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Applications of quantitative ^{1}H- and ^{13}C-NMR spectroscopy in drug analysis*

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Abstract: The usefulness of ^{1}H and ^{13}C Fourier transform (FT) nuclear magnetic resonance spectroscopy (^{1}H- and ^{13}C-NMR) as quantitative methods stems from the potential direct relationship between the area under an NMR peak and the number of the particular type of nuclei that give rise to the signal, though it is necessary, especially for quantitative ^{13}C-NMR, to take some precautions. The experimental limitations that have to be overcome in order to obtain quantitative ^{13}C-NMR spectra are associated with the relaxation time, the nuclear Overhauser effect (NOE), and the NMR instrument itself (filter characteristics, power level of the exciting pulse, dynamic range, digital resolution). Practical problems aside, ^{13}C-NMR has a greater potential than ^{1}H-NMR for the study of organic systems. The sensitivity of ^{13}C chemical shifts to small differences in molecular environment, coupled with a large chemical shift range, gives a "chromatographic" separation of resonances of interest, and has made ^{13}C-NMR an attractive method for analysing complex mixtures. Some applications of quantitative ^{1}H- and ^{13}C-NMR spectroscopy in drug analysis are discussed.

Keywords: *Quantitative ^{1}H-NMR; quantitative ^{13}C-NMR; drug analysis.*

Introduction

The first NMR signals were independently observed in 1945 by Bloch and Purcell who were jointly awarded the Nobel Prize for Physics in 1952. After the discovery of the phenomenon of chemical shift in about 1950, the development of NMR techniques has been very rapid. The first commercial continuous wave high-resolution ^{1}H-NMR spectrometer was produced in 1953. The proton is the most sensitive naturally occurring nucleus detectable by NMR. This property, coupled with its common occurrence in chemistry, particularly organic chemistry, has resulted in the proton being the most studied nucleus. The sensitivity of the ^{13}C nucleus is much lower, and the natural abundance is only 1.1%. The greater problems associated with ^{13}C-NMR were not satisfactorily overcome until the introduction of commercial Fourier transform (FT) spectrometers in about 1970. Despite practical problems, ^{13}C-NMR has a greater potential than ^{1}H-NMR for the study of organic systems. Some advantages of ^{13}C-NMR include the direct observation of molecular backbones, the sensitivity of ^{13}C chemical shifts to small differences in molecular environment, and the large chemical shift range

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compared with ^{1}H-NMR. This gives a high specificity, resulting in a "chromatographic" separation of resonances of interest. Other advantages of NMR spectroscopy include its direct applicability (no derivatization is required), and its non-destructive nature.

The usefulness of ^{1}H- and ^{13}C-NMR as quantitative methods stems from the potential direct relationship between the area under an NMR peak and the number of nuclei giving rise to it. Therefore, ^{13}C-NMR is an attractive method for analysing complex mixtures qualitatively as well as quantitatively. In principle, it is possible to identify and to determine quantitively all the individual compounds of even very complicated mixtures of organic compounds, without extensive and time-consuming extraction and separation procedures [1–4].

Quantitative NMR Spectroscopy

Unfortunately, extracting the desired quantitative information from a ^{13}C-NMR spectrum is hampered by several experimental and instrumental limitations. This is also true for ^{1}H-NMR, but in this case the conditions for obtaining quantitative results are generally easy to fulfil.

The large spread of ^{13}C relaxation times, from 0.1 to about 100 s, in conjunction with the usually short pulse repetition times (short in comparison with the relaxation time T_1) leads to preferential saturation of the more slowly relaxing nuclei.

The nuclear Overhauser effect (NOE) is a by-product of proton irradiation, leading to an almost threefold increase in intensity of the ^{13}C-NMR signals. However, the nuclear Overhauser effect observed in proton decoupled spectra does not always reach its theoretical maximum, and the NOE may be different for every carbon atom. Therefore, the NOE must be nullified in order to obtain quantitative results [5–7].

Two methods are commonly used for quantitating ^{13}C-NMR spectra. The addition of paramagnetic relaxation agents, such as chromium tris-acetylacetonate, to the sample solution shortens the relaxation times T_1 and the NOE decreases. The use of paramagnetic relaxation reagents should be strongly restricted because NOEs may not be completely suppressed in the general case. When the sample is to be recovered, the paramagnetic species must be separated from the sample. Therefore, the gated decoupling technique is generally chosen to eliminate the NOE [8, 9]. When the proton decoupler is on during the acquisition time, and off during the pulse delay (the interval time between two pulses), a completely decoupled ^{13}C-NMR spectrum, producing one resonance line for each carbon atom, without NOE is obtained. The pulse delay must be sufficiently long so that the NOE, built up during the acquisition time, can die out completely, and relaxation of all the excited nuclei is possible. It can be shown that, for a ^{13}C nucleus with $T_1 < 30$ s, the optimum pulse delay is 6.5 times T_1, using a pulse width of 90°. Long spectral accumulation times can be avoided by selecting ^{13}C nuclei with a short T_1 to perform quantitative measurements [10–14].

Another approach for quantitative ^{13}C-NMR is to ignore differences in T_1 and NOE. Samples are run under conditions of equal or near-equal concentration, temperature, pulse width and pulse repetition rate, and include a known amount of a standard compound. The essential condition required to achieve accurate intensity measurements using such standards is that the carbons of the standard and those to be measured must have very similar relaxation times and NOEs. If these conditions are fulfilled, then accurate intensity measurements can be obtained from routine proton-decoupled ^{13}C-NMR spectra. Otherwise weighting factors or calibration curves, which relate the

individual peak intensities and the standard peak intensity to the amount of each compound present, must be computed from standard solutions and applied to the solutions being analysed. The amount of each component is determined from the concentration of the standard by comparing peak intensities [15, 16].

Some instrumental aspects should also be considered. These instrumental problems occur in quantitative ^{1}H- as well as ^{13}C-NMR spectroscopy, but in ^{1}H-NMR they are generally easy to overcome.

The signal intensity is affected by filters, used prior to the analog-digital-converter (ADC) in order to reduce noise from outside the spectral range. If the bandwidth of the filters is too narrow, lines at the end of the spectrum are reduced in intensity. If the total filter bandwidth is set equal to the frequency range F, as is usually done, the signals at the end of the spectral range have only 70–80% of their true intensity. This problem can be solved by selecting a larger filter bandwidth, for instance $2F$ [11, 17, 18].

Another problem is the effect of finite pulse power, or non-uniform response across the spectral width. If the power level of the exciting pulse is too low, all the nuclei within the spectrum of interest are not subjected to the same excitation. It can be shown that, to have a uniform power spectrum, the 90° pulse, which has a characteristic value for each instrument, must be less than $1/4F$, where F is the frequency range of the spectrum. In this case intensity errors are less than 2% across the spectral width. In ^{1}H-NMR the spectral widths are quite narrow, so that this condition is easily fulfilled [1, 5, 11].

Another important criterion associated with the ADC concerns the dynamic range of the signals that are to be digitized. The ratio of the tallest to the smallest peak in a spectrum must not exceed a value determined by the number of bits in the ADC. A 10 bit ADC can represent a signal to 1 part in 2^{10}, or 0.1%. Although this ratio may seem very large, it must be remembered that, especially for samples run at low concentrations, the size of the solvent signal can be very abundant indeed. Dynamic range considerations also arise during the summation of the spectra in the computer memory of the NMR instrument. It is easily recognized that the dynamic range of the computer (the length of a computer word) must exceed that of the ADC. Since no overflow can be tolerated during the accumulation, most computers use a scheme of weighted averaging [1, 11].

Finally, digital resolution has to be considered. The computer of an NMR instrument may not have sufficient storage (data points) to define completely all the peaks. The maximum resolution obtained from an NMR spectrum is often determined by these limitations rather than magnetic field inhomogeneity. ^{13}C-NMR line widths can be quite narrow (about 1 Hz). If the digital resolution is such that a given signal is not sufficiently defined by the data points, the integration of the peaks will produce inaccurate results. The resonance definition can be improved by multiplication of the free induction decay (the NMR spectral data before Fourier transformation) with an exponential function, having a negative time constant, which broadens the lines, and improves the signal-to-noise (S/N) ratio. A high S/N ratio is necessary for a high level of accuracy. It was found that the minimum resonance definition for accurate results is approximately two data points above half maximum intensity for area determination, and three data points above half maximum intensity for peak height determination. A definition of about four to five points above half maximum intensity is a good criterion for broadening, but in practice this is not always possible for small peaks. The line broadening technique should be used with care when integrating peaks that are close to each other. As line broadening increases, resolution decreases, which can cause overlapping of closely lying resonances [1, 8, 19].

Nowadays sophisticated software has been developed for quantitative ^{1}H- as well as ^{13}C-NMR spectroscopy. These methods can result in an important saving of time. They are ideally suited to perform automatic integrations and series of quantitative determinations of a more routine nature [3, 20].

Although ^{1}H- and especially ^{13}C-NMR techniques are not as sensitive as, for instance, UV-spectrophotometry, they have already been applied to the quantitative analysis of many pharmaceutical products. The advantage of quantitative NMR spectroscopy is that several active components or impurities may be determined simultaneously in a relatively short time and that differentiation of closely related structures is possible. Some examples of the application of quantitative ^{1}H- and ^{13}C-NMR spectroscopy in drug analysis will be discussed below.

Applications of Quantitative ^{1}H- and ^{13}C-NMR in Drug Analysis

Amyl nitrite

The nitrites are the oldest and still the most important vasodilators. Amyl nitrite and nitroglycerine were the first members of this group introduced into medicine. Amyl nitrite acts slightly faster than nitroglycerine. In the USP XX amyl nitrite is defined as a mixture of the nitrite esters of 3-methyl-1-butanol and 2-methyl-1-butanol, containing not less than 85.0% and not more than 103.0% of $C_5H_{11}NO_2$. The ^{1}H-NMR spectrum of amyl nitrite, recorded in carbon tetrachloride, shows a resonance signal at 4.8 ppm, due to the α-methylene protons. Benzyl benzoate is used as an internal standard the methylene protons of which resonate at 5.3 ppm. The quantity, in mg, of amyl nitrite in the test preparation is calculated by the general formula

$$\text{weight (mg)} = W_s \frac{A_u}{A_s} \frac{E_u}{E_s}$$

where W_s is the weight, in mg, of the internal standard, A_s and A_u the average area of the internal standard peak and the analyte, respectively, and E_s and E_u the corresponding proton equivalent weights (i.e. the molecular weights divided by the number of protons giving rise to the measured signal). The equivalent weight of amyl nitrite is 117.15 divided by 2, i.e. 58.57, and of benzyl benzoate 212.24 divided by 2, i.e. 106.12. The same method is used for the assay of amyl nitrite inhalant [21].

Succinylcholine chloride

Succinylcholine chloride (Fig. 1) is a neuromuscular agent. It interrupts the transmission of nerve impulses at the skeletal neuromuscular junction and is used mainly in surgical anesthesia to obtain sustained skeletal muscular relaxation. It is commercially available as a sterile powder and as a sterile solution for injection. The official titrimetric assay method and other titrimetric procedures are lengthy, require numerous reagents and are not stability-indicating. The gas chromatographic assay of succinylcholine involves its conversion to a volatile product under closely controlled experimental conditions. In 1985 a simple, accurate and specific ^{1}H-NMR spectroscopic method was

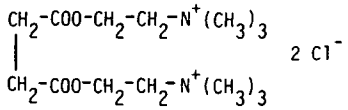


Figure 1
Succinylcholine chloride.

proposed by Hanna and Lau-Cam for the assay of succinylcholine chloride in drug substance and dosage forms. This method can also be used for monitoring the chemical stability of succinylcholine.

The ^{1}H-NMR spectrum of succinylcholine chloride, recorded in deuterium oxide (D_2O) after freeze-drying of the aqueous solution when analysing injections, shows a resonance signal at 3.27 ppm, due to the N-methyl groups. Acetamide is used as an internal standard the methyl group of which gives a resonance signal at 2.01 ppm. Therefore, the equivalent weight of succinylcholine chloride is the molecular weight divided by 18, the number of methyl hydrogens, and the equivalent weight of acetamide is the molecular weight divided by 3. The quantity of succinylcholine chloride, using the integral values for these resonance signals, is calculated by the same general formula as discussed for amyl nitrite. The precision of the method was found to be about 0.6% (relative standard deviation).

Both the analyte and the internal standard were found to be stable in D_2O solution for at least three days. However, aqueous solutions of succinylcholine chloride undergo hydrolytic cleavage to succinic acid and choline. This ^{1}H-NMR method provides evidence of degradation in commercial injections by an additional signal at 2.47 ppm, due to succinic acid. The choline moiety can also be recognized from some specific signals, especially the multiplet centred at 3.57 ppm. No interferences by certain additives to solutions for injection, for instance methyl paraben or benzyl alcohol, were observed. Thus, quantitative ^{1}H-NMR is an accurate, simple and specific method for the assay of succinylcholine chloride in injections [22].

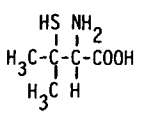
Penicillamine

A rapid and specific ^{1}H-NMR spectroscopic method for the determination of penicillamine in capsules was presented by Nelson *et al.* Penicillamine (Fig. 2) is a chelating agent forming soluble complexes with many heavy metals. It is used for the removal of copper in patients suffering from Wilson's disease, in the treatment of heavy metal poisoning, cystinuria, and rheumatoid arthritis. Because the L-isomer displays significantly greater toxicity, commercial preparations of penicillamine consist solely of the D-isomer.

The ^{1}H-NMR spectrum of penicillamine in D_2O exhibits two singlets at 1.58 and 1.64 ppm, due to the two non-equivalent methyl groups. Sodium saccharin is used as an internal standard. Its four aromatic protons resonate downfield between 7.4 and 7.9 ppm. The quantity of penicillamine, using the integral values for these signals, is calculated by a similar formula to that discussed for amyl nitrite.

Penicillamine can undergo oxidative transformation to penicillamine disulphide. The methyl groups of the disulphide produce two singlets, one appearing slightly upfield from the penicillamine singlet at 1.58 ppm, and the other overlapping with the penicillamine singlet at 1.64 ppm. Although small amounts of this product may remain undetected by NMR spectroscopy, concentrations greater than 2% will be detected, and concentrations of 5% or more can be determined by measuring the heights of the signals generated by the corresponding methyl groups of penicillamine and its disulphide.

Figure 2
Penicillamine.



An NMR method for assaying the enantiomer composition of penicillamine, using a lanthanide shift reagent, has also been described. Problems due to the insolubility of penicillamine in non-polar solvents are overcome by a two-step derivatization procedure. After addition of a suitable amount of a europium shift reagent, substantial chemical shift differences are observed for analogous protons in the D- and L-isomers. The optimum procedure derived for assaying DL-penicillamine involves comparison of the peak heights for the methyl peaks in the europium shifted spectrum after addition of 0.62 equivalents of shift reagent. The limit of the NMR assay method using a Fourier transform spectrometer is 0.4–0.5% of the L-isomer in commercial samples. Analyses using continuous wave NMR spectrometers enable a lower limit of 2.0–2.5% L-penicillamine to be quantitated [23, 24].

Cosmetic ingredients

Fifteen years ago, in 1974, a quantitative ^{1}H-NMR method for the evaluation of the iodine number, the ester value and the moles of ethoxylation in cosmetic raw ingredients such as oleyl alcohol, isopropyl palmitate, sesame oil, avocado oil, polyoxyethylene derivatives, etc., was presented by Kaplan and Laczynski. By using a similar procedure as described for the previous examples, the iodine value can be calculated by means of the ^{1}H-NMR signal due to the olefinic protons, and the ester value by means of the signal of the protons in the α-position of the oxycarbonyl group. The number of moles of ethoxylation for a compound such as polyoxyethylene stearyl ether can be determined with ease by ^{1}H-NMR spectroscopy. The terminal methyl signal of the stearyl moiety is used as a reference peak. The area of this resonance signal is divided by 3 to obtain the area per proton. Three protons must be subtracted from the ethoxylate peak, because the hydroxyl proton and the stearyl chain methylene group, bonded to the ethoxylate oxygen, show approximately the same chemical shift. The unit structure of an ethoxylate ($-CH_2-CH_2-O-$) reveals four protons per mole of ethoxylation. The area per proton times 4 yields the area responsible for each mole of ethoxylation. Thus, dividing the total area due to the ethoxylation (corrected for the hydroxyl and methylene protons) by the area per mole of ethoxylation will give the number of moles of ethoxylation in the compound under investigation. Chain-branching in ethoxylated materials disallows the use of the terminal methyl group as a reference peak [25].

Dextromethorphan and levomethorphan

The determination of the enantiomeric purity of a substance is an important aspect of drug analysis, especially in cases such as methorphan where the *levo-* and racemic forms of the drug are considered as narcotics, while the *dextro*-isomer is a widely used non-narcotic antitussive agent (Fig. 3). The standard method used to distinguish dextromethorphan from levomethorphan is polarimetry, if there is an adequate amount of sample and if there are no interfering compounds. Another method is the hanging

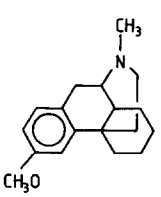


Figure 3
Methorphan.

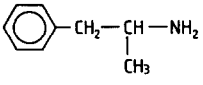
microdrop technique, with a sensitivity of 0.2 μg. However, these methods cannot accurately detect one isomer in the presence of the other. Wainer *et al.* have reported the application of chiral lanthanide nuclear magnetic shift reagents to the identification of dextromethorphan and levomethorphan, and the determination of one isomer in the presence of the other.

The addition of a europium or praseodymium shift reagent to an equimolar mixture of dextromethorphan and levomethorphan has multiple effects on the ^{1}H-NMR spectrum. The singlet associated with the N-methyl protons at 2.9 ppm broadens, and disappears at a substrate to reagent molar ratio of 80:1. This suggests that the nitrogen atom is the site of interaction between methorphan and the shift reagent. The singlet due to the methoxy protons is used to differentiate between dextromethorphan and levomethorphan. Addition of an adequate amount of shift reagent causes the signal associated with the *dextro*-isomer to move downfield, while the signal associated with the *levo*-isomer remains relatively constant. The maximum resolution of the signals occurs at a substrate to reagent molar ratio of 1:1. In conclusion, the chiral lanthanide shift reagents can be used to identify dextromethorphan and levomethorphan. This method rapidly and accurately differentiates between the two isomers, and allows the determination of as little as 10% of one product in the presence of the other [26].

Dexamphetamine and levamphetamine

A similar method is used for the determination of dexamphetamine and levamphetamine mixtures (Fig. 4). The official method for the determination of the enantiomeric purity of D-amphetamine sulphate involves the preparation of acetylamphetamine, followed by purification, and determination of the derivative's specific optical rotation. A more sensitive assay, using 1-fluoro-2,4-dinitrobenzene has also been developed. The direct quantitative measurement of the *dextro*- and *levo*-isomers can be accomplished by gas–liquid chromatography or NMR spectroscopy of different derivatives of D- and L-amphetamine. However, the use of a europium chiral NMR shift reagent for the determination of the enantiomeric purity of amphetamine in bulk drug form and pharmaceutical preparations avoids the synthesis of derivatives. The doublet associated with the methyl protons is used for the differentiation between D- and L-amphetamine. The doublet due to the *levo*-isomer is shifted further downfield than the doublet associated with the *dextro*-isomer, if an adequate amount of shift reagent is added. Optimum resolution is achieved at a 0.15 molar ratio of shift reagent to substrate. The resolution can be enhanced further by irradiation of the signal due to the methyne proton. This decouples the signal associated with the methyl protons, producing a pair of sharp singlets rather than a pair of doublets. The detection of as little as 5% L-amphetamine in the presence of D-amphetamine is possible [27].

Figure 4
Amphetamine.



Cephradine

Quantitative NMR spectroscopy has also been applied to the analysis of antibiotics. A ^{1}H-NMR method to determine quantitatively the presence of cephalexin in cephradine (Fig. 5) was described by Warren *et al.* Cephalexin may be present in a sample of

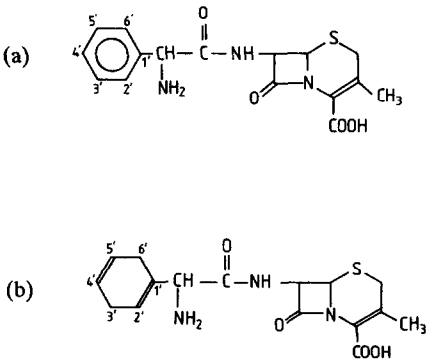


Figure 5
(a) Cephalexin; (b) cephradine.

cephradine as an impurity from the synthesis of cephradine, or as a decomposition product of cephradine. The quantitative NMR determination of cephalexin in cephradine is based on the integration of the five aromatic protons of the fully aromatic system in cephalexin at 7.5 ppm, vs the integration of the most downfield olefinic proton (2′) of cephradine at 6.2 ppm. This provides the data necessary to determine the percentage of cephalexin present. The precision at the 2% cephalexin level is ±0.18%. The method is applicable to the drug substance itself and to capsules and oral suspension formulations [28].

Phenethicillin

Quantitative proton, as well as ^{13}C-NMR, spectroscopy have been applied to the determination of the diastereoisomer ratio in phenethicillin (Fig. 6). Phenethicillin is a semisynthetic penicillin, prepared by N-acylation of 6-aminopenicillanic acid with racemic α-phenoxypropionyl chloride. This condensation introduces a new chiral centre. The isomer composition depends upon the solvent extraction and crystallization procedures. The two isomers show differences in their antibacterial spectra. Limits of between 55 and 75% of the more active L-isomer are specified. The British Pharmacopoeia allows certain optical rotation limits, corresponding to isomer compositions of 34–100% of the L-isomer. The optical rotation measurement, however, sums the effect of all five asymmetric centres and is not as specific as a microbiological assay. Wilson *et al.* have described a procedure for the determination of the diastereoisomer ratio in phenethicillin potassium and its formulations using ^{1}H- and ^{13}C-NMR.

The ^{1}H-NMR spectrum of L-phenethicillin shows a typical AB-splitting pattern at 5.4 and 5.6 ppm, due to the two β-lactam protons, whereas in the ^{1}H-NMR spectrum of D-phenethicillin these β-lactam protons appear as a singlet at 5.5 ppm. The percentage of the L-isomer is calculated from the integration of these signals. The accuracy of the quantitative ^{1}H-NMR method decreases as the proportion of D- and L-isomers deviates from a near equal mixture. However, excellent correlation was obtained in the region

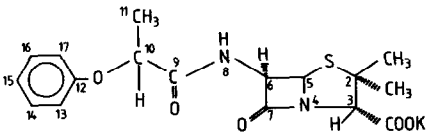


Figure 6
Phenethicillin.

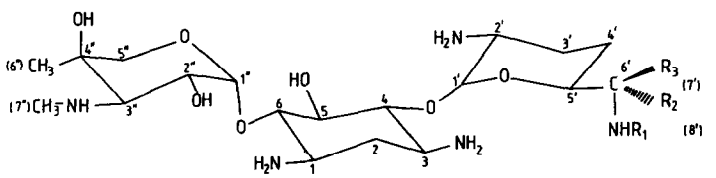
normally expected with commercial samples, i.e. 55–75% L-isomer. The relative standard deviation was found to be about 1.1%.

Because of the greater peak resolution of ^{13}C-NMR spectroscopy, its application to the determination of the diastereoisomer ratio in phenethicillin was also investigated. For the quantitative analysis of diastereoisomeric mixtures the two β-methyl groups on C-2 proved to be the most useful, since the chemical shift difference between the signals of the D- and L-isomers, at 31.8 and 32.4 ppm, respectively, was the largest (0.6 ppm), compared with the other signals. The ^{13}C-NMR method was as accurate and as precise as the ^{1}H-NMR assay, and could be applied over a wider range of D to L ratios [29].

Gentamicin

Quantitative ^{1}H- and ^{13}C-NMR have also been applied to the analysis of gentamicin. Gentamicin sulphate is a mixture of the sulphates of antimicrobial substances produced by *Micromonospora purpurea*. The proportions of the main components present in the gentamicin sulphate, gentamicins C_1, C_{1a} and C_2, can be monitored by ^{1}H-NMR spectroscopy. Gentamicin 2_a and 2_b are only minor components (Fig. 7).

Some characteristic signals in the ^{1}H-NMR spectrum of a typical sample of gentamicin sulphate, recorded in D_2O, include peaks at 2.95 ppm, due to the N-methyl group present in all three components, at 2.75 ppm, due to the second N-methyl group present in C_1, at 1.35 ppm, due to the C-methyl group present in all three components, and at 1.25 ppm. The signal at 1.25 ppm is in fact one peak of the methyl doublet for the CH-methyl group present in C_1 and C_2. The other peak of the doublet is very close to, or overlaps with, the 1.35 ppm signal. The quantitative ^{1}H-NMR spectroscopic method depends on measurement of the peak heights of the signals for the N-methyl and C-methyl groups present in all three components, and of those present in C_1 and C_2 only, followed by calculation of peak height ratios to control composition within acceptable limits. Originally this method was developed for use with spectra from a 60 MHz continuous wave (CW) instrument. An estimation of the composition of the different gentamicins in the analysed sample was computed by using empirical formulae [30]. The British Pharmacopoeia test controlling the composition of gentamicin sulphate is also based on a 60 MHz CW instrument [31]. The original ^{1}H-NMR method was modified by Reuter *et al.* in 1982 for application to a 90 MHz instrument [32]. Busson *et al.* have



GENTAMICIN C

		R_1	R_2	R_3
gentamicin	C_1 :	Me	H	Me
gentamicin	C_2 :	H	H	Me
gentamicin	C_{1a} :	H	H	H
gentamicin	C_{2a} :	H	Me	H
gentamicin	C_{2b} :	Me	H	H

Figure 7
Gentamicin.

evaluated the application of the ^{1}H-NMR method on a 90 MHz Fourier transform instrument [33]. Largely due to the highly subjective and critical nature of baseline adjustment and also to the fact that impurities may interfere with the signals to be measured, the use of quantitative ^{1}H-NMR as a general and reliable test for controlling the composition of gentamicins remains questionable. ^{1}H-NMR is not a specific assay method for gentamicin, but it controls within broad limits the ratios of the three main components.

Quantitative ^{13}C-NMR spectroscopy has also been used for the analysis of gentamicin sulphate. Two procedures for applying ^{13}C-NMR spectroscopy to the quantitative determination of the C_1, C_{1a} and C_2 components of gentamicin sulphate were described by Kountourellis *et al.* in 1983 [34]. One was based on the use of calibration plots of peak height ratios of analyte to standard (dioxane) resonance intensities recorded under conditions of full relaxation, the other upon a steady-state experiment and the use of weighting factors. A modified ^{13}C-NMR procedure as a potential routine assay method was published by Busson *et al.* in 1986 [33]. Ratios of gentamicin components were obtained from peak height measurements of selected resonance signals in spectra recorded under steady-state conditions. For the analyses, the following clearly separated resonance signals were selected: C-7′ (10.2 ppm), C-8′ (31.9 ppm) and C-6′ (58.3 ppm) for C_1; C-7′ (13.1 ppm) for C_2; C-7′ (15.0 ppm) for C_{2a}; C-6′ (43.5 ppm) for C_{1a}; C-8′ (34.3 ppm) and C-6′ (53.0 ppm) for C_{2b}. If the analysis is limited to component ratio rather than actual composition, addition of an internal standard can be omitted and a knowledge of only the relative response factors for each of the pertinent resonances is required. This implies that a reference mixture of accurately known composition is available. The lower limit of detection of the ^{13}C-NMR method was about 2%.

Heparin

High-field ^{1}H-NMR spectroscopy has been employed for monitoring the purity of pharmaceutical heparin preparations. Heparin, a potent anticoagulant of natural origin, is a highly sulphated glycosaminoglycan (averaging 2.5 sulphate groups per disaccharide unit), composed of 1–4 linked, alternating residues of α-D-glucosamine and α-L-iduronic acid or β-D-glucuronic acid. Heparin preparations may be contaminated with the related glycosaminoglycan dermatan sulphate (chondroitin B, β-heparin). High-field ^{1}H-NMR spectroscopy (300 MHz) is able to differentiate heparin from dermatan sulphate. The percentage of dermatan sulphate can be determined using the integrals for the acetamidomethyl (3-proton) signal of dermatan at 2.10 ppm, for the acetamidomethyl (3-proton) signal of N-acetylated residues in heparin at 2.07 ppm, and for the H-2 (1-proton) signal of the N-sulphated residues in heparin at 3.32 ppm. The error in these measurements is estimated to be 5–10%. ^{1}H-NMR spectroscopy can also be used to detect other sources of contamination, for instance ethylenediamine tetracetic acid (EDTA) [35].

Pilocarpine

The presence of isopilocarpine, the epimer of pilocarpine, and of pilocarpinic acid, a hydrolytic degradation product of pilocarpine, was detected and all three substances were assayed in various commercial ophthalmic formulations of pilocarpine hydrochloride by ^{13}C-NMR spectroscopy (Fig. 8). The assay is based upon the integration of selected resonances calibrated against tetramethylammonium bromide, used as an external reference. The normalized intensities are then related to those of a reference

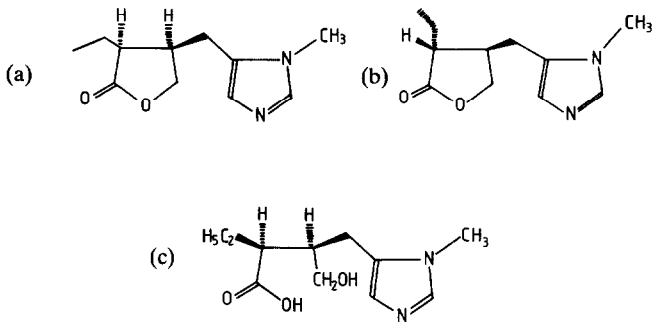


Figure 8
(a) Pilocarpine; (b) isopilocarpine; (c) pilocarpinic acid.

solution of pilocarpine hydrochloride, in order to eliminate any factor arising from differences in relaxation time. The N-methyl group shows the same chemical shift for all products, and provides a convenient basis for the assay of the total alkaloid content, whereas the resonance signals of C-8 (the CH_2-group next to the oxygen atom) are best suited for assaying the individual products. The percentage composition is determined with an experimental error of 5% [36].

Ephedra alkaloids

The oriental crude drug, *Ephedra herba*, contains the sympathomimetic amines L-ephedrine and D-pseudoephedrine (Fig. 9). Due to some differences in biological activity, separate analysis of these alkaloids is necessary. A ^{13}C-NMR procedure has been described for this purpose by Yamasaki and Fujita. Seven or eight specific peaks for each compound are used. After obtaining calibration curves for both alkaloids relative to the internal standard, dioxane or alternatively morfoline, the amount of L-ephedrine and D-pseudoephedrine is calculated by comparison of their integration values with the internal standard. The results compared favourably to those obtained by gas–liquid chromatography [37].

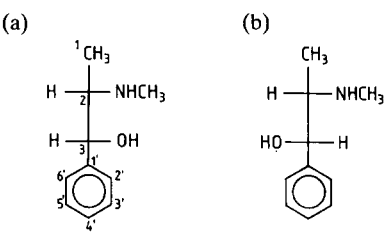


Figure 9
(a) L-Ephedrine; (b) D-pseudoephedrine.

Drug mixtures

Quantitative ^{1}H- as well as ^{13}C-NMR spectroscopy has been employed for the analysis of commercial antipyretic preparations containing aspirin, caffeine and phenacetin or ethenzamide [38, 39]. ^{13}C-NMR spectroscopy has also been applied in forensic drug analysis. The chemical shifts of 14 substances of abuse were assigned by Alm *et al.*, and quantitative analysis was performed on four of them in complex forensic drug mixtures

(amphetamine, methamphetamine, phendimetrazine, and ephedrine) with 2,4-dimethylpentane as the internal standard. The results were compared with gas chromatography, and good agreement was found between the two methods [40].

Conclusion

Only a few selected applications of quantitative ^{1}H- and ^{13}C-NMR spectroscopy in pharmaceutical analysis have been discussed in this review. It is obvious that both techniques have great potential, and may be successful where other methods fail, especially when differentiation of closely related structures is required. ^{13}C-NMR is even more specific than ^{1}H-NMR, but both methods, especially ^{13}C-NMR, have low sensitivity. There are no general rules for predicting if an analytical problem can be solved by quantitative ^{1}H- or ^{13}C-NMR spectroscopy: every case has to be considered separately. A careful design of the experimental protocol is necessary in order to achieve the desired accuracy and precision [41].

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