NMR-Spectroscopy in Drug Analysis
Quantitative NMR Spectroscopy

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Part I: NMR Spectroscopy in Drug Analysis - Principles

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Content:

a) Basic principles
b) NMR spectroscopy as a primary method of measurement
c) Optimization of the experimental parameters
d) Processing the spectra

a. **Basic principles of quantitative NMR spectroscopy**

The intensity of a NMR-signal is given by the area under the specific signal. This area is directly calculated by modern spectrometer software. For this purpose all intensities (in arbitrary units) for each data point over the whole signal are added. The intensity $I_A$ of a signal is directly proportional to the number of nuclei $N$ evoking the signal. Due to this relation the intensities of NMR signals can be used for quantitative purposes.

The relation between the signal intensity $I$ and the number of observed nuclei is given by:

**Equation 1**

$$I = c_s \cdot N$$

The proportionality constant $c_s$ results from parameters of the spectrometer and the sample. It is called “spectrometer constant”. So there is a linear relation between the intensity of a NMR signal and the number of nuclei evoking it.

This relation could also be derived from the description of a signal $S(\omega)$ as pure Lorentzian line resonating at frequency $\omega$ (the lineshape of NMR signals drawn as pure absorption mode signals, cf. Fig. 1 (taken from [1], [2]) by integration from $-\infty$ until $+\infty$. Except of the number of the nuclei which contribute to that signal all parameters of the NMR system are constant.

A disadvantage of NMR spectroscopy as a quantitative method of measurement is the low sensitivity. Since NMR signals are based on the small difference of the spin populations in either $\alpha$- or $\beta$-state only this little amount of nuclei is taken in concern in NMR experiments. While in UV spectroscopy a limit of detection in the µM range can be achieved, in NMR spectroscopy concentration of 0.1 to 1 mM are required. In addition, quantification is only possible if the signals being integrated are well separated from others. In contrast to chromatographic methods there is only little possibility to influence this separation because the chemical shift is directly related to the molecular structure. By changing the solvent or by adding auxiliary reagents, the so called “shift reagents” the separation of the signals can be changed. In addition, problems concerning the line shape may occur and the integration of the signals will get worse.

![Figure 1: Lorentzian line of an NMR-Signal; the width at half height ($\Delta \nu_{1/2}$) is marked (from [1]).](image)
These disadvantages can be partly compensated. The decisive advantage of NMR spectroscopy over chromatographic and electrophoretic methods is due to the fact that almost no preparation time exists. Whereas in HPLC much time has to be spent for equilibration of the columns or derivatization of the analyte for UV or fluorescence detection, the NMR spectrometer is always ready to measure. The substance has to be dissolved in a proper deuterated solvent only. The amount of solvent needed is about 0.7 ml (using common 5mm probe heads). So, facing the larger solvent volume in HPLC the higher costs for deuterated solvents are compensated.

b. NMR spectroscopy as a primary method of measurement

Another great advantage of NMR spectroscopy is its suitability as a primary method of measurement. In 1971 the “mole” was introduced into the SI-system (Système Internatioal d’Unités) as the basic unit of the amount of substance. The responsible committee, the Consultative Committee for Amount of Substance (CCQM, Comité Consultatif pour la Quantité de Matière) within the International Committee for Weights and Measures (CIPM, Comité International des Poids et Mesures) has defined a primary method of measurement to be:

“A primary method of measurement is a method having the highest metrological qualities, whose operation can be completely described and understood, for which a complete uncertainty statement can be written down in terms of SI units, and whose results are, therefore, accepted without reference to a standard of the quantity being measured.” [3]

In other words, when using a primary method of measurement the amount of substance can be determined directly from the physical context of the measurement without referencing to another substance. For example, the amount of substance can be determined coulometrical under constant current and assuming a single-electron transition as \( n = I t F^{-1} \). In the case the current \( I \), the time \( t \), and the Faraday constant \( F \) are expressed in SI-units, the amount of substance results directly in the SI-unit “mol” [4]. Primary methods of measurement are coulometry, gravimetry, titrimetry, the group of colligative methods* and the isotope dilution mass spectrometry [3]. NMR spectroscopy also fulfils these requirements.

The signal-to-noise ratio in NMR spectroscopy can be expressed as:

\[ R(\omega) = \frac{S_p(\omega)}{2N(\omega)} \]

In equation 2 \( S_p \) represents the pure absorption mode signal at the frequency \( \omega \) and \( N \) the noise. The line shape of NMR signals is given theoretically, as mentioned before, in pure absorption mode by a pure Lorentzian line. Both signal and noise can theoretically be derived from fundamental constants, properties of the NMR sample and parameters of the receiver. By this and some algebraic conversion, Equation 2 can be rewritten as:

\[ R(\omega) = \left[ \frac{\hbar^2 I(I+1)}{24kT} \right] \left[ \frac{N \omega^{3/2}}{T^{3/2}} \right] \left[ \frac{T^{3/2}}{\lambda F} \right] \left[ \frac{2 \xi^2 \rho QV_c}{\lambda F} \right] \sin \alpha \]

Without going into detail the following is necessary to be mentioned. While the fundamental constants like the gyromagnetic ratio \( \gamma \) and the Boltzmann constant \( k \) and the sample proper-

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* Colligative properties are those properties (of a liquid) that depend only on the amount of dissolved particles, but not on the type of them. Examples of properties that fall into this category are melting and boiling points.
ties such as the Larmor frequency $\omega$ or the temperature $T$ are known or can easily be determined. This is not the case for the most parameters of the receiver, e.g., the ratio of effective inductance to total inductance of the coil $\rho$, the filling factor of the receiver coil $\xi$, or the quality factor of the receiver coil $Q$; only the volume enclosed by the receiver coil $V_c$ can be easily determined.

Due to this expression on fundamental constants, sample properties and receiver parameters, NMR spectroscopy can be used as a primary method of measurement when the conditions, explained in the forthcoming parts, are considered [4].

Concerning the vector model of NMR spectroscopy, only the part of the macroscopic magnetization being along the $y$-axis of the rotating frame of reference can be detected. Its intensity is determined first by the total intensity of macroscopic magnetization along the $z$-axis of the un-excited sample (i.e., the small difference of nuclei in the $\alpha$- and $\beta$-state) and second by the part of it which is placed in the $xy$-plane (i.e., by the tip angle).

When NMR spectroscopy is used as a quantitative method, there are higher requirements in comparison to a qualitative method. The main aim is to have the detectable part of magnetization along the $y$-axis of the rotating frame as large as possible. Therefore, some parameters of the experiment have to be optimized.

**c. Optimization of the experimental parameters**

*Transmitter frequency and spectral width*

The excitation pulse is symmetrical to the transmitter frequency $\nu$ and its width is proportional to $\tau^{-1}$ ($\tau$ is the duration of the pulse). The power is strongest at the center frequency $\nu$ and decreases to the edges. Thus, the excitation pulse is not an exact rectangular pulse.

If the FID is sampled with only one detector, it is impossible to discriminate whether the signal resonates at higher or lower frequency with respect to the transmitter frequency. The transmitter frequency has to be placed at the edge of the spectrum. This means, that half of the transmitter power is unused and the part with the same high transmitter power becomes even smaller. Therefore, in current spectrometers two phase sensitive detectors are used in order to record the $M_x$- and the $M_y$-component of the magnetization vector at the same time. This “quadrature detection” avoids the aforementioned disadvantages. Since the pulse is not an ideal rectangular pulse, the transmitter frequency has to be as near as possible, ideally in equal distance to the signals of interest.

The spectral width must be chosen large enough to record the whole area of interest. If the width is too small, signals of the spectrum outside this window will be aliased (or “folded”) and will appear at a wrong frequency. This usually is not the case in modern spectrometers working with digital filters. The principle is named “oversampling” and means the FID is sampled at a faster rate than required. The signals outside the window will be mathematically eliminated (cf. Fig. 2). An additional effect of this method is the minimization of noise depending on the digitization [5]. The use of digital filters enables to observe only a part of the whole spectrum without aliasing any signals.

The Fourier transformation generates two data sets, the “real” and “imaginary” spectrum. Thus, the “real” spectrum which is usually displayed contains only half the number of data points than the original FID (in absence of any manipulation like zero filling, see below) resulting in half the number of data points (TD) of the FID.
Consequently, the digital resolution is given as the reciprocal of the acquisition time (AQ):

\[ R_D = \frac{\text{total frequency window}}{\text{total number of data points}} = \frac{SW}{SI} = \frac{2SW}{TD} = \frac{1}{AQ} \]

Due to relaxation effects the FID decays while the noise remains constant. Thus, with longer acquisition time the interferogram contains an increasing part of noise. To avoid truncation effects, it is recommended to sample the FID until it has decayed to almost zero. Longer sampling does not provide further information but the noise might cause distortions. The digital resolution can be artificially improved by appending zeros to the original FID, a procedure known as “zero filling”.

Taken together the length of acquisition time have to be chosen long enough to sample the whole FID and to reach the desired digital resolution but to minimize distortions due to noise.

**Relaxation delay and tip angle**

Since the part of the magnetization along y-axis can be detected only, the part should be as large as possible. Therefore, the total bulk magnetization vector has to be rotated out of the z-direction into the xy-plane by applying a 90° tip angle. Thus, the time needed for total relaxation compared to the usual applied 30° tip angle increases.

The relaxation process follows exponential behavior:

\[ \frac{dM_z}{dt} = \frac{(M_0 - M_z)}{T_1} \]

After applying some pulses (“dummy scans”) equilibrium is reached and the bulk magnetization along the z-axis is little less than \( M_0 \), and \( M_z \) is now only 99% of \( M_0 \). Setting \( M_0 \) at 1 and \( PR \) as pulse repetition period (the time from the pulse until the next pulse of a single pulse experiment), it is:
The signal-to-noise ratio is directly proportional to the amount of magnetization in the xy-plane. Furthermore, the signal-to-noise ratio after \( n \) repetitions is equal to the signal-to-noise ratio after one experiment multiplied with square root of the number of repetitions. The number of repetitions multiplied with the pulse repetition period results in the total experimental time \( t_{\text{tot}} \). Thus, the signal-to-noise ratio is:

\[
(S / N)_n = k \cdot \frac{1}{T_1} \cdot 0.99 \sin \alpha \sqrt{n \cdot 0.99 \cos \alpha}
\]

To optimize the signal-to-noise ratio this equation can be differentiated with respect to \( \alpha \) (holding \( T_1 \) and \( t_{\text{tot}} \) constant). The derivative equation is then set equal to zero and solved numerically. The optimum of the tip angle yielded is 82.865 degrees [6]. In order to reach the desired 99% bulk magnetization in z-direction before the next pulse, a pulse repetition period has to be of the 4.5fold the size of the longest \( T_1 \) relaxation time [6].

Furthermore a relation between the signal-to-noise ratio and the pulse angle on the one hand and the integral accuracy on the other hand can be established: at the expense of integral accuracy an improvement of signal-to-noise ratio can be obtained. Using a smaller tip angle and a smaller pulse repetition period, the S/N improves [7].

For quantitative analysis purposes the integral values of the signals must be as correct as possible. Thus, qNMR experiments should be performed using a 90° tip angle which provokes a maximum of detectable magnetization in the xy-plane. Using this tip angle it is also required to apply a pulse repetition period of at least five times the \( T_1 \) relaxation time constant.

In order to determine the \( T_1 \) relaxation time exactly, the ‘inversion recovery’ pulse sequence (Fig. 3) can be used [1]. This sequence starts with a 180°-x-pulse inverting the spin populations. During relaxation the macroscopic magnetization aligned with the –z-axis shrinks towards the xy-plane and establishes to the +z-axis again. Applying a 90°-x-pulse the partially recovered bulk magnetization is rotated into the xy-plane and can be detected. Several experiments of this kind have to be performed using varying times \( \tau \) between the two pulses. As mentioned before, relaxation follows an exponential behaviour, the relationship between the magnetization in equilibrium \( M_0 \) and the magnetization at the time \( t \) \( M_t \) is given by (the factor 2 is needed because the magnetization is – \( M_0 \) at the beginning of the relaxation):

\[
M_t = M_0 \left(1 - 2e^{-\frac{\tau}{T_1}}\right)
\]

The same relation can be established for the intensities of the signals at each time \( t \) (taking \( A \) and \( B \) as constants):

\[
\text{Figure 3: The „Inversion-Recovery“ sequence}
\]
\begin{equation}
I_t = I_o - 2I_o e^{-\frac{t}{T_1}} = A + Be^{-\frac{t}{T_2}}
\end{equation}

Thus, there is a linear correlation between the natural logarithm of signal intensity and the time between the two pulses. The slope is $1/T_1$. The calculation can usually be done using software packages.

Transversal relaxation describes the decay of magnetization in the xy-plane. It influences the width of signals. In order to describe the width of NMR signals the width at half height, $b_{1/2}$ is used. While ideal NMR signals can be described by the Lorentzian lineshape, $b_{1/2}$ can also be written:

\begin{equation}
b_{1/2} = \frac{1}{\pi \cdot T_2^*}
\end{equation}

In equation 10 $T_2^*$ represents the observed transversal relaxation time which is composed of the real relaxation time $T_2$ and is influenced by field inhomogeneties.

Due to the relation between $T_1$ and $T_2$, $T_2$ can never be greater than $T_1$: the transversal magnetization – described as $T_2$ – might have already completely decayed while the longitudinal magnetization – described as $T_1$ – has not completely re-established. However, the magnetization along field direction can be maximal only in the case the transversal magnetization has completely decayed (for example see the T1-plot of fluvoxamine in Fig. 4. [8])

\textbf{Number of scans}

There are two possibilities to improve the signal-to-noise ratio. The first one is at the expense of integral accuracy as already explained. For quantitative measurements, of course, this way...
is not recommended. The other possibility for improving (S/N) is using a higher number of scans (n):

**Equation 11**

\[
\frac{(S/N)}{n} = \sqrt{n \cdot (S/N)}
\]

It has to be taken into account that the (S/N) only improves with the square root of the number of scans. Applying 64 instead of 16 scans doubles (S/N) while applying 112 instead of 64 improves with the factor 1.32 only. In both cases the same number of scans (48) was added and, thus, in both cases the total experimental time was extended in the same way (assuming a pulse repetition period of 5 seconds, there would be an extension of 3 minutes).

Usually the concentration of the analyte should be adopted in a way (in an order of 10\textsuperscript{-3} M, depending on the specific problem) that 128 to 256 scans provide an adequate signal-to-noise ratio.

d. **Processing the spectra**

Different methods can be applied in order to improve the spectra in the frequency domain upon transforming the spectra into the time domain (which is the FID) by Fourier Transformation (FT).

The simplest one is applying a window function on the FID prior to FT. After the FID has decayed almost to zero, noise can be detected only. Applying an *exponential* window function the natural decay of the FID is imitated and forces the detected FID finally to zero. Spectra manipulated in this way show an improved *sensitivity* because the influence of the noise at the end of the FID is minimized [1]. Unfortunately, enhancing the sensitivity as described worsens the resolution. In NMR software packages the parameter which has to be set is not the exponent of the desired function, it is the line broadening factor.

An enhancement of the resolution can be achieved by applying a *Lorentz-Gauss transformation* [9]. The transformation of the natural line shape (Lorentzian) into a gaussian line shape narrows the signals. Signals located close together will be better separated. The parameters of this function are usually set as a fraction of the total acquisition time.

**Integration of signals**

As described earlier, the principle of quantitative analysis of NMR spectra is based on the fact that the intensity of a signal corresponds to the number of nuclei evoking the signal. The natural line shape – the Lorentzian line – of NMR signal requires an integration of the area of at least the 20fold the width at half the height of the signal in order to cover at least 99% of the whole intensity [10].

**13C-satellites**

In ¹H-NMR spectra problems due to couplings of ¹H with ¹³C may occur. Due to the natural abundance of carbon-13 (98.9% ¹²C and 1.1% ¹³C) the coupling occurs only at 1.1% of the molecules in the sample. The two additional signals at each side are named “satellites”. The height of each is 0.56% of the height of the main signal and the distance of them to the main signal is due the ¹J(H,C)-coupling constant of 209 Hz (equal to 0.5 ppm in a 400 MHz spectrum, Fig. 4). For the quantitative evaluation it is important to integrate the satellites together with the main signal either for all signals which should be compared or for none of them.
On the other hand these satellites can be taken in account for quantifying impurities with a proportion less than one per cent. The size of the satellites is due the natural abundance of $^{13}$C-nuclei always 0.56 % of the main signal. Thus, the satellites can be used as reference signals for impurities in this order of magnitude.

**Rotation side bands**

Similar to $^{13}$C satellites rotation side bands might split the main signal. Rotation side bands are due the rotation of the sample tube. Rotation is done in order to improve field homogeneity along x and y axis. It is easy to discriminate between $^{13}$C satellites and rotation side bands when changing the rotation frequency: the distance between the side bands and the main signals changes according to the rotation frequency while it is unchanged for the satellites. For accurate measurements side bands should not be present.

Reasons for side bands are mostly due to bad field correction (shim) along the x- and y-axis. Applying good field correction (better shim) it is possible to eliminate the side bands completely. Another reason for the appearance of side bands can be the low quality of tubes. In order to have ideal field homogeneity the sample tubes must be of ideal straightness. Furthermore, they must have equal wall thickness along the whole length. Since the tubes can be damaged upon heating, NMR tubes should never go into an oven for drying.

Taken together, when considering the advices given above and shimming with sufficient accuracy it is possible to renounce the rotation of the sample tube.

**Phase correction**

The phase of the receiver coil does not necessarily match the bulk magnetization vectors. This leads to the necessity of phase correction after the Fourier transformation. Applying phase correction should result in pure absorption mode signals of the real part of the spectrum. If phase correction is done in an improper way – leading to no pure absorption mode signals – the integrals will be wrong. Thus, an exactly applied phase correction prior to integration is important.

**Baseline correction**

Even after correct phase correction distortions of the base line may still occur. These distortions can be corrected after Fourier transformation by applying a proper baseline correction. Usually, a polyonomic function is added to the spectrum in order to balance the baseline distortions. When two similar signals will be compared, in most cases a global baseline correction (i.e. a correction taking the whole spectrum into consideration) leads to the desired aim. In the case signals of very different size or signals which are located close to each other have to be integrated, it is recommended to restrict the baseline correction to the desired region only. Furthermore, if the two signals are far apart from each other it may be better to perform baseline correction independently in the two regions.
Correction of the integrals

Finally, even a correction of the integrals themselves could be applied. This can be done in order to correct distortions (like fluctuations or noise) in the baseline and at the edge of the integrals (like convoluted signals). Such errors show integration lines which do not run out straightly. There are different correction functions available for balance. The software package XWIN-NMR (Bruker Biospin) provides the two functions “SLOPE” and “BIAS”. The BIAS-function adds a constant function to the spectrum resulting in an influence on the baseline in vertical direction. The SLOPE-function adds a linear function to the spectrum resulting in an influence on the slope of the spectrum [11].

Poorly separated signals can be better separated applying a procedure known as “deconvolution”. This mathematical operation improves the separation by dividing the overlapping signals into its components. The experimentally derived signals are adapted to either the optimal theoretical line shape of a Lorentzian line or the smaller Gaussian line or a mixture of both [11]. Thus, small signals aside to larger ones can be better integrated.

However, when applying such corrections it might happen that those integral values are more influenced by the desired result than by the experimental data. Furthermore, such corrections must be often performed manually resulting in even greater influence of the operator.

Special problems concerning $^{13}$C-spectra

In chapter c the dependence of the intensity of a signal on the relaxation of the specific nuclei was discussed. When measuring hydrogens (with a $T_1$-relaxation time of about one second) a pulse repetition period of ca. 5 to 7 seconds is usually enough to make sure that all hydrogens of the maximal intensity can be detected.

However, carbon-13 shows longer $T_1$-relaxation times, usually in the order of 10 to 30 seconds. Quaternary carbons may even have $T_1$-times longer than 300 seconds. In order to have a total relaxation before the next pulse, pulse repetition periods of up to 2.5 minutes and more may be necessary. Taking at least 512 to 1024 scans into consideration (because of the low natural abundance and small gyro magnetic ratio), this gives a total experimental time of more than 20 to 40 hours. If the time between two pulses is only a few seconds (in routine spectra 1 to 2 seconds are usually applied) the nuclei are only partly relaxed prior to the next pulse. The detected intensities are therefore for nuclei with longer $T_1$-times relatively smaller in comparison to those with smaller $T_1$-times. Such spectra cannot be used for quantitative purposes.

Another problem arises from the nuclear Overhauser effect (NOE). Excitation of a nucleus influences the relaxation behavior of the nuclei in the neighborhood [12]. In order to simplify spectra the $^{13}$C-spectra are usually measured under “decoupling” conditions, i.e. the couplings between $^1$H and $^{13}$C are eliminated and, thus, the $^{13}$Cs appear as singlets. Under this conditions a heteronuclear NOE enhances the $^{13}$C-signal intensities for signals which belong to C atoms carrying one or more hydrogens. If $^{13}$C-spectra will be evaluated quantitatively the NOEs must be suppressed (e.g. by adding paramagnetic ions or applying special pulse sequences). In decoupled spectra only similar carbons of enantiomers can be compared with sufficient accuracy.
Part II: NMR Spectroscopy in Drug Analysis - Applications

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Content:
a) Introductory remarks
b) NMR spectroscopy in International Pharmacopoeias
c) Evaluation of impurities resulting from the synthesis pathways
d) Evaluation of impurities from decomposition
e) Hyphenation of HPLC and NMR
f) Chemometrics in drug analysis
g) Conclusion
h) References

a) Introductory Remarks

$^1$H and $^{13}$C NMR spectroscopy is famous for the elucidation of structures of newly synthesized compounds, natural products and semi-synthesized compounds. Utilizing COSY, HMBC, HSQC, TOCSY, NOESY, and ROESY experiments the constitution, configuration and conformation of small molecules as well as polymers, such as peptides, sugars, and nucleotides, can be elucidated. $^{19}$F, $^{15}$N, and $^{31}$P were additionally employed in structure determination.

However, NMR spectroscopy can also be used

- to identify a drug
- to evaluate the level of impurities and to elucidate their structure
- to observe the course of decomposition
- to evaluate the content of residual solvents
- to determine the isomeric composition:
  \[ \rightarrow \text{the ratio of diastereomers} \]
  \[ \rightarrow \text{the enantiomeric excess (ee) by means of chiral additive} \]

For some years the term quantitative NMR spectroscopy and the abbreviation qNMR has been frequently used indicating the growing importance of NMR spectroscopy in the field of quantitative analysis.

b. NMR Spectroscopy in International Pharmacopoeias

As a consequence of the aforementioned remarks International Pharmacopoeias make increasingly use of NMR spectroscopy for identification purposes and quantitative NMR spectroscopy (qNMR) for evaluation of composition of polymers and impurities in drugs.

The European Pharmacopoeia (PhEur 4th edition) describes the method of NMR spectroscopy only in principle using continuous wave (no longer in use nowadays) and pulsed spectrometry. Applying this monograph allows a qualitative analysis only. Hence, most of the applications are found in the identity section (see Table 1). In contrast, beside the description of the physical background of NMR spectroscopy, the apparatus, the general method and the interpretation of a spectrum the United States Pharmacopoeia 26 (2003) gives detailed information about procedures to be applied for qualitative and quantitative purposes. In the section for qualitative analysis 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 is given.
Consequently, the NMR spectroscopy is used in the USP for identification of the drugs and their impurities and for quantification purposes.

Table 1: Examples of NMR applications in various pharmacopoeiae

<table>
<thead>
<tr>
<th>Identity</th>
<th>PhEur 4.7</th>
<th>Buserelin, Goserelin, Tobramycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP 1998</td>
<td>Hydrocortisone Sodium Phosphate</td>
<td></td>
</tr>
<tr>
<td>USP26</td>
<td>Amylnitrite isomers</td>
<td></td>
</tr>
<tr>
<td>PhEur 4.7</td>
<td>Heparins low-molecular-mass, Haemophilus Type b conjugate vaccine,</td>
<td></td>
</tr>
<tr>
<td>Tests</td>
<td>PhEur 4.7</td>
<td>Poloxamer: ratio of oxypropylene/oxyethylene</td>
</tr>
<tr>
<td>USP26</td>
<td>Hydroxypropylbetadex: molar substitution</td>
<td></td>
</tr>
<tr>
<td>Assay</td>
<td>USP26</td>
<td>Orphenadrine citrate: meta/para isomer</td>
</tr>
</tbody>
</table>

**Identity**

The agonistic nonapeptide analogues of human gonadotrophin-releasing hormones (GnRH) gonadorelin, i.e. buserelin and goserelin, are identified by a simple $^1$H NMR spectroscopy (see buserelin spectrum in Fig. 1).

![Fig. 1: $^1$H-spectrum of buserelin acetat in D$_2$O/acetic acid-d$_4$](image)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse repetition period</td>
<td>28 sec</td>
</tr>
<tr>
<td>Spectral width</td>
<td>4401 Hz</td>
</tr>
<tr>
<td>Digital resolution</td>
<td>0.08 Hz/pt</td>
</tr>
<tr>
<td>Temperature</td>
<td>300 K</td>
</tr>
<tr>
<td>Solution</td>
<td>3.2 mg buserelin acetat in 800 µl D$_2$O/acetic acid-d$_4$ (80/20)</td>
</tr>
<tr>
<td>Referencing</td>
<td>centre of the solvent peak: 4.79 ppm</td>
</tr>
</tbody>
</table>
Due to heavy signal overlapping, the spectra of *tobramycin* and *hydrocortisonesodium phosphate* are very complicated (cf. tobramycin spectrum Fig. 2) [16] and could be assigned only by means of 2D experiments. Applying homo- and heteronuclear shift correlations the $^1$H and $^{13}$C NMR spectra of tobramycin at varying pH values could be fully assigned [13]. However, the $^1$H NMR spectrum demanded by the PhEur 4.5 for identification is used in the same manner as IR spectra. Hence a sort of pattern recognition is applied.

![Fig. 2: Structural formula and $^1$H NMR spectrum (400 MHz, D$_2$O) of tobramycin](image)

The USP XXVI identifies *amyl nitrite* being a mixture of mainly isoamyl nitrite ((CH$_3$)$_2$CH-CH$_2$-CH$_2$-O-N=O) beside other isomers by means of a $^1$H NMR spectrum. Among other peaks the NMR spectrum is characterized by a doublet at ~ 1 ppm and a multiplet centered at ~ 4.8 ppm representing the methyl and methylene hydrogens in $\alpha$-position to the nitrite group. In the assay the absolute method using benzyl benzoate as an internal standard was applied. The quantity of amyl nitrite is calculated from the signal area of the $\alpha$-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 monograph “heparins of low-molecular-mass” employs $^{13}$C NMR spectroscopy, 75 MHz, for identification of the various heparins, e.g. dalteparin, enoxaparin, parnaparin, tinzaparin, certoparin and nadroparin. Due to the varying ways of depolymerization of heparin the heparins of low-molecular-mass differ in the structure of the non-reducing end (2-O-sulfo-α-L-idopyranosuronic acid or 4-enopyranose uronate) and the reducing end (6-sulfo-2,5-anhydro-D-mannose, the corresponding mannitol or 2-sulfamoyl-2-deoxy-D-glucose-6-sulfate) as well as in the molecular weight.

As a representative the structural formula and the $^{13}$C NMR spectrum of tinzaparin is given below [14]. According to Neville [15] the spectra of the various heparins differ especially in the region between δ = 80 and 92 ppm showing signals corresponding to C3 (~ 82 ppm), C2 (~ 86 ppm), C4 (~ 87 ppm) and C1 (~ 90 ppm) of the anhydromannose moiety and the hydrated aldehyde group (~ 90 ppm). Using the pattern of $^{13}$C signals the various heparins of low mass can be recognized. Details are reviewed in [16].

Correspondingly chondroitin sulfate [17, 18] as well as bovine mucosal heparin and porcine mucosal heparin were characterized by $^1$H and $^{13}$C NMR spectra [19, 20, 21, 22]. The manufacturer of dextran sulfate was determined by comparison of the pattern of 300 MHz $^1$H NMR spectra [23, 24].

For some years glycoconjugate vaccines are increasingly used e.g. against Haemophilus Influenzae, meningococcal Group C disease and many pneumococcal serotypes. These conjugate vaccines consist of saccharide and protein components which can be characterized by $^1$H NMR spectroscopy.
NMR spectroscopy. Identity tests were validated to control the identity and content of acetyl groups of capsular polysaccharides of *Neisseria meningitis* in vaccine manufacture [25,26], of *Haemophilus influenzae* Type b in bulk ware [27], and of *Salmonella typhi* Vi [28]. In addition it is possible to determine the size of the capsular oligosaccharide by means of NMR spectroscopy [29]. Jones and Lemercier were also able to validate NMR identity tests (500 MHz) with regard to specificity between structurally related polysaccharides, reproducibility between batches from the same manufacturer and identical material from different manufacturer, and robustness to variations in experimental conditions [30]. Moreover, Ravenscroft was able to track the preparation of semi-synthetic glycoconjugate vaccines by means of NMR spectroscopy [31]. Consequently the monograph of *Haemophilus type b conjugate vaccine* employs $^1$H NMR spectroscopy for identification purposes as an alternative to immunochimical methods.

**Tests**

Most of the impurities are originated from the synthesis pathways, i.e. starting products, intermediates and synthesis by-products, and products of decomposition of the drug. Both are evaluated as related substances mostly by means of high performance liquid chromatography (HPLC). In addition, reagents used during the course of the synthesis pathway, heavy metals and residual solvents can occur as impurities. NMR spectroscopy can be employed for quantification of related substances and residual solvents.

The molar substitution of hydroxypropylbetadex, known as hydroxypropyl-β-cyclodextrin, being a poly(hydroxypropyl)ether of β-cyclodextrin of random substitution in 2, 3 and 6 position is characterized by $^1$H NMR spectroscopy. Since no quantification procedure is described in PhEur 4.7, details of recording and processing the spectrum are given in the monograph. The spectrum shown below is produced considering the monograph. Frequency, gain, digital resolution, sample rotation, probe tuning, receiver gain etc are given in the parameter box. The molar substitution MS can be obtained by calculating the ratio of the area of the methyl groups of the signal of the hydroxypropyl group at $\delta = 1.2$ ppm and three times the area of the signal of the glycosidic hydrogen at $\delta = 5.4$ ppm. The MS has to be in the range of 0.40 and 1.50 and within 10% of the value stated on the label.

$$MS = \frac{A_1}{3 \cdot A_2} = \frac{300}{3 \cdot 91.10} = 1.10$$

$A_1$ = area of the signal due to the 3 protons of the methyl groups, which are part of the hydroxypropyl groups (1.2 ppm)  
$A_2$ = area of the signals due to the glycosidic protons (5-5.4 ppm)  
Degree of substitution = 7 · MS = 7.7

The sample of hydroxypropylbetadex, evaluated here, is in agreement with the requirements of the PhEur.
Bruker Avance 400 MHz operating at 400.13 MHz equipped with BBO-head for (1H-channel, X-channel). The data processing was performed using BRUKER X-WIN NMR 3.5 software under Microsoft Windows.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse repetition period</td>
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<td>Spectral width</td>
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<tr>
<td>Digital resolution</td>
<td>0.14 Hz/pt</td>
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<tr>
<td>Temperature</td>
<td>300 K</td>
</tr>
<tr>
<td>1H-Flip angle</td>
<td>90°</td>
</tr>
<tr>
<td>Transmitter offset</td>
<td>3.1 ppm</td>
</tr>
<tr>
<td>Rotation</td>
<td>no</td>
</tr>
<tr>
<td>Solution</td>
<td>20 mg hydroxypropylbetadex in 750µl D₂O</td>
</tr>
<tr>
<td>Referencing</td>
<td>centre of the solvent peak: 4.79 ppm</td>
</tr>
</tbody>
</table>

A corresponding method for determination of molar substitution and hydroxypropyl content in hydroxypropyl cellulose was developed and proved to be robust by Andersson et al. [32]. The hydroxypropyl content can also be determine with [33] and without [34] acetylation prior to NMR measurement. In turn, the determination of the degree of deacetylation of chitosan, a natural polysaccharide obtained by partial deacetylation of chitin, could be validated by 1H NMR spectroscopy [35].

According to the USP 26 (2003) and PhEur 4.7 the content of oxypropylene in poloxamer, an ethylene oxide/propylene oxide block copolymer (Pluronic F 68®) can be determined by means of a 1H NMR spectrum using the integrals of CH₂O/CHO signals between δ = 3.2 and 3.8 ppm and the CH₃ signal appearing at δ ~ 1.3 ppm (see Fig.5).

The percentage can be calculated from the following equations:
bruker avance 400 MHz operating at 400.13 MHz equipped with BBO-head for (1H-channel, X-channel). The data processing was performed using BRUKER X-WIN NMR 3.5 software under Micro-
soft Windows.
Pulse repetition period 10 sec Number of scans 16
Spectral width 4789 Hz Transmitter offset 2 ppm
Digital resolution 0.16 Hz/pt 1H-Flip angle 90°
Temperature 300 K Rotation no
Solution 50 mg Pluronic in 700µl CDCl3
Referencing centre of the solvent peak: 7.26 ppm

Fig. 5 1H NMR spectrum (400 MHz, CDCl3) of poloxamer

\[
% \text{oxyethylene} = \frac{3000 \cdot \alpha}{33 \cdot \alpha + 58} \quad \text{with } \alpha = \frac{\text{area (CH}_2\text{O/CHO)}}{\text{area (CH}_3)}
\]

with \( \alpha = \frac{859.71}{108.55} = 7.92 \) \% oxyethylene = 81.84% Applying the integrals found for the sample of pluronic measured here the content of oxyethylene was found to amount to 81.8%. This is in agreement with the requirements of the USP and the PhEur which are 75 to 85 %.

The USP XXVI makes use of the relative method of quantification in order to determine the content of \( m\)- and \( p\)-methylphenyl isomer in the \( o\)-methylphenyl substituted orphenadrine citrate. The signals of the benzylic methine hydrogen atoms are well separated. Hence, from the areas of the signal of the \( m/p\)-methyl substituted compound appearing at about 5.23 ppm and of the orphenadrine signal appearing at 5.47, the content of the impurities can be determined.
Beside the drug substances the chemical reference substances (CRS) have to be characterized with regard to identity and purity. Beside high performance liquid chromatography NMR spectroscopy is often used, e.g. in the case of the malathion impurity B which can be evaluated by means of $^{31}$P NMR spectroscopy.

**Assay**

Beside the assay of amyl nitrite no assay utilizing NMR spectroscopy is described in the International Pharmacopoeias yet.

**Perspective**

The aforementioned examples clearly demonstrate the suitability of NMR spectroscopy for drug analysis purposes. Thus, further applications can be expected in the Ph. Eur. and national European pharmacopoeias as well as the JP and the USP in the near future. The high potential of NMR spectroscopy in terms of identification and quantification of drugs and evaluation of their impurities resulting from synthesis pathways or degradation will be demonstrated in the following sections.

**c. Evaluation of impurities resulting from the synthesis pathways**

Most of the related substances are originated from the synthesis pathways. Normally the synthesis pathway is known and logically, the impurities are known and can be easily evaluated by means of HPLC. In the case different or new synthesis pathways are used for production of a drug, new related substances can occur which cannot be properly quantified by the HPLC method described in the international pharmacopoeias. In this context NMR spectroscopy can help to find, identify and often quantify the “new” impurities. Moreover, NMR spectroscopy is a powerful tool for impurity profiling and tracking the production ways from manufacturer to different brokers and finally to the drug manufacturer (cf. gentamicin, Chapt. III and [36, 37]). Drugs are licensed by competent authorities with regard to a defined synthesis pathway. Thus, drugs produced on a different way has to be considered as counterfeit drugs [38, 39]. Due to an increasing number of counterfeit drugs NMR spectroscopy is of growing importance in the field of drug analysis and fake.

In the following paragraph representative examples of quantitative NMR spectroscopy will be given

- where the NMR methods have not yet found the way into the international pharmacopoeias
- where NMR spectroscopy has helped to identify unknown impurities of known drugs produced by a known pathway.
- where NMR spectroscopy is a good orthogonal method to HPLC.

Lankhorst et al. [40] were able to evaluate 0.1 % dihydrolovastatin in *lovastatin* by means of a 600 MHz $^1$H NMR spectrum. A 400 MHz spectrum is shown below (Fig. 6)
Bruker Avance 400 MHz operating at 400.13 MHz equipped with BBO-head for (H-channel, X-channel). The data processing was performed using BRUKER X-WIN NMR 3.5 software under Microsoft Windows.

<table>
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<th>Value</th>
</tr>
</thead>
<tbody>
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<td>Pulse repetition period</td>
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</tr>
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<td>Spectral width</td>
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</tr>
<tr>
<td>Digital resolution</td>
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</tr>
<tr>
<td>Temperature</td>
<td>300 K</td>
</tr>
<tr>
<td>Transmitter offset</td>
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</tr>
<tr>
<td>1H-Flip angle</td>
<td>30°</td>
</tr>
<tr>
<td>Rotation</td>
<td>Yes</td>
</tr>
<tr>
<td>Solution</td>
<td>20 mg lovastatin in 750 µl CDCl₃</td>
</tr>
<tr>
<td>Referencing</td>
<td>centre of the solvent peak: 7.26 ppm</td>
</tr>
</tbody>
</table>

However, the relevant signals of the octahydronaphthalene ring system of dihydrolovastatin used for quantification between $\delta = 5.0$ and 6.2 ppm interfere with the $^{13}$C satellites of hexahydronaphthalene moiety of lovastatin when using less than 600 MHz field strength [16]. Thus, the method was not introduced in the USP. However, when applying $^{13}$C decoupling the method works with a 400 MHz spectrometer.
Dequalinium chloride is usually obtained by conversion of 4-aminoquinaldine with 1,10-dihalodecane. The impurity pattern of dequalinium chloride varies depending on synthesis conditions. The formerly DAB9 restricted the content of non-quaternary amines only. However, apart from residual aminoquinaldine (also a product of photolysis [41]) and a product consisting of three quinaldine molecules connected with two decanes, as well as another over-alkylation products, which were found by HPLC; the 400 MHz $^1$H NMR analysis exhibits a further impurity, 4-amino-1-[10-[(2-methylquinolin-4-yl)amino]decyl]-2-methylquinolinium chloride [42], the later quaternary compound exhibiting high activity against trypanosomiasis and being used to treat cattle. Thus, this impurity has to be quantified and limited in a pharmacopoeia monograph.

![Dequalinium chloride](image)

Fig. 7: $^1$H NMR spectrum of dequalinium chloride (400 MHz, $D_2$O)
Bruker Avance 400 MHz operating at 400.13 MHz equipped with BBI-head (inverse broadband observer for heteronucleus, inner coil tuned to $^1$H). The data processing was performed using BRUKER X-WIN NMR 3.5 software under Microsoft Windows XP.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse repetition period</td>
<td>20 sec</td>
</tr>
<tr>
<td>Number of scans</td>
<td>128</td>
</tr>
<tr>
<td>Spectral width</td>
<td>3592 Hz</td>
</tr>
<tr>
<td>Transmitter offset</td>
<td>4.5 ppm</td>
</tr>
<tr>
<td>Digital resolution</td>
<td>0.13 Hz/pt</td>
</tr>
<tr>
<td>$^1$H-Flip angle</td>
<td>90°</td>
</tr>
<tr>
<td>Temperature</td>
<td>300 K</td>
</tr>
<tr>
<td>Rotation</td>
<td>No</td>
</tr>
<tr>
<td>Solution</td>
<td>Dequalinium chloride saturated (&lt;1mg) in 700µl D₂O</td>
</tr>
<tr>
<td>Referencing</td>
<td>centre of the solvent peak: 4.70 ppm</td>
</tr>
</tbody>
</table>

Inspection of the $^1$H NMR of the methyl region (2.6 to 2.9 ppm, Fig: 7 right insert) revealed distinct signals for at least one of the methyl groups of each aforementioned compound. Integration of these methyl signals allows the determination of the (molar or weight) composition of the samples. Some commercially available samples were found to consist of 75.7 % dequalinium chloride, 18.2 % overalkylation impurity, 5.8 % of the new impurity and 0.3 % 4-aminoquinaldine [42,43]. These results prompted the revision of the pharmacopoeia monograph on the one hand in order to additionally limit the quaternary impurities and the improvement of the synthesis on the other hand. Nowadays batches provided by Ravensberg GmbH consist of some 96 % of dequalinium chloride, less than 2 % of the overalkylation product (a), some 1.5 % of 4-amino-1-[10-[(2-methylquinolin-4-yl)amino]decyl]-2-methylquinolinium chloride (b) and less than 0.5 % of aminoquinaldine (c) and, thus, fulfil the requirements of the PhEur 4 (see Fig. 8 upper expansions).

The $^{13}$C NMR satellites having a natural abundance of the $^{13}$C-isotope of 0.56% intensity of the main signal are labelled (see Fig. 8); they can be utilized to estimate the percentage of the three impurities.

Fig. 8 Expansions of the $^1$H NMR spectra of two different lots of dequalinium chloride
Vitamin E, +α-tocopherol as free phenol, acetate or succinate ester, always consists of small amounts of β-, γ-, and/or δ-tocopherol. Owing to partially poor resolution and the requirement of authentic reference standard, HPLC and gas chromatography analysis turned out to be not satisfactory. Baker and Myers [44] could prove that both 1H and 13C NMR are suitable to quantify the composition of vitamin E by integration of corresponding signals.

Lacroix et al. [45] was able to determine more impurities of fenofibrate by means of a 400 MHz spectrometer than were observed with HPLC. Fenofibrate raw materials were found to contain a huge number (> 20) of impurities. With HPLC using a RP 18 symmetry ODS column, a mobile phase consisting of acetonitrile/H2O/CF3COOH (700:300:1) and a UV detector set at 280 nm, about 11 known and unknown impurities could be quantified at a trace level of 0.05 to 0.1%. With the NMR method 12 known and several unknown impurities could be detected. Additionally, upon increasing the number of scans the sensitivity of the NMR method could be enhanced to detect some impurities which were not detectable by HPLC.

Interestingly two different manufacturers of sulfasalazine supplied two impurities of different structure which co-elute in the HPL chromatogram [46]. Since the mass and NMR spectra of these impurities were identical the structure was likely to be the same. The structure could be elucidated by means of 1H and 13C NMR spectra in addition of homonuclear and heteronuclear two-dimensional experiments, i.e. COSY and HMBC to be the same.

Innumerable examples of NMR spectroscopy being involved in the elucidation of the structure of impurities are reported in the literature, e.g. the isomers of lisinopril and enalapril and their diketopiperazine degradation products [47, 48], bromination side products of the bromocryptine production by fermentation of claviceps purpurea [48], and cimitidine oxidation products [48].

d) Evaluation of impurities from decomposition

Due to the existence of ester, amide or lactams many drugs tend to undergo hydrolysis. NMR spectroscopy is not only able to identify and quantify the amount of decomposition products, but it is also appropriate to observe the pathway of the reaction, to detect intermediates and to follow the kinetics. Since a couple of water suppression methods are available in the software packages of each spectrometer, e.g WATR (= water Attenuation by T2 Relaxation) or WATERGATE (water suppression by gradient-tailored excitation), the hydrolysis of drugs can be observed in aqueous (physiological) media. Applying the WATR suppression method Waigh and coworker were able to determine the rate constants of the ester hydrolysis of atropine, acetylcholine, carbachol and procaine at temperatures in a range of 45 to 90 °C and at various pH values applying the Arrhenius equation [49]. The obtained results were in good agreement with data reported in the literature.

The ester propantheline, a (2-hydroxyethyl)diisopropylmethylammoniumbromide of xanthene carboxylate, tends to hydrolyze in commercial tablets when they are not stored in tightly sealed containers below 30 °C and protected from moisture. Hanna and Lau-Cam were able to determine 0.1 to 0.9 per cent free xanthanoic acid in presence 97.1 to 99.8 per cent of the drug by 1H NMR spectroscopy. In addition, they observed the course of hydrolysis at pH 2.0, 7.0 and 11.0 in aqueous solution after extraction with CDCl3 [50].

Benzodiazepine compounds consist of groups, i.e. the ester function and the imide group, which can be hydrolyzed. In physiological media the imide hydrolyzes to give the ring-opened benzophenone whose amide function decomposes to glycine and an anthranil acid derivative. Dawson and coworkers studied the reaction of flurazepam by applying 1H, 13C and 19F NMR spectroscopy in different media and at various temperatures [51, 52].
Popovic et al. investigated the deprotonation reaction of oxazepam, lorazepam, nitrazepam and clonazepam and were able to determine the acidity constant as well as the equilibrium constants [53].

\[
\begin{align*}
\text{HN}^+(\text{C}_2\text{H}_5)_2 & \quad + \text{H}_2\text{O} \quad \text{H}^+ \\
\text{OH} & \quad \text{NH}_3^+ \\
\end{align*}
\]

Fig. 9: Representative hydrolysis pathway of a benzodiazepine

The chemistry of β-lactam antibiotics can be monitored by $^1\text{H}$ and $^{13}\text{C}$ NMR spectra in different media. Since the spectra are rather simple the epimerisations and the decompositions of penicillins resulting in penicilloic acid and related products can be easily observed. Details were already reviewed by Branch et al. [54, 55]. In addition, the conversion of 6-aminopenicillanic acid to 8-hydroxyenicillic acid accelerated by carbonate can be followed by $^1\text{H}$ NMR using the signal of the methyl hydrogens in position 2 [56]. The alkaline hydrolysis of cefotaxime was also elucidated and the kinetic of the reaction observed by means of $^1\text{H}$ NMR spectroscopy [57]. Imming et al. found the hydrolysis kinetics of lactams strongly depending on the ring size. γ-Butyrolactam hydrolyzes considerably slower than β-propiolactam, and δ-valerolactam and β-propiolactam had the same reactivity [58]. β-Lactam antibiotics were more reactive than both which explains the rate of reaction with the serine residue of the transpeptidase.

Using the WATR technique method, signals in the region of $\delta = 4-5 \text{ ppm}$ can be easily observed. Thus, the kinetics of hydrolysis of neostigmine bromide in weak acid aqueous solution can be monitored [59].

The decomposition process of bispyridinium aldoximes TMB-4, an acetylcholine esterase reactivator, were observed in D$_2$O solutions of different pD values and various temperatures [60]. Whereas the aldoximes turned out to be rather stable, corresponding ether and cyano derivatives were found to convert to the pyridone in alkaline medium at high temperatures.

The process of photodecomposition of the calcium channel blocker nifedipine to the 4-(2′-nitrophenyl)pyridine compound can be observed by $^1\text{H}$ NMR spectroscopy [61] monitoring the singlets between 2.3 and 2.6 ppm representing the methyl groups and 3.3 and 3.7 which belong to the methoxy groups and are well separated for each compound.

Erythromycin A and clarithromycin belong to the class of macrolide antibiotics being in clinical use for a long time. Erythromycin is known to decompose in acidic media via a hemiketal and enolethers to give an anhydride structure. In comparison, clarithromycin is more stable due to the fact that the alcohol group being involved on the formation of the ketal in erythromycin, is replaced with an methyl ether. However, recent NMR studies [62] employing two-dimensional diffusion-ordered (DOSY) spectra revealed the decomposition pathway of Clarithromycin in acidic aqueous solution, starting off with the cleavage of the cladinose sugar, whose signals show a much fast diffusion than the rest of the molecule. After a longer time...
the remaining macrolide skeleton tend to form an enolether whose double bond shows isomer-
ism. Erthromycin B, a natural component of erythromycin, showed a similar decomposition.

The cancerostatic oxazaphosphorine drugs, such as ifosfamide, are known to be unstable. A
$^{1}$H, $^{13}$C, and $^{31}$P NMR spectroscopy study [63] shed some light into the complex degradation
pathways. In the first acidic hydrolysis the endocyclic P-N bond is cleaved. In the second
stage of hydrolysis the remaining former exocyclic P-N bond is broken and subsequently a 2-
hydroxyoxazaphosphorine ring formed which releases chloroethylamine. The final step leads
to a phosphoric acid monooester.

Fluorouracil which is administered in high doses via infusion pumps and injections tend to
hydrolyze in this media. Since the degradation products, i.e. fluoracetaldehyde, fluoromalonic
acid semialdehyde and fluoroacetate, involve the risk of cardiotoxicity, is important to know
the degree of degradation of a given injection solution. $^{19}$F NMR spectroscopy has been
proved to be a suitable method [64, 65].

e) **Hyphenation of HPLC and NMR**

Often, the hyphenation of HPLC and NMR can help to find, identify and quantify impurities
of drugs or the composition of a complex drug. E.g. Albert and coworker [66] were able to
separate and assign the configuration of vitamin A cis/trans-isomers by means of an online
HPLC-NMR separation by employing a cyanopropyl-modified silica gel column and hexane
as a mobile phase. The structure of the related carotenoids, lutein and zeaxanthin isolated
from ox retina, could be completely elucidated by means of a COSY stopped-flow NMR
spectrum of zeoxanthin [67].

A representative instrument for shown below (see Fig. 10)

![Fig. 10: Bruker Biospin LC-NMR-coupling](image)

Details of the LC-NMR coupling technique, as well as hyphenation
of NMR with other chromatographic methods such as gel
permeation chromatography, and solid phase extraction on the one
hand and other spectroscopic methods like mass spectrometry
as well as applications in drug analysis, drug metabolism, natural
drug analysis, environmental analysis were recently summa-
rized by Albert [68].

f. **Chemometrics in drug analysis**

In recent years NMR spectroscopy is increasingly used in the characterization of biological
samples, e.g. metabonomics [69], mixtures of compounds, e.g. drugs, and kinetic studies. The
large multispectra datasets obtained from automatic sample preparation and NMR acquisition need an automated data analysis.

First, stable operating conditions, regarding the field homogeneity (i.e. shim), sample temperature, pH values and composition of the background matrix of the sample, have to be ensured because these parameters influence the chemical shift sensitively. In addition, suppression techniques have often to be applied to get rid of water signals or signals of other solvents, e.g. water and ethanol in beverages such as beer, which might interfere with signals of interest [70]. Second, a principle component analysis (PCA) can help to solve the problems related with phase, frequency shifts, area and linewidth [71, 72, 73, 74], which has to be done prior to pattern recognition and classification techniques. However, the peak alignment necessary for the automated comparison of spectra is still in the focus of chemometric research. Beside the unfavorable bucketing resulting in a loss of resolution and information genetic algorithms are also applied for peak alignment [75, 76].

Finally, the complex spectra obtained may lead to difficulties sorting out the important signals. Thus, multivariate analysis methods, e.g. PCA, partial least squares (PLS) or nonlinear techniques such as neural networks, can be applied for interpretation of the samples.

Forshed et al. tried to determine the content of the impurity aminophenol in paracetamol (acetaminophen) although the signals of interest of both components were overlapping [77]. All NMR parameters including the different methods of preprocessing the NMR data were carefully examined. Zero-filling and multiplication by a negative exponential function (line broadening) of the free induced decay were applied prior to the Fourier transformation. These data were automatically phased and shifts adjusted by means of a genetic algorithm. The baseline correction was performed by multiplicative scatter correction and the spectra were compressed using a wavelets and sequential zeroing of weights variable selection. Neural networks were employed for variable selection and building of the calibration model. Using the model 15 ppm of aminophenol could be evaluated.

Artificial neural networks were used to classify and quantify binary mixtures of alditol compounds [78]. Multivariate analysis using the PLS discriminant analysis or PCA can be employed to classify components of e.g. a saponin extract of Quillaja saponaria into structurally related groups [79], to differentiate between bottom- and top-fermented beers and between fruit juices of different geographical origin and to detect additives or supplements based on other fruit types [70]; the latter examples were studied using the AMIX software of Bruker Biospin. PCA maps can be used to visualize clustering patterns of urine associated with metabolic response e.g. to the nephrotoxin bromoethanamine [80]. Furthermore, Nicholson and his group perform NMR-based metabonomic toxicity classification by means of PCA of metabolite profiles found in urine [81, 82, 83, 84].

**d) Conclusion**

The advantages of quantitative NMR spectroscopy over HPLC techniques can be summarized as follows:

- Integration of signals is more precise and accurate than HPLC analysis,
- often, no isolation of the impurity necessary,
- no expensive chemical reference substances necessary,
- additional structural information of impurities, isomers etc.,
• NMR can be quicker (no equilibration time), easy to perform and more specific ⇒ high reproducibility.

However, a quantification of components is only possible in the case the signals of the compounds are separated and due to less sensitivity the sample size and the concentrations needed are often higher in comparison to liquid chromatography hyphenated with UV spectroscopy or mass spectrometry. Nevertheless, there are some possibilities to overcome the sensitivity problem:

• Using spectrometer of high field strength (> 400 MHz).
• Gradient shimming techniques increases the quality of spectra.
• Inverse and cryo probes increase the signal-to-noise ratio by > 5.
• Maximizing the concentration of a sample and minimizing the solvent/analyte ratio (microcoil technology, e.g. Varian nano.nmr probe or Bruker MicroCryoProbes, 10^{12}).
• The higher the number of scan (accumulation) the higher the S/N ratio.

Since sensitivity is not only needed for quantitative purposes but also for protein NMR, in drug discovery and many other areas, the development of cryo and nano probes has made great progress in the last years, the problem of sensitivity is more and more disappearing. Limits of detection and quantification in the nanomolar range are often easy to reach.
References


