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Review

Quantitative NMR spectroscopy—Applications in drug analysis

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Abstract

NMR spectroscopy being a primary ratio method of measurement is highly suitable to evaluate the quality of drugs. NMR spectroscopy can be used for the identification of a drug substance, the identification and quantification of impurities arising from the synthesis pathway and degradation, or residual solvents as well as the determination of the content in the assay. This review gives an overview of the application of quantitative NMR spectroscopy in International Pharmacopoeias and for licensing purposes.

Keywords: qNMR; Drugs; Identification; Quantification; Impurities

Contents

1. Introduction

NMR spectroscopy can be considered as a *primary ratio method of measurement* [1,2] being characterized by the fact that the ratio of substances can be determined directly from the physical context of the measurement *without* referencing to another substance. The absolute amount of substances can be determined by using simple reference substances. Further primary methods of measurement are, e.g. coulometry, gravimetry or titrimetry.

Since the intensity I_A of a signal is directly proportional to the number of nuclei N evoking the signal, the intensities of NMR signals (=areas under specific signals) can be taken for quantitative investigations. The linear relationship between the signal intensity I and the number of observed nuclei (in case of single pulse excitation) is given by:

$$I = c_S \times N$$

The proportionality constant c_S results from parameters of the spectrometer, termed "spectrometer constant", and the sample.

The precision of the integrals determines the accuracy of quantification depending (a) on the noise level of the spectrum, (b) on the line shape, (c) quality of shimming, (d) choice of the window function and (e) phase-, baseline- and drift cor-

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rections [3]. Considering these parameters the qNMR methods can be validated.

However, NMR spectroscopy suffers from the disadvantage of low sensitivity based on the small difference of the spin populations in either α- or β-state. The population difference is given by the Boltzmann equation

$$\frac{N_\alpha}{N_\beta} = \exp\left(\frac{-\gamma h B_o}{2\pi k T}\right)$$

Since the population difference $N_\alpha - N_\beta$ depends on the magnetic field strength at a field of 60 MHz the population excess is only 0.001%, and at a field of 600 MHz the population excess is 0.01%, resulting in a LOD in a nanomolar range. Beside the high field strength, the low sensitivity can be overcome by

1. the increase of the number of scans (accumulation) the higher the S/N ratio;
2. precise (gradient) shimming techniques which increases the quality of the spectra;
3. using inverse and cryo probes which increase the signal-to-noise ratio (by a factor of about 10), e.g. for ^{1}H-measurements;
4. maximizing of concentration and optimizing solvent volume (microcoil technology, e.g. nano-NMR probes or microcryo probes).

A quantification is only possible in case the signals being integrated are well separated. 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. However, by changing the kind of solvent, the pH value or by adding auxiliary reagents ("shift reagents"), a separation of the signals can be often achieved. Overlapping signals from compounds exhibiting the same molecular hydrogen ratios might be corrected by subtraction of calculated integrals. In contrast, overlapping signals from (isomeric) compounds exhibiting the same molecular hydrogen ratio could not be used for quantification. This is one of the main limitations of the method. In some cases, the temperature influences the signal separation. Thus, it is important to set the temperature to a defined value.

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 in the case of UV, fluorescence or electrochemical detection, the NMR spectrometer is always ready to measure and needs experimental time depending on analyte concentration and experimental mode only. The substance has to be dissolved in a proper deuterated solvent. The amount of solvent needed is about 0.7 ml (using common 5 mm probe heads). So, facing the larger solvent volume in HPLC the higher costs for deuterated solvents are compensated. Sample amounts of 20 mg are approximately needed to achieve a good signal-to-noise ratio and an adequate experimental time.

Another advantage of NMR is the fact that for the purity assay of a drug not all impurities (used for the 100% method in HPLC) need to be identified.

NMR spectroscopy is widely used in pharmaceutical analysis to identify a drug and the accompanying impurities; qNMR can be used in drug analysis in order

1. to evaluate the level of impurities, to elucidate their structure and/or to observe the course of degradation resulting in the related impurities;
2. to evaluate the content of residual solvents;
3. to determine the isomeric composition, i.e. the ratio of diastereomers and the enantiomeric excess (ee) by means of chiral additives;
4. to determine molar ratios of (protonated) basic drugs and (deprotonated) organic acids in respective salts.

Thus, countless examples of application of quantitative NMR spectroscopy are reported which are summarized, e.g. in ref. [4]. Logically, International Pharmacopoeias make increasingly use of qualitative NMR spectroscopy for identification purposes and of quantitative NMR spectroscopy for evaluation of the composition of polymers and impurities in drugs. The review will focus on the application in International Pharmacopoeias and related examples.

2. NMR spectroscopy in International Pharmacopoeias

The European Pharmacopoeia (PhEur fifth edition [5]) describes the method of NMR spectroscopy only in principle using continuous wave, which is 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 identification section (see below). Thus, a revision of the general monograph on NMR spectroscopy is under progress.

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 both qualitative and quantitative purposes. In the section for qualitative analysis, the correlation between chemical shifts and coupling constants, on 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 qualitative and quantitative purposes more frequently. Table 1 gives the examples of monographs where NMR spectroscopy is currently applied.

2.1. Identification of drugs

Due the structural complexity of nonapeptide analogues of the human gonadotropin-releasing hormones (GnRH) go-

Table 1
The application of NMR spectroscopy in monographs of International Pharmacopoeias

Identification	
PhEur 5.0	Buserelin, goserelin, tobramycin
BP 1998	Hydrocortisone sodium phosphate
USP26	Amylnitrite isomers
PhEur 5.0	Heparins low-molecular-mass, haemophilus Type b conjugate vaccine, meningococcal Group C conjugate vaccine, pneumococcal polysaccharide conjugate vaccine (adsorbed)
Tests	
PhEur 5.0	Poloxamer: ratio of oxypropylene/oxyethylene
PhEur 5.0	Hydroxypropylbetadex: molar substitution
USP26	Orphenadrine citrate: meta/para isomer
Assay	
USP26	Amylnitrite isomers

PhEur, European Pharmacopoeia; BP, British Pharmacopoeia; USP, United States Pharmacopoeia.

nadorelin, buserelin and goserelin, the aminoglycoside tobramycin (see Fig. 1), the steroid hydrocortisone, the low-molecular-mass heparins, such as dalteparin, enoxaparin, parnaparin, tinzaparin, certoparin and nadroparin (^{13}C NMR), and the acetylated haemophilus and other vaccines the ^{1}H and ^{13}C NMR spectra are full of partially overlapping signals which can be assigned by means of two-dimensional experiments only. However, the pharmacopoeiae make use of a sort of pattern recognition for identification of the drugs corresponding to the application of IR spectra.

In addition, the PhEur described the identification of conjugate vaccines, such as the Haemophilus Vaccine (see Fig. 2), Meningococcus Group C Vaccine and Pneumococcus Vaccine. Fig. 3 impressively demonstrates the differences of Meningococcal Group A and C Conjungate Vaccine spectra. Further, NMR spectroscopic methods were applied and validated in order to control the identity and content of acetyl groups of capsular polysaccharides of *Neisseria meningitis* in vaccine manufacture [6,7], of *Haemophilus influencae* Type b in bulk ware [8], and of *Salmonella typhi* Vi [9] as well as to determine the size of the capsular oligosaccharide [10]. These methods were accepted from the licensing authorities.

Furthermore, the USP 26 identifies amyl nitrite being a mixture of mainly isoamyl nitrite ($(CH_3)_2CH–CH_2–CH_2–O–N=O$) beside other isomers by means of an ^{1}H NMR spectrum which is characterized by a doublet at ∼1 ppm and a multiplet centered at ∼4.8 ppm representing the methyl and methylene hydrogens in α-position to the nitrite group.

2.2. Tests

Most of the impurities are originated from the synthesis pathways, i.e. starting products, intermediates and synthesis

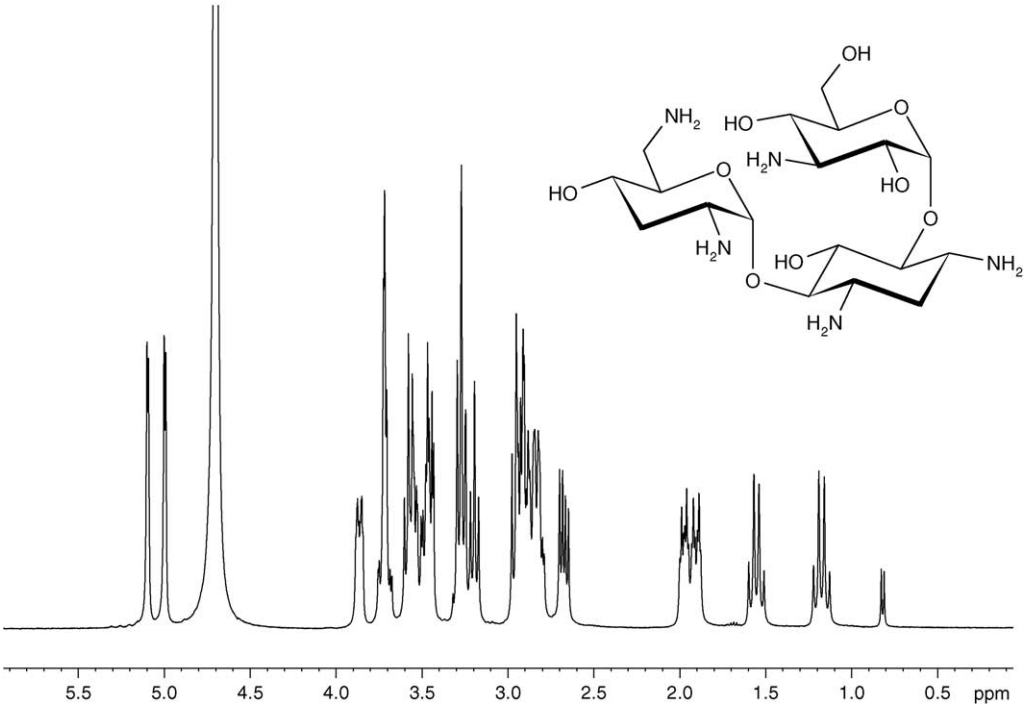

Fig. 1. Structural formula and ^{1}H NMR spectrum (400 MHz, D_2O) of tobramycin.

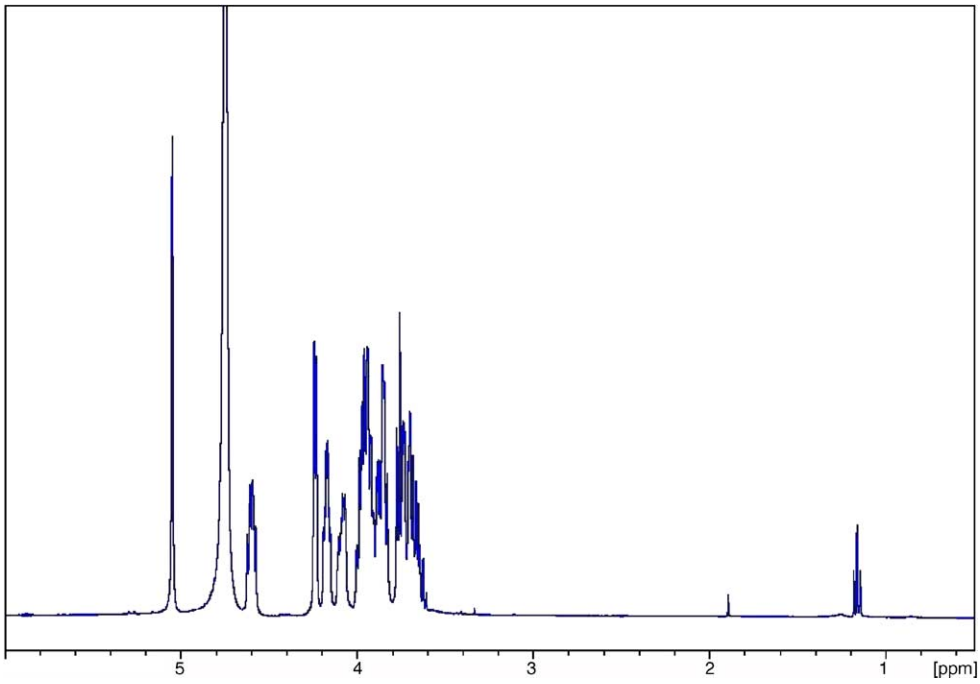

Fig. 2. ^{1}H NMR spectrum (400 MHz, D_2O) of Haemophilus Type b Conjugate Vaccine.

by-products, products of decomposition of the drug as well as residual solvents. The related substances are mostly evaluated by means of high performance liquid chromatography (HPLC). NMR spectroscopy can be employed for quantification of related substances [11] and residual solvents [12]. Interestingly the European Pharmacopoeia makes use of the NMR spectroscopy in order to characterize the composition of an oligo- and a polymer, i.e. hydroxypropylcyclodextrin and poloxamer, respectively.

The molar substitution (MS) of hydroxypropylbetadex, known as hydroxypropyl-β-cyclodextrin being a poly(hydroxypropyl)ether of β-cyclodextrin of random sub-

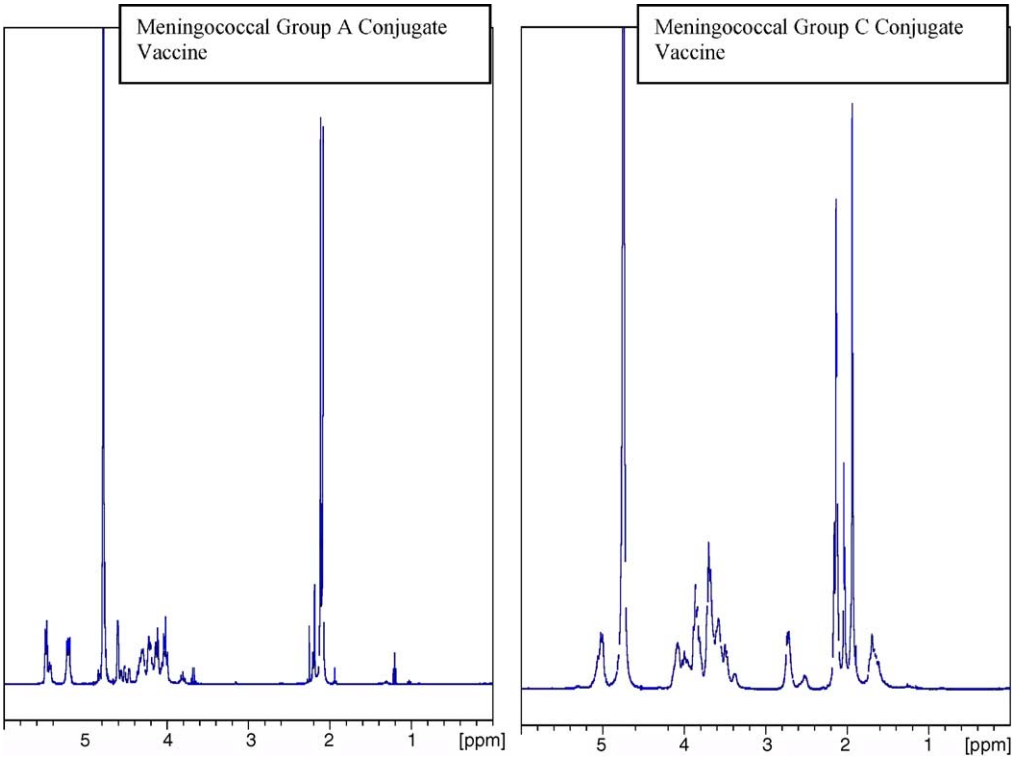

Fig. 3. ^{1}H NMR spectra (400 MHz, D_2O) of Meningococcal Group A and Group C Conjugate Vaccines.

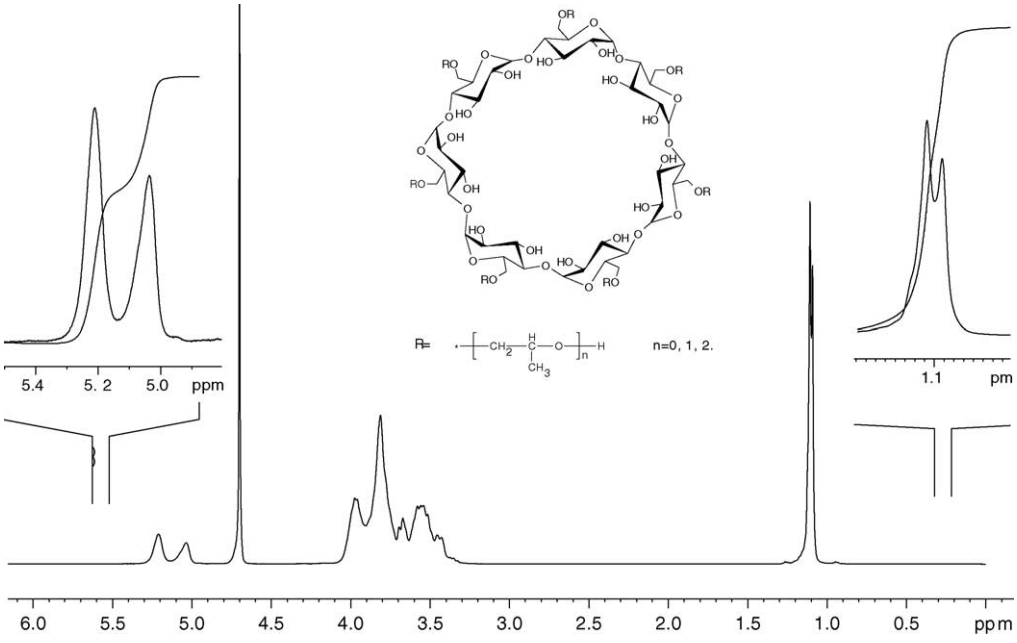

Fig. 4. Structural formula and ^{1}H NMR spectrum (400 MHz, D_2O) of hydroxypropyl-β-cyclodextrin.

stitution in 2, 3 and 6 position is characterized by ^{1}H NMR spectroscopy. The 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 by means of the following equation:

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

A_1 = area of the signal due to the three protons of the methyl groups, which are part of the hydroxypropyl groups (12 ppm); A_2 = area of the signal due to the glycosidic protons (5–5.4 ppm); degree of substitution = 7MS = 7.7.

The MS has to be in the range of 0.40 and 1.50 and within 10% of the value stated on the label. As can be seen from the calculation the sample of hydroxypropylbetadex, evaluated here (see Fig. 4), meets the requirements of the PhEur.

Correspondingly the USP 26 and PhEur 5.0 determine the content of oxypropylene in *poloxamer*, an ethylene oxide/propylene oxide block copolymer (Pluronic F 68®) by means of an ^{1}H NMR spectrum using the signals the integrals of CH_2O/CHO signals between $\delta = 3.2$ and 3.8 ppm and the CH_3 signal appearing at $\delta \sim 1.3$ ppm. The percentage can be calculated from the following equations:

$$\mathrm{HO}\left(\mathrm{CH_2CH_2O}\right)_a\left(\mathrm{CH_2\underset{\mathrm{CH_3}}{\underset{|}{C}HO}}\right)_b\left(\mathrm{CH_2CH_2O}\right)_c\mathrm{H}$$

$$\%\,\text{oxyethylene} = \frac{3000 \times \alpha}{33 \times \alpha + 58} \quad \text{with}$$

$$\alpha = \frac{\text{area}(CH_2O/CHO)}{\text{area}(CH_3)}$$

Applying the integrals found for a sample of pluronic evaluated in our laboratory, the content of oxyethylene was found to be 81.8% [4]. This is in agreement with the requirements of the USP and the PhEur, which are 75–85%.

The USP 26 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 and 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. However, no batch could be collected from the market, which contains the *m*- or *p*-methyl substituted isomer [4].

2.3. Assay

In the assay of amyl nitrite, the absolute method using benzyl benzoate as an internal standard was applied in the USP 26. The quantity of amyl nitrite is calculated from the signal area of the α-methylene group of the drug (at 4.8 ppm) and the signal area of the methylene hydrogens of benzyl benzoate at 5.3 ppm. Besides this application, no assay utilizing NMR spectroscopy is described in the International Pharmacopoeias yet.

3. Further application

As already mentioned, most of the related substances are originated from the synthesis pathways. Normally, the syn-

Table 2
Examples of qNMR used for the evaluation of impurities, which are either synthesis by-products or products of degradation

Drug	Impurity and by-products evaluated	Reference
Lovastatin	0.1% dihydrolovastatin	[17]
Dequalininium chloride	Aminoquinaldine, 4-amino-1-[10-[(2-methylquinolin-4-yl)amino]decyl]-2-methylquinolinium chloride and overalkylation products	[18,4]
Vitamin E (α-tocopherol)	β-, γ- and/or δ-tocopherol	[19]
Fenofibrate	12 known and unknown impurities	[20]
Sulfasalazine	Identification and evaluation of 2 co-eluting impurities	[21]
Propantheline	Products of hydrolysis	[22]
Flurazepame	Ring-opened products of hydrolysis	[23,24]
Cefotaxime	Products of alkaline hydrolysis	[25]
Neostigmine	Kinetic of hydrolysis	[26]
TMB-4, acetylcholinesterase reactivator	Degradation to corresponding ethers, cyano compounds and to pyridones	[27]
Nifidipine	Photodecomposition	[28]
Erythromycin	Decomposition to hemiketal and enolether	[29]
Clarithromycin	Decomposition releasing the cladinose	[29]
Fluorouracil	Degradation to fluoroacetaldehyde, fluoromalonic acid semialdehyde and fluoroacetate	[30,31]
Ifosfamide	Elucidation of the complex degradation	[32]

thesis pathway is known and logically, the impurities are known and can be easily evaluated by means of HPLC. In this 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 because these methods are developed for a defined production procedure. In this context, NMR spectroscopy can help to trace, identify and often quantify the "new" impurities. Moreover, NMR spectroscopy is a powerful tool for impurity profiling and tracing production ways from manufacturer to different brokers and finally to the drug manufacturer which was impressively demonstrated for gentamicin [13,14]). Marketing of drugs is authorized by the competent authorities with regard to a defined synthesis pathway. Thus, drugs produced on a different way have to be considered as counterfeit drugs [15,16]. Due to an increasing number of counterfeit drugs NMR spectroscopy is of growing importance in the field of drug analysis and fake.

Representative examples of qNMR spectroscopy used for quantification of impurities, which result from synthesis pathways and from degradation of the drugs are given in Table 2.

4. Perspective

The advantages of quantitative NMR spectroscopy over HPLC techniques can be summarized as follows: (a) qNMR analysis is often more accurate and precise than standard HPLC methods [33]; (b) normally, no isolation of the impurity is necessary; (c) no expensive chemical reference substances are necessary; additional structural information of impurities, isomers, etc. are available; and (d) NMR can be less time consuming (no equilibration time), easy to perform and more specific leading to a high reproducibility.

The aforementioned examples clearly demonstrate the suitability of NMR spectroscopy for drug analysis purposes. Thus, further applications can be expected in the PhEur and National European Pharmacopoeias as well as the JP and the USP in the near future. However, in order to apply NMR spectroscopy more often, the general monograph on NMR spectroscopy especially in the European Pharmacopoeia needs adoption to the modern techniques.

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