be viewed individually without overlap [10]. These experiments yield values for both sets of parameters. The alternative "correlated" experiments are different in that they provide connectivity information between various nuclei in a molecule. For example, given the assignments of all CH,

CH_2, CH_3 carbons in a ^{13}C NMR spectrum, it is possible to obtain all the respective ^1H chemical shifts by carrying out the appropriate ^{13}C–^1H correlation experiment [11]. For ease of visualization the data are normally presented in the form of a two-dimensional contour plot, but in the case quoted, although the correlation is achieved *via* the one-bond ^{13}C–^1H spin coupling constant, this parameter is not measurable in the experiment. Another much used example replaces the need for extensive spin-decoupling experiments in ^1H NMR by correlating all the ^1H nuclei which are spin-coupled to each other [12]; a similar experiment correlates nuclei which have an nOe between them [13]. The whole subject is too large to give further details here, but advances in the technique are so rapid that each issue of the *Journal of Magnetic Resonance* contains several articles devoted solely to technique development. The *Journal of the American Chemical Society*, for example, carries such articles in most issues, often giving structural analysis of natural products, hormones, metabolites, peptides and proteins. A non-exhaustive list would include glycosphingolipids [14], peptides [15], prostaglandins [16], proteins [17], steroids [18], nucleotides [19] and polysaccharides [20].

HPLC detection by NMR

One new area where NMR spectroscopy, mainly of the sensitive nuclei such as ^1H or ^{19}F, promises to be of much use in pharmaceutical analysis is the ability to couple the output of a liquid chromatograph to an NMR spectrometer. This was first demonstrated in 1978 using a stopped-flow technique and one year later using continuously flowing samples. A considerable number of papers have been published in subsequent years showing the viability of the technique [21]. The principal advantages of the method include the high information content available in the ^1H spectrum of the detected peak and the non-invasive nature of the detection procedure. The major disadvantages associated with HPLC–NMR are the high cost of the instrumentation, the limited choice and high cost of acceptable solvents, which are normally deuterated for NMR spectroscopy. However, with modern spectrometers and pulse-sequence suppression schemes this restriction is much alleviated and the relatively low sensitivity of the NMR detector, due again to technological advances, is becoming less of a hindrance. Dorn [21] has quoted a detection limit, using ^1H NMR on a 200 MHz (4.7 T) instrument, of 10–20 μg for compounds with molecular weights up to 300 Daltons using a chromatographic time window of 15–30 s. Other groups have used higher field strengths up to 9.4 T where the chromatographic column was placed inside the bore of the superconducting magnet to reduce dead volume [22]. Other NMR-sensitive nuclides can be used effectively in liquid chromatography, in which case the need for deuterated solvents no longer applies. A recent example is the separation and analysis of fluorinated steroids using ^{19}F HPLC–NMR [21].

Analysis of body fluids

Moving away from chemical to the more biomedical applications of NMR analysis, ^1H NMR has fairly recently been shown to be very useful for monitoring small molecule metabolites in tissue fluids such as blood, plasma or urine. Applications have included the measurement of natural endogeneous compounds, drugs and their metabolites. High-resolution ^1H NMR spectra of blood and other fluids are normally dominated by the H_2O resonance, but this can be suppressed by saturation techniques or pulse methods as noted above. The largest resonances remaining derive from the macromolecules, such as haemoglobin in blood or albumin in serum, but these broad

resonances can also be effectively suppressed using the spin-echo technique initiated by the group at Oxford [23] and developed by others [24, 25]. For urine the spin-echo technique is not really necessary because of the lack of protein; a simple water-saturation technique allows the facile monitoring and quantitation of numerous metabolites such as creatine, citrate, hippurate, glycine, lactate, etc. [26].

Abnormalities in human metabolism can easily be picked up by this technique, as for example in the case of diabetes, where the high concentration of glucose present in the urine is detected. In addition, non-endogenous materials such as ethanol or paracetamol have been detected together with their metabolites. A typical spectrum on a high field instrument (400 MHz for ^{1}H) takes about 30 min on 0.5 ml of a specimen untreated except for the addition of a few percent of D_2O for magnetic field stabilization. Alternatively, it is possible to lyophilize specimens and redissolve in D_2O at a 10-fold greater concentration to obtain results more rapidly and without the need for water resonance suppression techniques. Other nuclei can be used for detection, an example being the analysis of blood and plasma for sodium using ^{23}Na NMR. Separate analysis of intracellular and extracellular sodium in blood is thereby possible [27].

Analysis of metabolism in single cell organisms

Moving up to a more complex type of sample, i.e. single-cell organisms or tissue culture, many studies have been undertaken using NMR spectroscopy to try to elucidate the metabolic fluxes present. Quantitation and analysis usually relies on feeding the cells ^{13}C-labelled substrate, e.g. glucose or alanine; the ^{13}C label is then monitored during the biochemical cycles, either glycolysis or gluconeogenesis. Examples using ^{13}C NMR include gluconeogenesis from alanine in rat hepatocytes [28] and glucose metabolism in *Escherichia coli* [29]. Phosphorus NMR has also been well used in this area for monitoring the phosphate pools, such as nucleotide phosphates, as a function of glucose metabolism in bacteria [30, 31]. Also ion transport has been studied in yeast cells using ^{39}K, ^{23}Na and ^{31}P NMR [32]. A general article on the cellular applications of ^{13}C and ^{31}P NMR spectroscopy has appeared in *Science* [33].

Analysis of intact tissues including in vivo *applications*

This is a rapidly expanding field which is based upon the observation of various types of phosphate in solution inside living tissues held inside an NMR spectrometer magnet. Usually sugar phosphates, inorganic phosphate, creatine phosphate and ATP can be distinguished and quantified and the effect of various drugs or other physiological perturbations can be determined. The scientific advances in the subject have been paralleled by new technology, such as the availability of specially designed magnets with a horizontal bore of sufficient diameter to admit a whole adult human for observation. The ^{31}P NMR detector consists of a coil placed on a surface of the tissue and the particular organ is then brought into focus, using pulses which give different effects at different depths below the surface.

The problem is that r.f. does not penetrate well, and this means that in general only organs and tissues near the surface can be studied. The pioneering paper published in the early 1970s on excised muscle showed that soluble phosphate gave rise to relatively sharp ^{31}P NMR peaks, but that macromolecules and lipids, being immobile, gave only a broad background. From this the technique has developed and can be applied to perfused organs, such as heart and kidney, using oxygenated buffers to keep them viable; the method is also applied to the study of whole animals, first using conventional NMR

probes and then using surface detector coils placed over the organ of interest. A large number of reviews has appeared describing the techniques and applications in detail [34, 35] and a book by Gadian is also recommended [36].

A typical example includes the rapid analysis of high energy phosphate flux in live rats [37, 38]. With the ability to use very wide-bore magnets it became possible to insert human limbs into the NMR spectrometer in order to monitor phosphate levels in normal and diseased states. The abnormal profiles revealed have, in a number of cases, led to clinical diagnosis of human enzyme disorders [39, 40] and show promise for early diagnosis before pathological symptoms develop. ^{31}P NMR is not the only feasible nuclide for such studies; results have also been obtained with ^{13}C and ^{1}H NMR.

These latter techniques using large-bore magnets, surface detector coils and also magnetic field gradients to give sharp NMR lines only in the spatial region of interest, are collectively described as 'Topical Magnetic Resonance' (TMR) techniques [41]. This same equipment can be adapted to allow the ultimate application of NMR spectroscopy in the clinic, to obtain images of internal structures in the body.

NMR imaging

This is not really an analytical application at all, but rather it is a clinical diagnostic tool to complement X-ray computer tomography, which gives X-ray pictures of internal body structures, primarily of the hard substances such as bone. Soft internal organs require the administration of special X-ray opaque agents. Together with the associated risk of exposure to ionizing radiation, the X-ray technique has a number of drawbacks. Conversely NMR imaging uses no apparently harmful radiation and is complementary in studying the soft tissues in the body; this is because it is the ^{1}H resonance of water which is usually detected. The spatial location is obtained by applying known magnetic field gradients and using the radio frequency–magnetic field ratio constancy condition to give a linear measure of distance. Application of gradients in three dimensions then allows, via computer tomography techniques, the clinician to build up a two-dimensional cross-section of any of the three orthogonal planes. This equipment is now available commercially and is slowly going into hospitals. The high cost, however, typically in the region of £1 million, is obviously a barrier to its widespread use.

The early literature on the subject was usually found in physics journals, but now many applications have been published, mainly in journals such as *Radiology* or the *Journal of Computer-Assisted Tomography*. Indeed, the technique now has its own journal, *Magnetic Resonance Imaging*, which includes a recent review [42]. It is no longer necessary to be restricted to the use of the H_2O chemical shift for building up images. Complementary information is available in images built up from the H_2O relaxation time distribution or from the use of other nuclei such as ^{31}P or ^{23}Na. Increases in sensitivity have resulted in fast scans such that, recently, examples have been shown of real-time imaging on parts of the body in motion, such as during a heartbeat. The possibility now exists to obtain simultaneously both images of internal organs and their high resolution NMR spectra of ^{1}H, ^{13}C or ^{31}P; the physiology and biochemistry can be studied at the same time.

Summary

In the early 1970s it seemed that the advances given by Fourier transform techniques, superconducting magnets and microcomputers had led to the subject of NMR reaching a

plateau. Since about 1975 the whole breadth of NMR application has developed mainly because of a greater understanding and exploitation of spin physics. Thus NMR can no longer be regarded as a single subject. This is reflected, for example, in the fact that no longer are there just NMR conferences, but a number of meetings devoted to a variety of specialized applications of NMR have arisen, some as outlined in this article. Nonetheless the capital cost of research NMR equipment is not inexpensive, being typically of the order of £200,000, and much higher for imaging devices. Although the cost in real terms of the equipment relative to performance has never been lower, cognisance must be given to the question of cost effectiveness, especially in industrial organizations.

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