Review

NMR spectroscopy in pharmacy

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Abstract

Since drugs in clinical use are mostly synthetic or natural products, NMR spectroscopy has been mainly used for the elucidation and confirmation of structures. For the last decade, NMR methods have been introduced to quantitative analysis in order to determine the impurity profile of a drug, to characterise the composition of drug products, and to investigate metabolites of drugs in body fluids. For pharmaceutical technologists, solid state measurements can provide information about polymorphism of drug powders, conformation of drugs in tablets etc. Micro-imaging can be used to study the dissolution of tablets, and whole-body imaging is a powerful tool in clinical diagnostics. Taken together, this review covers applications of NMR spectroscopy in drug analysis, in particular, methods of international pharmacopoeiae, pharmaceutics and pharmacokinetics. The authors have repeated many of the methods described in their own laboratories. © 1998 Elsevier Science B.V. All rights reserved.

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1. Iintroduction

Since the development of the high resolution NMR spectrometer in the 1950s, NMR spectra have been a major tool for the study of both newly synthesised and natural products isolated from plants, bacteria etc. In the 1980s a second revolution occurred. The introduction of reliable superconducting magnets combined with newly developed, highly sophisticated pulse techniques and the associated Fourier transformation provided the chemist with a method suitable to determine the 3-dimensional structure of very large molecules, e.g. biomacromolecules such as oligopeptides [1], in solution.

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mainly used for the elucidation and confirmation of structures. For the last decade, NMR methods have been introduced to quantitative analysis in order to determine the impurity profile of a drug, to characterise the composition of drug products, and to investigate metabolites of drugs in body fluids. For pharmaceutical technologists, solid state measurements can provide information about polymorphism of drug powders, conformation of drugs in tablets etc. Micro-imaging can be used to study the dissolution of tablets, and whole-body imaging is a powerful tool in clinical diagnostics. Taken together, this review will cover applications of NMR spectroscopy in drug analysis, in particular methods of international pharmacopoeiae, pharmaceutics and pharmacokinetics. The authors have repeated many of the methods described, in their own laboratories.

1.1. The instrument

Organic compounds are composed basically of the elements hydrogen, carbon, phosphorus, nitrogen and oxygen. Additionally, there are the halogens fluorine, chlorine, bromine and iodine and sometimes metal atoms. Each of these elements has an isotopic nucleus which can be detected by the NMR experiment. The low natural abundance of ^{15}N and ^{17}O in nature prevents NMR being routinely applied to these elements without the use of labelled substances, but ^{1}H-, ^{13}C-, ^{19}F- and ^{31}P NMR spectroscopy are daily routine work. Many instruments are equipped with a so-called QNP (quattro nuclei probe) for sequential NMR analysis of ^{1}H, ^{13}C, ^{31}P and ^{19}F, without the hardware having to be changed. Modern NMR spectrometers are available up to field strengths of 18.8 Tesla or a proton resonance frequency of 800 MHz. Routine analysis is made at proton frequencies between 300 and 500 MHz. Depending on the kind of experiments, the high field instruments allow analysis of concentrations down to some µg ml^{-1}, but the 'normal' case is a concentration of 1–100 mg ml^{-1}. The data are recorded using 32 bit ADCs (analog-digital-converter). This results in a high spectral dynamic range.

1.2. Principles

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

1.3. Spectra

The frequency at which an NMR signal appears depends mainly on the magnetic field strength. For example, protons have a resonance frequency of 300 MHz at 7.05 Tesla. The chemical environment of an active nucleus leads to a small shift in the resonance frequency, the 'Chemical Shift'. Functional groups find their expression in the Chemical Shift. The result is an intensity-/frequency-diagram, the NMR spectrum. This collection of more or less separated NMR signals is analogous to intensity/time diagrams in chromatography, in which one component is represented by one signal. In ^{1}H NMR spectroscopy, each H-atom leads to at least one signal. Since most molecules of analytical interest contain more than one H-atom, spectra are more complex than chromatograms. What is crucial to the information taken from NMR spectra is the spectral dispersion, which is a linear function of the magnetic field strength. The homonuclear spin coupling of protons leads to a low dispersion of ^{1}H NMR spectroscopy. In ^{1}H NMR spectra of complex mixtures, it is often not possible to detect single components, but the sum of functional groups in the mixture can be determined. In ^{13}C NMR spectra, the dispersion is much higher. The low natural abundance of the NMR active ^{13}C-isotope has a detrimental effect to the sensitivity, but signals are singulets after heteronuclear decoupling. The high spectral dispersion makes parts of the ^{13}C NMR spectra directly comparable to chromatograms. An example is the carbonyl-

region in a ^{13}C NMR spectrum of a lipid mixture, where each fatty acid is represented by a specific signal.

^{31}P NMR spectroscopy is the method of choice for phospholipids or any other phosphorus containing compound. Most phospholipids contain only one phosphorus atom, so the ^{31}P NMR spectrum of lecithin reads like an HPLC chromatogram. There are some advantages in comparison with HPLC: specific detection of the phosphorus nucleus, high dispersion and high dynamics. The role of ^{31}P NMR spectroscopy will be discussed later in detail.

1.4. Response

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

1.5. Reproducibility

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

1.6. Calibration

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

1.7. Experimental

Methods marked with [SSL] [11] have been developed by Spectral Service and have not been published previously. To illustrate the published NMR-methods, most spectra shown in the figures were recorded by Spectral Service and replace those of the original studies. The 300 MHz NMR spectra were measured at Spectral Service GmbH, Cologne (Germany) on a NMR-Spectrometer AC-P 300, at 7.05 Tesla (BRUKER, Karlsruhe, Germany) equipped with automated sample changer and QNP-head for nuclei ^{1}H, ^{13}C, ^{19}F and ^{31}P. The data processing was performed using BRUKER WIN NMR 5.0 software under Microsoft Windows 95.

2. Analysis of drugs

2.1. Quantitative NMR spectroscopy

European pharmacopoeiae mostly use NMR spectroscopy for the identification of drugs and
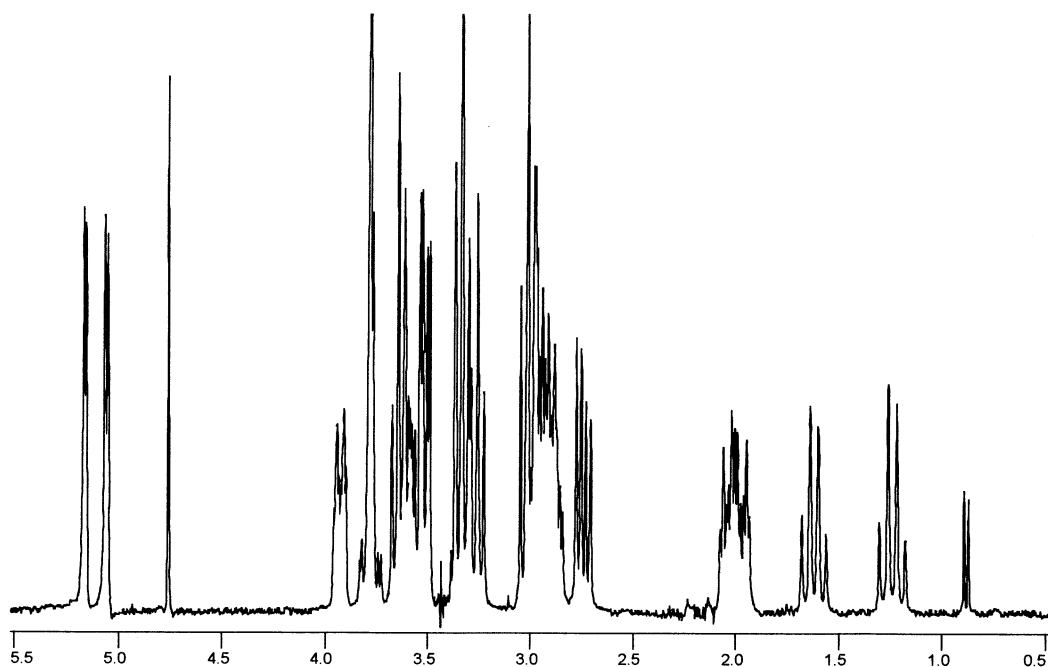
Fig. 1. Expansion of the ^{1}H NMR spectrum of tobramycin, 300 MHz, solvent D_2O.

reagents: In the cases of tobramycin (Pharmaco-poeia Europaea, Ph. Eur.) and hydrocortisone sodium phosphate (BP 93), the NMR spectra replace the normally used IR spectra. Due to heavy signal overlapping, the spectra of these compounds are very complicated (see Figs. 1 and 2). Their assignment is only possible by 2D experiments. Thus, the ^{1}H NMR spectra are used in the same manner as IR spectra, which can be described as a sort of pattern recognition. An increasing number of reagents, e.g. adenine, butoxycaine, aesculin etc., are identified by ^{1}H and ^{13}C NMR spectra in national and international pharmacopoeiae.

2.1.1. Heparins

Low molecular weight (LMW) heparins ($M_w <$ 8000), attracting interest in the management of thromboembolic diseases, are obtained from different depolymerization procedures of heparin, a polysulfated glycosaminoglycan. The group of Neville [2] was able to exactly characterise those compounds by means of ^{1}H and ^{13}C NMR spec-

troscopy. Depending on the depolymerization methods, various amounts of the contaminant dermatan sulfate (chondroitin sulfate) were found by means of 300 MHz ^{1}H NMR spectra [3,4]. The molecular weight was determined using ^{13}C NMR [5]. Here, the signal intensities of the reducing end and internal anomeric carbons, having distinct chemical shifts, were compared in DEPT spectra. The method is as exact as size exclusion chromatography, which is often used for the purpose of weight determination of huge molecules. Hence, ^{13}C NMR spectroscopy is used in international pharmacopoeiae to identify LMW heparins (see general monograph, Ph. Eur. III). Six different forms of LMW heparins are under discussion for introduction to the Ph. Eur. They differ in the procedure of depolymerization of heparin: Certoparin sodium is obtained by isoamyl nitrite depolymerization of heparin sodium from porcine intestinal mucosal, parnaparin by radical-catalysed decomposition (H_2O_2 and cupric salts), dalteparin sodium and nadroparin calcium by nitrous acid depolymerization, tinzaparin by enzy-
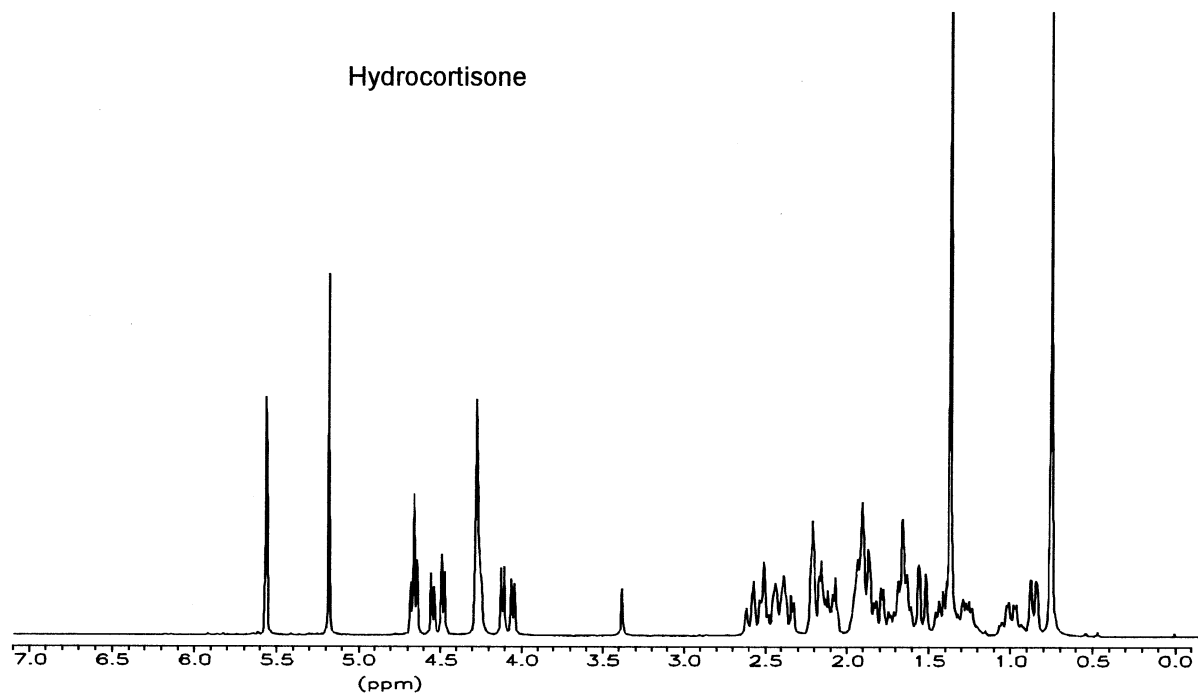
Hydrocortisone

Fig. 2. ^{1}H NMR spectrum of hydrocortisone sodium phosphate 300 MHz, solvent DMSO-d_6.

matic degradation using heparinase, and enoxaparin by alkine depolymerization of heparin benzyl ester. According to the different procedures, the LWM heparins 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 (Fig. 3).

Thus, signals of those rings appearing in the range between 80 and 92 ppm can be taken to identify the corresponding heparin. For example, the ^{13}C NMR spectrum of certoparin is characterised by four distinct signals corresponding to C3 ($\sim$ 81.6 ppm), C2 ($\sim$ 86.3 ppm), C4 ($\sim$ 86.6 ppm), and C1 ($\sim$ 90.4 ppm) for the anhydromannose skeleton, and a signal at 90.4 ppm for the hydrated aldehyde functional group. ^{1}H and ^{13}C NMR spectra of tinzaparin and dalteparin are displayed in Figs. 4 and 5. As expected, the signals of the anhydromannose moiety of dalteparin are similar to those of certoparin.

It is noteworthy that bovine mucosal heparin and porcine mucosal heparin can be distinguished by ^{1}H and ^{13}C NMR spectra [6–8]. Since they differ in the extent of sulfation, they show a different 'fingerprint'. In addition, the chemical shift of H1 and H5 in iduronic acid as well as the magnitude of separation of the glucosamine/iduronic acid H1 pair are appropriate to discriminate between the counter ion sodium or calcium [6]. In a similar manner, the extent and site of sulfation of dextran sulfate could be described by 300 MHz ^{1}H NMR spectra. Additionally, the manufacturer could be determined [9,10] by comparison of the pattern.

To record ^{13}C NMR spectra of bovine or porcine mucosal heparin, the accumulation of a large number of transitions is necessary, which leads to very long measuring times up to 20 hours. The higher the magnetic field strength of the NMR spectrometer the better the results. Alternatively ^{1}H NMR spectroscopy at higher temperature improves the spectral resolution and allows the differentiation between bovine and
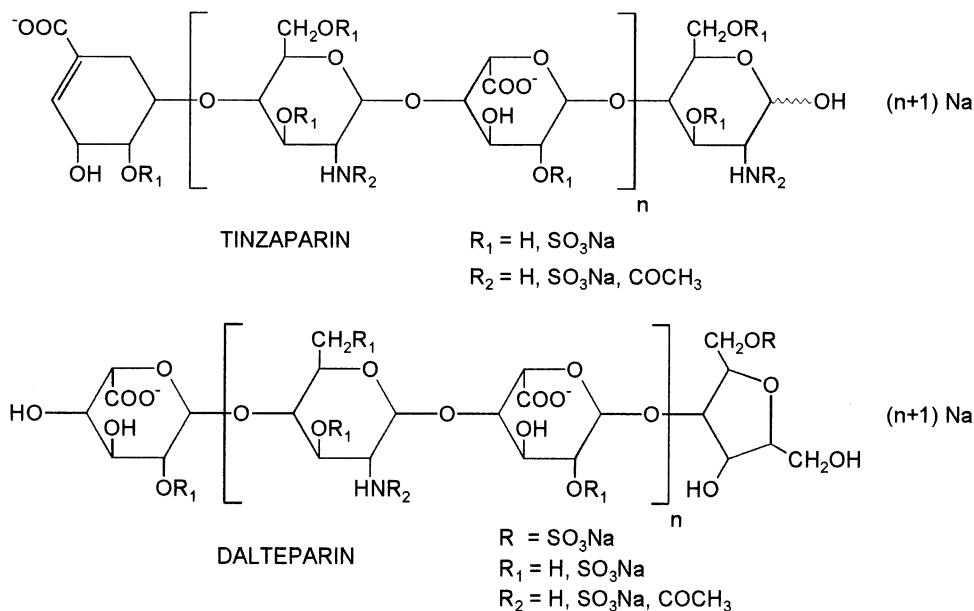
Fig. 3. Types of heparins.

porcine mucosal heparin and other kinds of heparinoides. Fig. 6 shows the high temperature ^{1}H NMR spectra (353 K) of bovine and porcine mucosal heparin (SSL) [11].

The sucrose octasulfate anion, which is believed to be the active component of sulcrafate, an antiulcer drug, is hypothesised by means of FAB-MS to consist of large amounts of hepta- and hexasulfate derivatives. The technique of 2D and deuterium-induced, differential-isotope-shift (DIS) ^{13}C NMR spectroscopy (chemical shift induced by H/D exchange of the OH groups) provides a picture of sample identity and purity [12]. Without going into detail, it should be emphasised that undersulfation of sucrose did not occur before hydrolysis; all drug samples were found to be pure. Consequently, the hexa- and heptasulfated sucroses postulated by FAB-MS experiments were artefacts of this method!

2.1.2. Polymers

In poloxamer, a synthetic block copolymer of ethylene oxide (EO) and propylene oxide (PO) (USP XXIII), the oxypropylene/oxyethylene ratio is determined using a ^{1}H NMR spectrum measured in $CDCl_3$. The oxypropylene units are char-

acterised by a narrow signal at about 1.1 ppm due to the CH_3 group. The CH_2O/CHO units cause a multiplet between 3.2 and 3.8 ppm. The percentage of oxyethylene can be calculated from the expression

$$\% \text{ oxyethylene} = \frac{3300 \times \alpha}{33\alpha + 58}$$

$$\text{with } \alpha = \frac{\text{area}(CH_2O/CHO)}{\text{area}(CH_3))} - 1$$

The content of oxypropylene in the sample examined (Pluronic F 68; Fig. 7) amounts to 81 percent which is in accordance with the requirement of the pharmacopoeia.

In addition to a quantitative determination of EO and PO taken from ^{1}H NMR spectra, ^{13}C NMR spectra (Fig. 8) enable us to see the type of polymerisation, e.g. block- or mixed-polymerisation, the type and ratio of endgroups (EO or PO) and the stereochemistry in polyethyleneglycols (tactic or atactic) [13].

The stereochemistry of polylactides is important to the physical and chemical behaviour. Pure tactic polymerisation can be differentiated from atactic or mixed polymers by ^{13}C NMR analysis (Fig. 9).
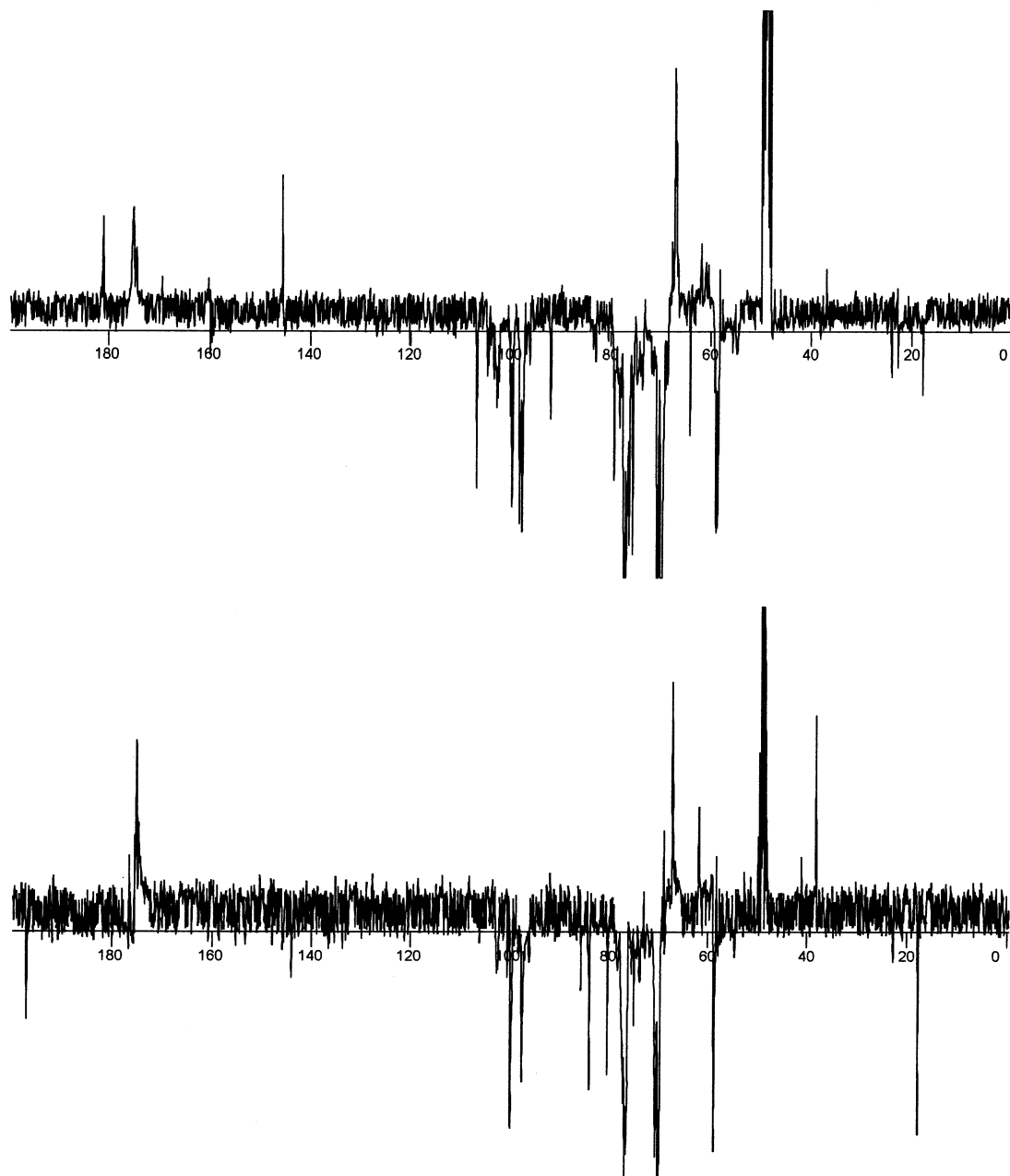
Fig. 4. ^{13}C NMR DEPT spectra of tinzaparin (above) and dalteparin (below), 75 MHz, solvent D_2O.

In contrast to the Ph. Eur. the United States Pharmacopoeia (USP) describes quantitative methods in addition to the qualitative analyses. The relative method of quantitation is used in the orphenadrine monograph in order to determine the content of *m*- and *p*-methylphenyl isomer in the *o*-methylphenyl substituted drug (see Fig. 10).

The signals of the benzylic methine hydrogen atoms are well separated. Hence, from the areas of the signal of the *m/p*-methyl substituted com-
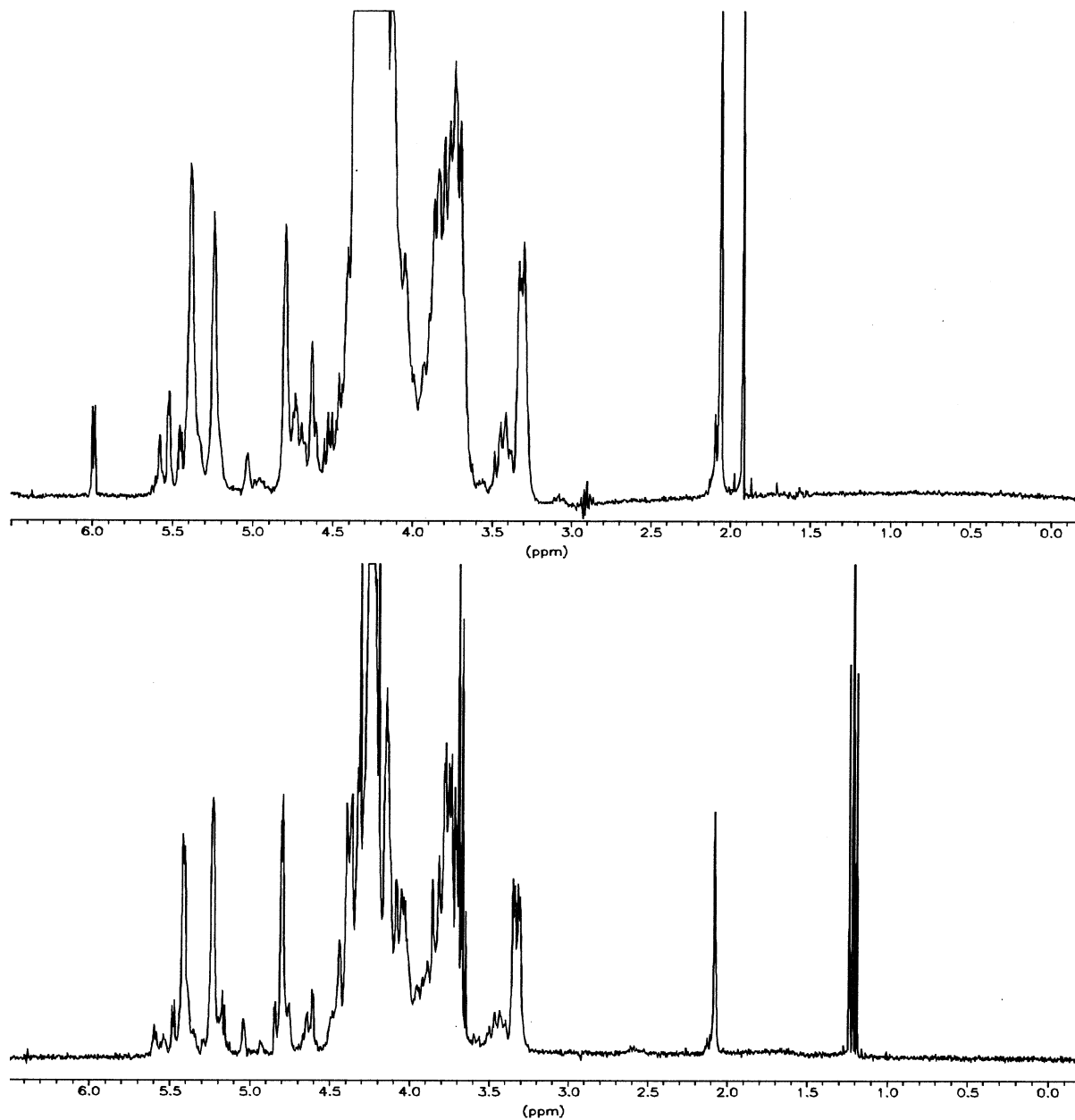
Fig. 5. ^{1}H NMR spectra of tinzaparin (above) and dalteparin (below), 300 MHz, solvent D_2O at 353 K.

pound appearing at about 5.23 ppm and of the orphenadrine signal appearing at 5.47, the content of the impurities can be determined. The limit for both isomers is 3 percent.

The USP monography of orphenadrine employs NMR spectroscopy for determination of the isomeric *m*- and *p*-methyl compounds. After an alkaline extraction the sample is measured in CCl_4. The limit of quantitation using the CW method (continuous wave method) is specified to be 3%. Due to their higher magnetic flux density and the ability to accumulate pulses, modern FT
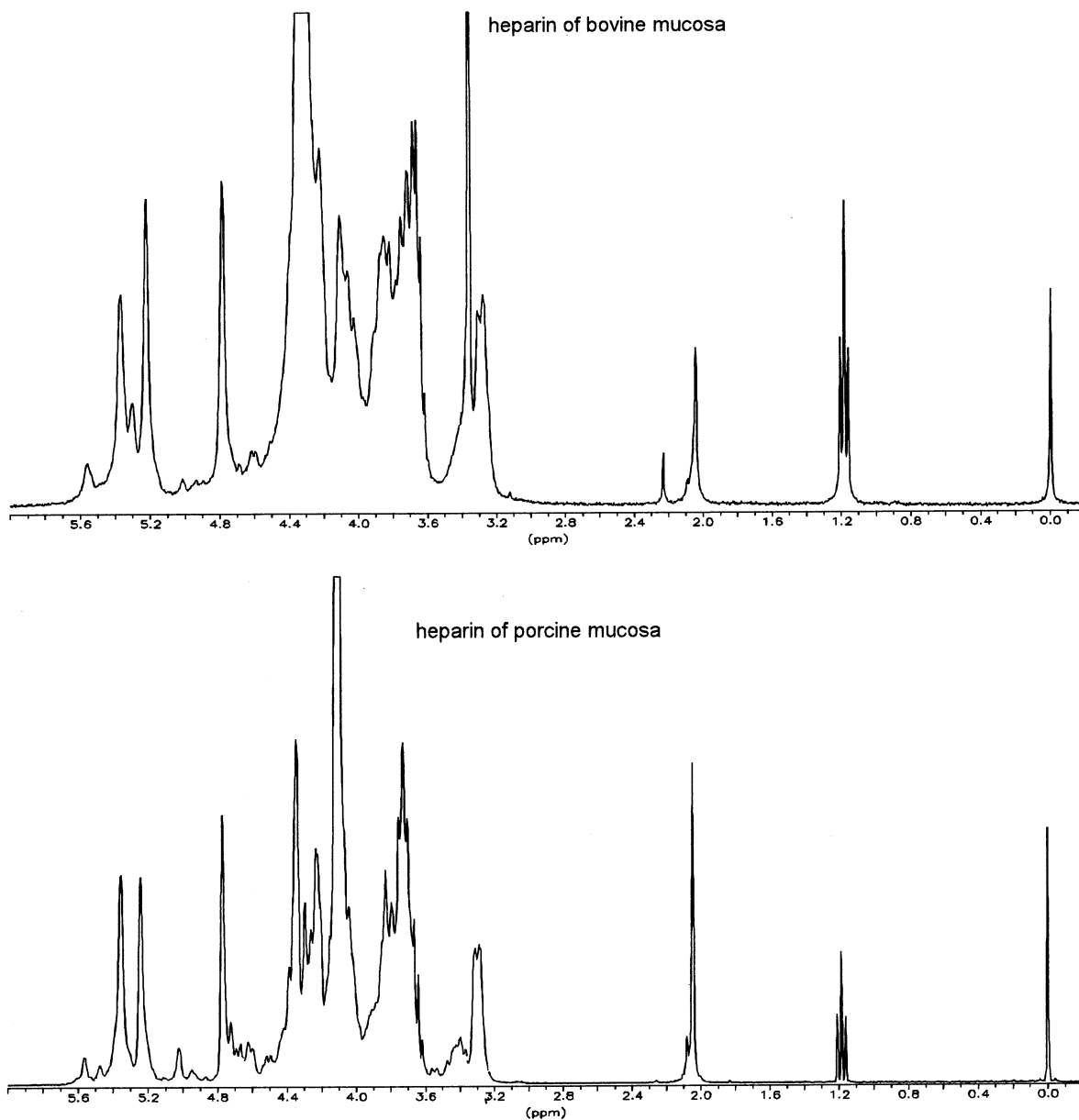
Fig. 6. Heparin of bovine (above) and porcine (below) mucosa, 300 MHz, solvent D_2O at 353 K.

NMR spectrometers have increased sensitivity and signal resolution and with that a remarkably lower limit of quantitation.

The new technology allows a direct determination of orphenadrine citrate in the aqueous formulation. However, in the following section the CW method is applicable to the modern FT method:

The method was tested with a sample of Norflex®. Two ^1H NMR spectra are shown: Fig. 11a shows the ^1H NMR spectrum of a CCl_4 extract (free base) prepared according to the USP instruction. A capillary inside the NMR tube, which is filled with benzene-d_6, serves as external deuterium lock. In Fig. 11b the injection solution
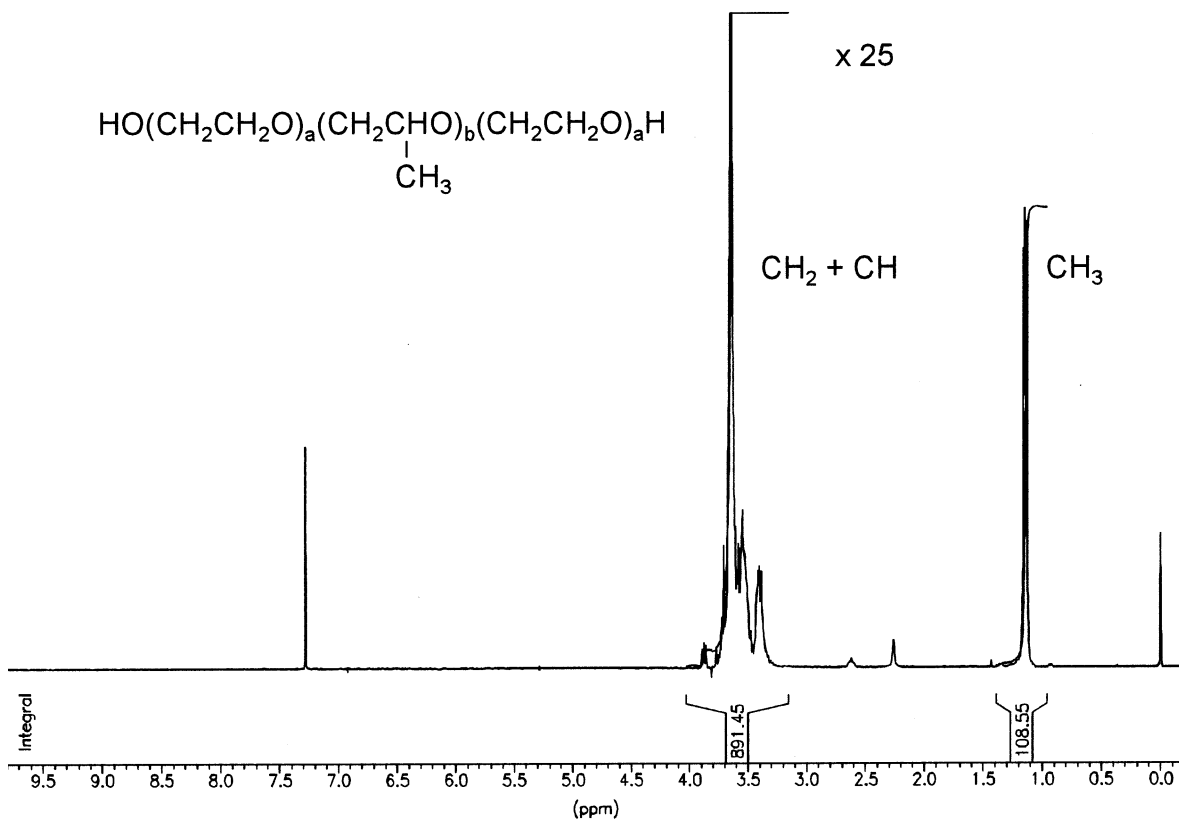
x 25

$$HO(CH_2CH_2O)_a(CH_2CHO)_b(CH_2CH_2O)_aH$$
$$\underset{CH_3}{|}$$

CH₂ + CH

CH₃

Integral

891.45

108.55

9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0

(ppm)

Fig. 7. ¹H NMR spectrum of pluronic, 300 MHz, solvent CDCl₃.

(0.5 ml) was treated with 0.2 ml D₂O and measured directly. Due to the fact that orphenadrine citrate was observed, considerable changes in the chemical shift of the signals are found. However, this has no influence on the analysis of the isomeric distribution. Additionally, the characteristic AB system of citric acid is found. The signals labelled with an asterisk are the corresponding ¹³C NMR satellites, which have, due to the natural abundance of the ¹³C-isotope, an intensity of 0.56% of the main signal. This demonstrates that the detection limit is in fact lower than 0.5% with all three methods. With respect to an estimated quantitation limit of 0.2% no m- or p-methyl compounds were found in the sample studied.

Amyl nitrite (USP XXIII), which is a mixture of nitrite esters of 2- and 3-methyl-1-butanol, is identified by an ¹H NMR spectrum showing two

multiplets, one at 4.8 ppm representing the α-methylene group and one at 1 ppm belonging to all other hydrogens. The absolute method of quantification using benzyl benzoate as an internal standard is taken as an assay. The quantity of amyl nitrite is calculated from the signal area of the α-methylene group of the drug (at 4.8 ppm) and the signal area of the methylene hydrogens of benzyl benzoate at 5.3 ppm.

The advantage of higher magnetic field strength is demonstrated in case of lovastatin. 600 MHz spectra [14] are compared with 300 MHz spectra(SSL) [11] in Figs. 12 and 13)

A ¹H NMR method using a 600 MHz instrument is described to quantify dihydrolovastatin in lovastatin with a limit of quantitation at 0.1%. Some preliminary remarks are made to understand the comparison of a 300 MHz spectrum with a 600 MHz spectrum. All ¹H NMR signals
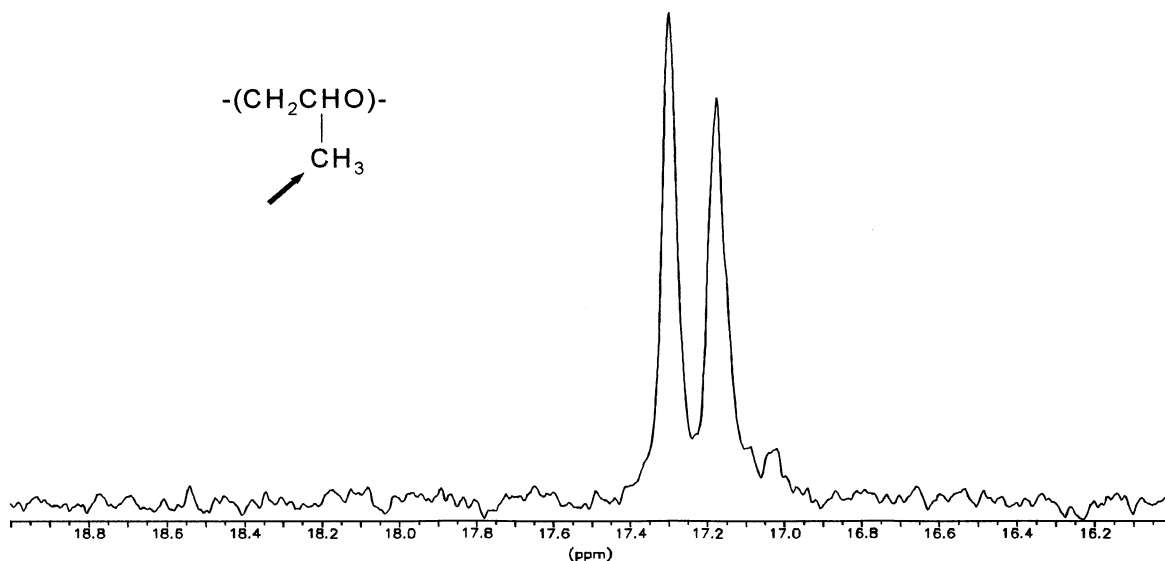
Fig. 8. ^{13}C NMR spectrum of pluronic (16–19 ppm), methyl groups of PO), 75 MHz, solvent CDCl$_3$.

of protons, which are bound directly to a C-atom, show ^{13}C-satellites of 0.56% intensity of the main signal. In practice, this theoretical ratio is found with good precision demonstrating the linearity of NMR signals over two orders of magnitude. Thus, the ^{13}C-satellites can be used for NMR calibration.

The chemical shift δ [ppm] of a proton is the same on a 300 MHz instrument and a 600 MHz spectrometer. But the distance of the ^{13}C NMR satellites to the parent signal is halved changing from 300 MHz to 600 MHz. This becomes evident in the comparison of spectra in Fig. 13. The H1 proton of dihydrolovastatin retains its chemical shift independent to the magnetic field, but in the 300 MHz spectrum it appears between the main signal and the satellite, whereas in the 600 MHz spectrum its position is high field (right) of the satellite. At 400 MHz the signals interfere, an evaluation is practically impossible. Modern spectrometers are able to avoid this conflict using ^{13}C decoupling. Using this technique, the satellite signals are removed completely, the main signal represents the protons bound to ^{12}C and ^{13}C.

It is expected that further application of NMR spectroscopy will be introduced to the Ph. Eur. and the USP in the future. The following section describes some proposals:

2.1.3. Identification and quantification

The characterisation of drugs in dosage forms by means of NMR spectroscopy is an interesting goal, because the identity and quantity can be determined simultaneously. Hanna et al. [15] describe a method for analysis of chlorpheniramine maleate in tablets and injection. After extraction of the drug, an internal standard (*t*-butanol) is added and a 90 MHz spectrum is recorded. Using the integrals of the signals, which appear in a range between 1.95 to 2.70 ppm, representing the aliphatic hydrogens of chlorpheniramine, and the integrals of the signal at 1.25 ppm belonging to the methyl groups of the internal standard, the quantity of the drug can be calculated. Even when using only a 90 MHz spectrometer, the method was found to be as accurate as a HPLC analysis. Thus, it is a simple and reliable means of quantifying a substance no matter whether it exists in a drug or in dosage forms.

Furthermore, it is possible to identify and quantify a mixture of ingredients in a dosage form. Caplets of Extra Strength Excedrin®, consisting of 250 mg acetylsalicylic acid, 250 mg paracetamol and 65 mg caffeine, can be easily examined after dissolution in Unisol® (a mixture of DMSO-d$_6$, CDCl$_3$, CD$_2$Cl$_2$), filtration, and
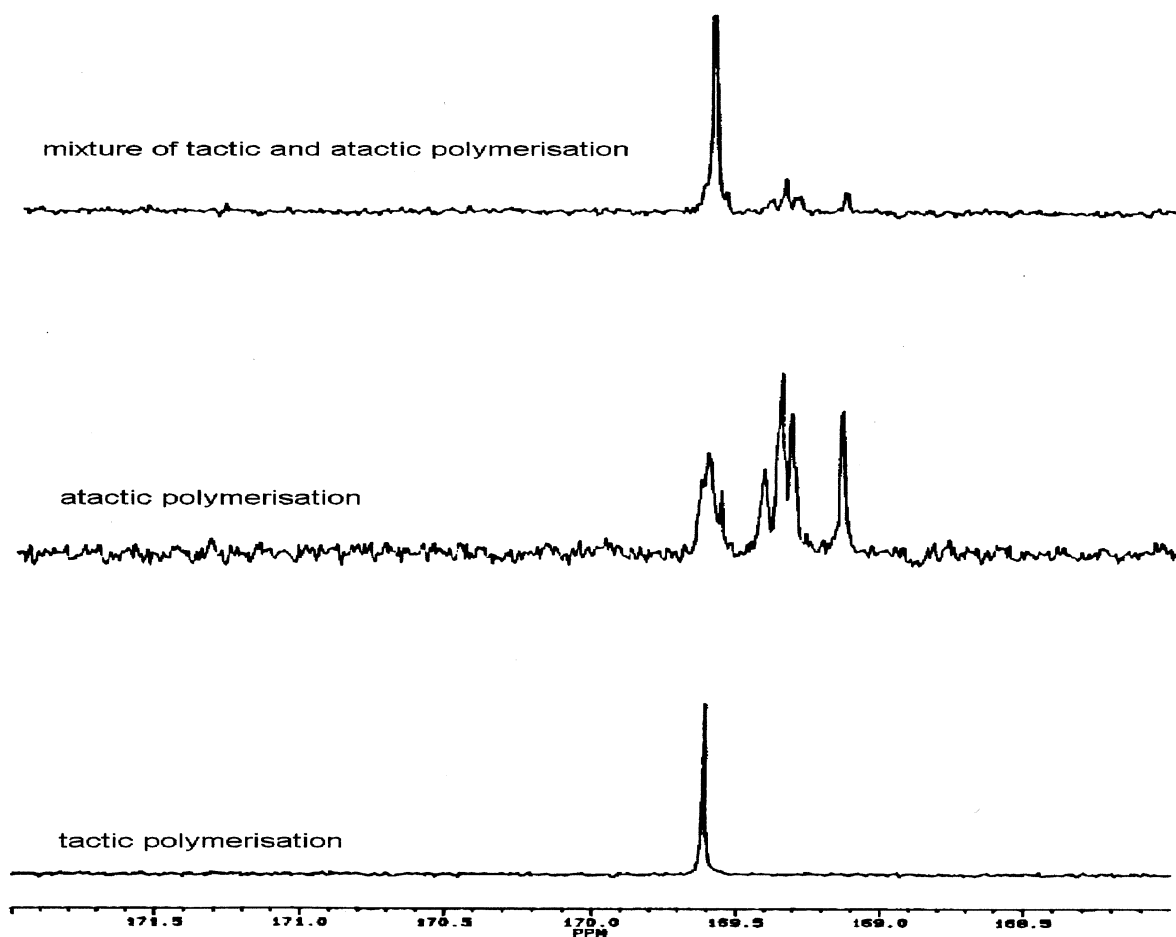
Fig. 9. Tactic and atactic polymerisation, 75 MHz, solvent CDCl$_3$.

recording an inverse gated decoupled ^{13}C NMR spectrum (after optimisation of the relaxation time), using acetophenone as an internal standard [16].
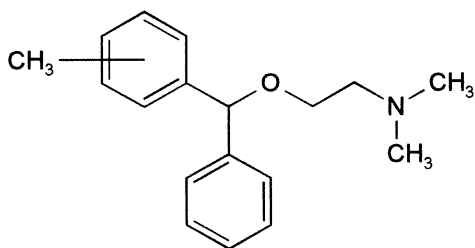

Fig. 10. Orphenadrine.

The power of ^{1}H NMR spectroscopy in the analysis of pharmaceutical formulations containing different concentrations of drugs is demonstrated in the ^{1}H NMR spectra of Grippostad C®, the method is similar to that applied to Extra Strength Excedrin®. The amount of the four drugs paracetamol, caffeine, ascorbic acid and chlorphenamine hydrogen maleate including the maleic acid can be analysed in one spectrum (see Fig. 14). Details of this spectrum are shown in Figs. 15 and 16.

2.1.4. Phospholipids

Many HPLC methods for phospholipids have been developed, but chromatographic resolution

(a)

Orphenadrine extract in CCl₄

(b)

Orphenadrine in D₂O
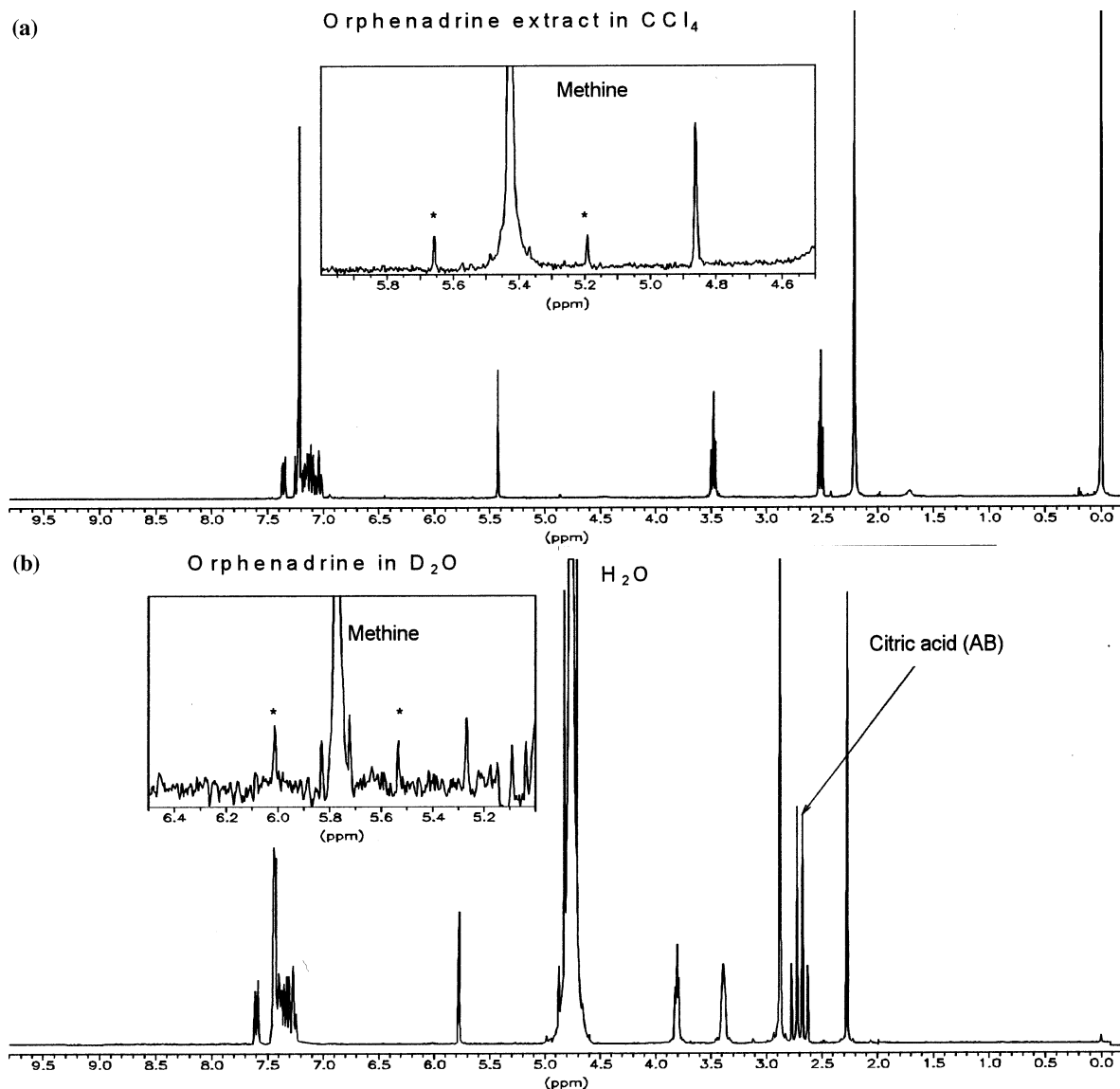

Fig. 11. (a) and (b) ¹H NMR spectra of orphenadrine citrate (Norflex®), 300 MHz.

and dynamics of detection are not always satisfactory. For each source of phospholipids, special standards are needed due to the different distribution of fatty acids. These standards are expensive and in some cases they are not available. Another problem is represented by the analysis of phospholipids in complex matrices. In many cases, separation is impossible or very difficult, not least due to the surface activity, which is desired in the

application of phospholipids, but which complicates the analysis of these compounds. Therefore, a method is needed which is selective in the detection of phospholipids in order to avoid a separation from the matrix. The ^{31}P NMR spectroscopy of phospholipids meets these requests. The I.L.P.S. (International Lecithin and Phospholipid Society) has chosen the ^{31}P NMR method as the reference method [17–19]. It has been tested
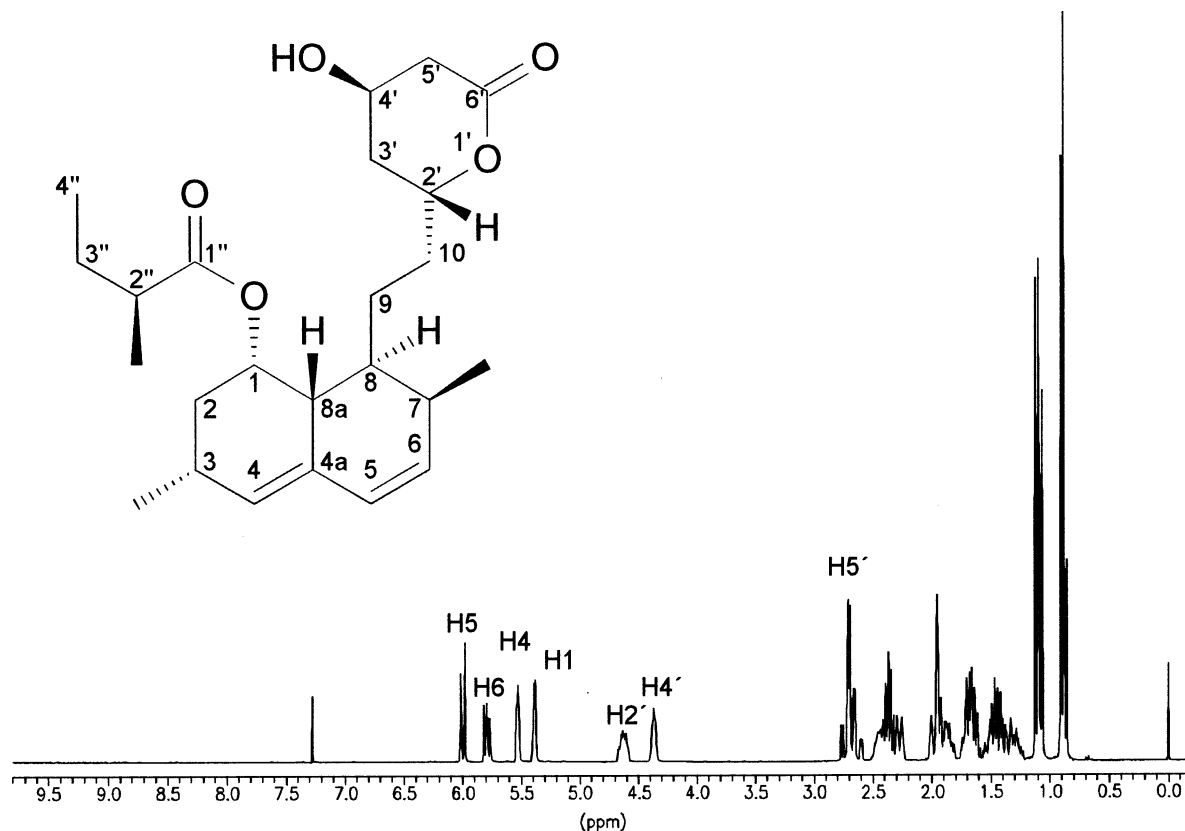
Fig. 12. ^{1}H NMR spectrum of lovastatin, 300 MHz, solvent CDCl$_3$.

world-wide by round robin tests in comparison to various HPLC and TLC methods. With triphenylphosphate as internal standard, a pulse angle of 15°, 10 s relaxation delay, and 32–256 accumulations, the method has a precision of < 0.5%.

The ^{31}P NMR spectroscopy differentiates between the various phospholipids and has high dynamics in quantification. Only phosphorus containing substances are detected by this method, the analysis is not disturbed by other non-phosphorous components. The resonance frequency of phosphorus depends on the chemical environment within the molecule. Phospholipids with different chemical structures are therefore recorded at distinct frequencies. Frequency can be measured very precisely, even small differences in the chemical structure of phospholipids can be detected easily. Each phospholipid is represented by a single sig-

nal. Separation of the various phospholipids is not necessary as the phospholipids are characterised by their different resonance frequencies. Fig. 17 shows a ^{31}P NMR spectrum of soy lecithin.

The selective quantification of the PC degradation products 1-LPC, 2-LPC and GPC (Fig. 18) makes ^{31}P NMR spectroscopy the ideal method for testing the stability of liposome containing formulations in pharmaceutical products. The amount of the different phospholipids can be calculated from their integral areas.

AL721 is a 7:2:1 mixture of neutral glyceride, phosphatidylcholine and phosphatidylethanolamine, which is considered to be valuable in HIV therapy. In order to determine the ratio of phosphatidylcholine and phosphatidylethanolamine based on the comparison of signal intensities, a pulse sequence has to be developed which minimises the influence of T_1 relaxation times and NOE differences [20].
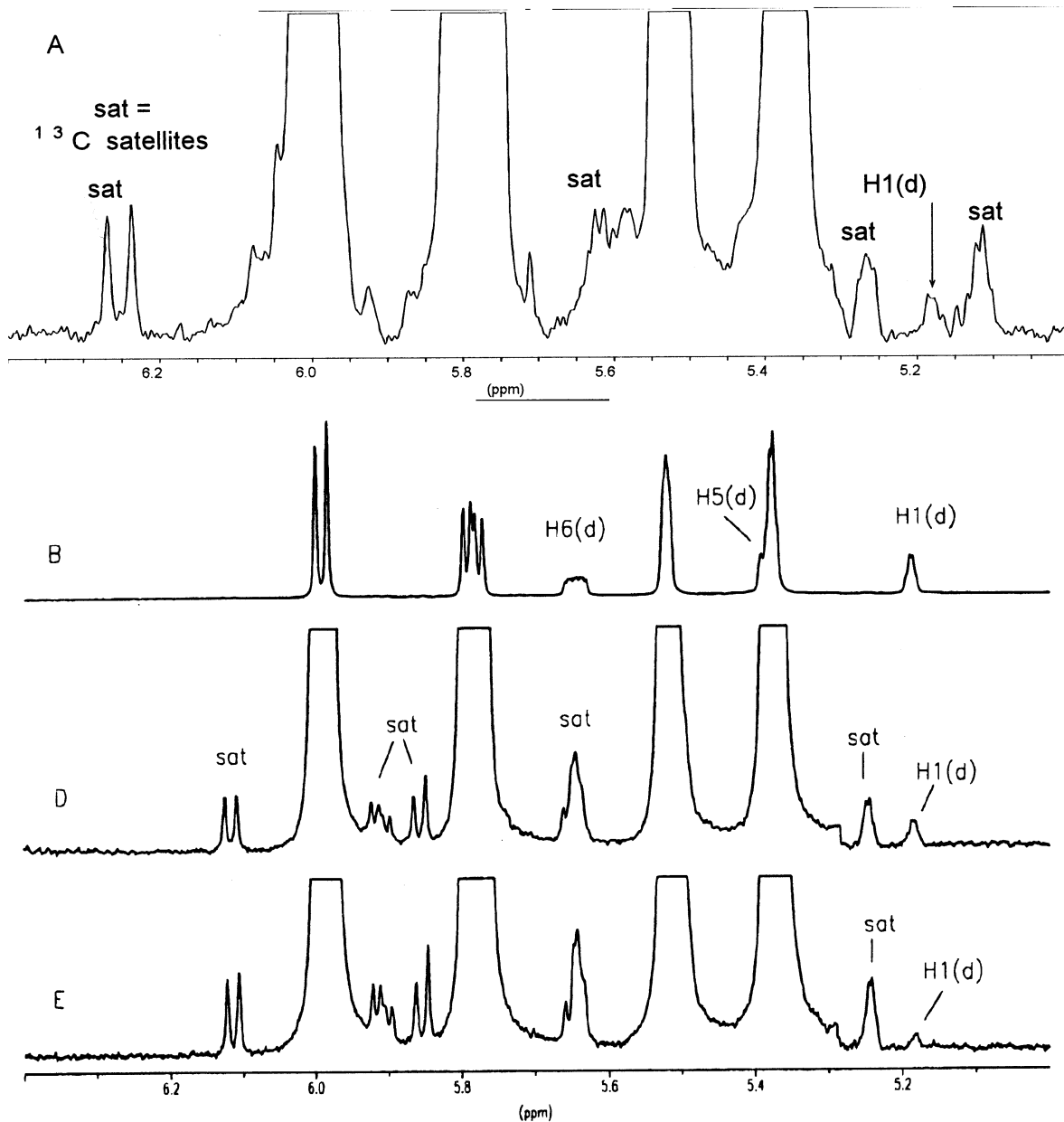
Fig. 13. (B), (D), (E) 600 MHz ^{1}H NMR spectrum taken from [14], Copied with permission from Pharmacopoeial Forum, 22(3). All rights reserved ©1996. The United States Pharmacopoeial Convention, Inc.; B: dihydrolovastatin enriched sample, D: Gist-brocades pilot product, E: 2 parts lovastatin USP and 1 part Gist-brocades pilot product, A: 300 MHz ^{1}H NMR spectrum of lovastatin.

The analysis of silicone products like simethicone can be performed with good selectivity, using ^{1}H NMR spectroscopy. The NMR-method was cross-validated[SSL] [11] against the official IR-method [21] (Fig. 19).

The comparison of IR and ^{1}H NMR spectra demonstrates the superiority of the NMR method, it provides more precise quantitative results and more information about structural details of silicones as shown in the following ex-
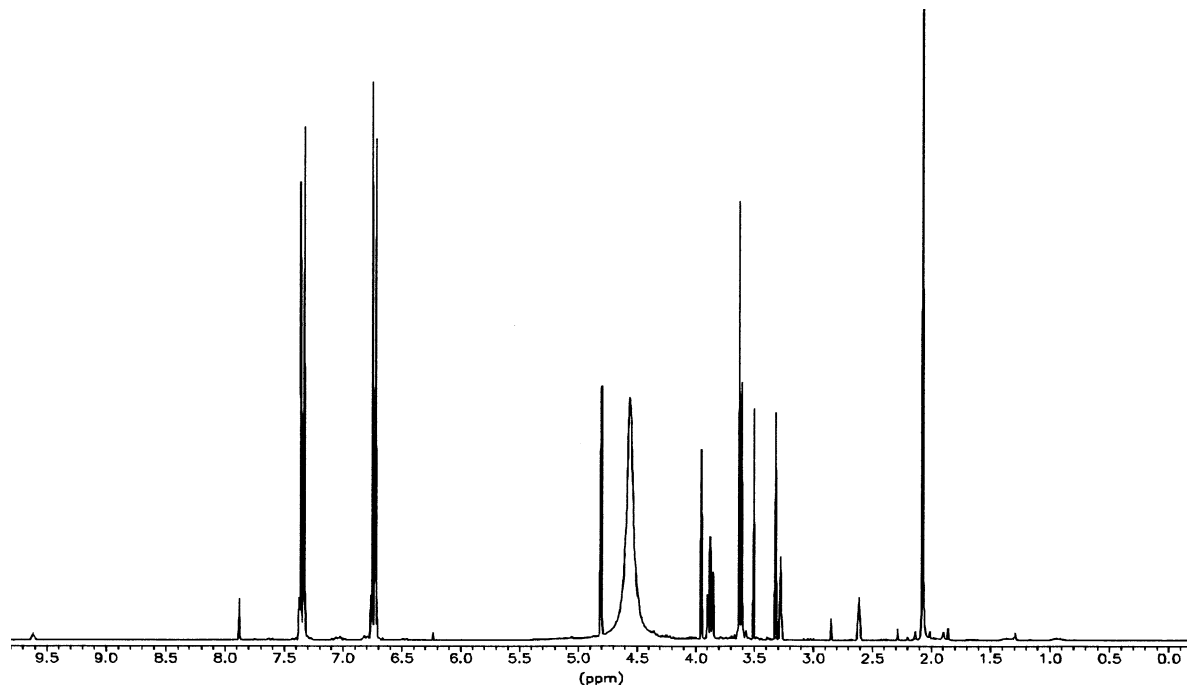
Fig. 14. ^{1}H NMR spectrum of Grippostad C, 300 MHz, solvent methanol-d$_4$.

panded spectra Fig. 20. Quantification can be done from the integral area by using an internal standard which does not interfere the silicone signals e.g. 2-(methyldiphenylsilyl)-ethanol. Structural data, e.g. chain length (and with that the viscosity), end groups (OH, TMS, OCH$_3$ etc.) or functional groups within the silicone chains, can be detected and quantified by means of ^{1}H NMR spectroscopy. As an example, the expansion of an ^{1}H NMR spectrum of silicone is shown in Fig. 21. The end groups Si(CH$_3$)$_2$–OH and three more links within the chain are well separated from the inner chain signals. The integrals can be used for the determination of the total chain length [11].

2.1.5. Fluorine NMR

Fluorocarbons like perfluorodecaline, perfluorooctane and perfluorobromooctane are expected to have an increasing number of medical applications. Because of the toxicity of oxidised metabolites, the amount of proton containing molecules is a critical parameter. In this case, the ^{1}H NMR spectroscopy is used to show the ab-

sence of protons and partly protonated fluorocarbons (Fig. 22) [11]. The measurement is done in neat liquid, and a capillary filled with benzene-d$_6$ is used as an external lock. The signals of protonated benzene and water are used for qualitative and quantitative calibration. The broad signals at 5.0 and 1.8 ppm are probe characteristics.

Especially in the field of dental care products inorganic phosphates and fluorides are used. The combination of ^{19}F NMR spectroscopy and ^{31}P NMR spectroscopy makes it possible to simultaneously determine the content of fluoride and monofluorophosphate in presence of ortho-, meta- and pyrophosphate.

The standard addition method is used for quantitation. After determination of the phosphates with ^{31}P NMR (Fig. 23) a calibration of the ^{19}F NMR spectrum is not necessary. The amount of monofluorophosphate is known from the ^{31}P NMR analysis. The fluoride content is determined from the integral areas of the fluoride and the monofluorophosphate signals in the ^{19}F NMR spectrum. Differences in the response of both
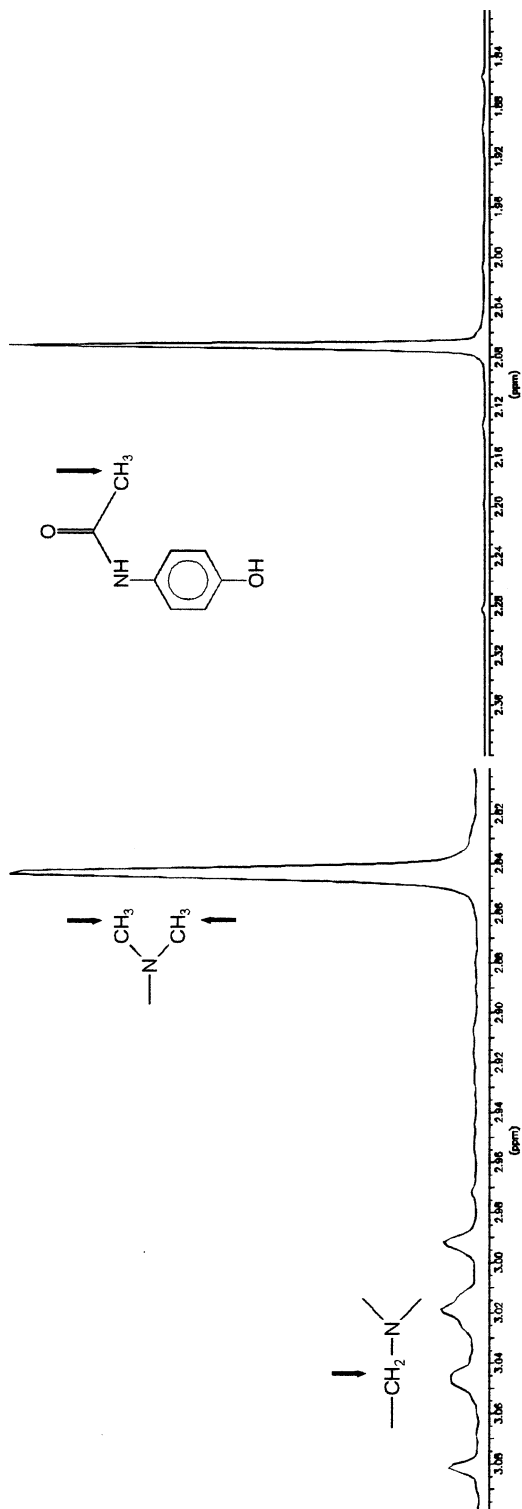
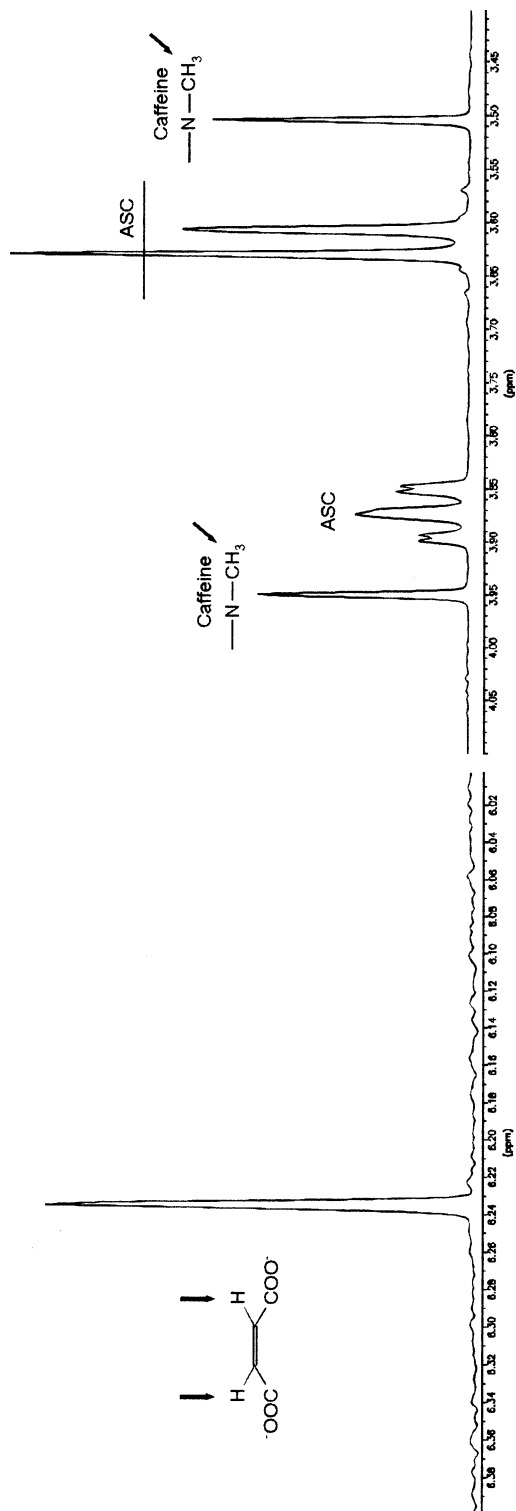Fig. 15. ^{1}H NMR spectrum (expansion; 1.82–3.10 ppm) of Grippostad C, 300 MHz, solvent methanol-d$_4$.

Fig. 16. ^{1}H NMR spectrum (expansion; 3.4–8.1 ppm) of Grippostad C, 300 MHz, solvent methanol-d$_4$.
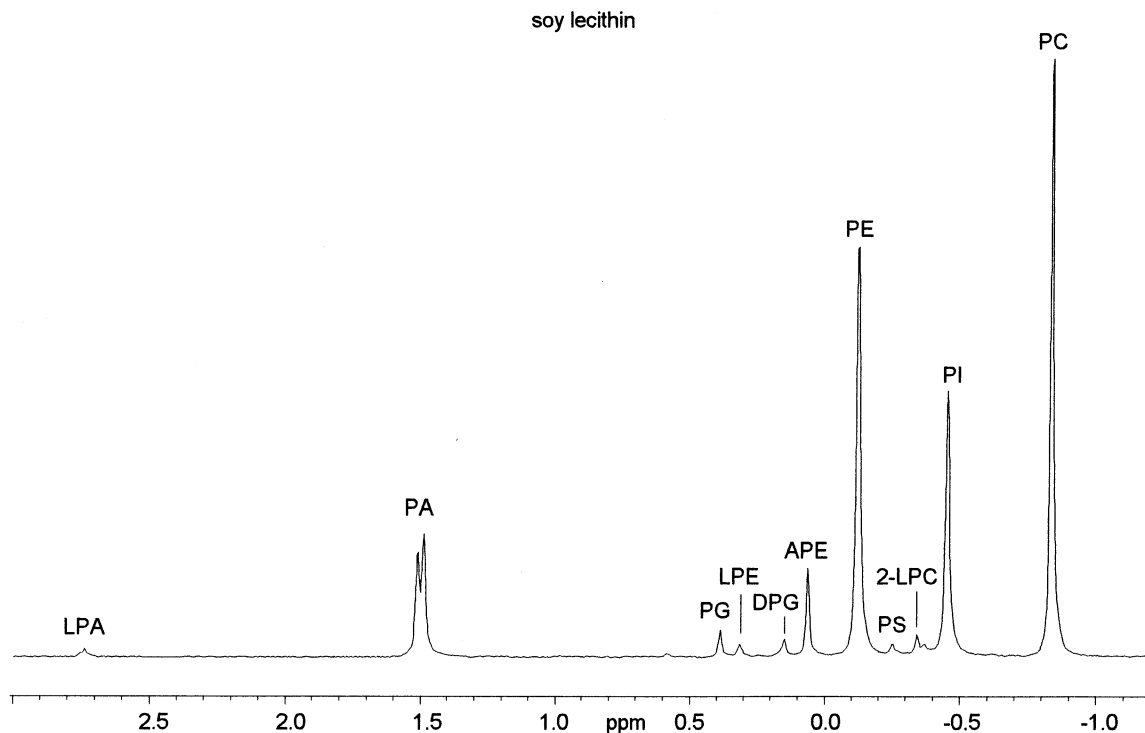
soy lecithin

Fig. 17. ^{31}P NMR spectrum of soy bean lecithin; (L)PA, (lyso)phosphatidylic acid, (D)PG, diacyl.

nuclei are determined by measurement of standardised solutions.

The sample preparation of dental care products depends on the kind of formulation. Organic components such as emulsifiers, surfactants and thickeners have to be removed as well as the water insoluble abrasives. In case of very low fluoride and phosphate content, it is recommended to evaporate the aqueous extract. The chemical shift of the phosphate signals depends considerably on the pH-value of the solution, therefore a buffer of pH-value of 8–9 should be applied. With strong acidic or basic medium, hydrolysis of meta- and pyrophospate occurs. The ring opening of the metaphosphate producing the linear triphosphate is easily detected in the ^{31}P NMR spectrum (Fig. 23). The outer P-atoms show a doublet at $\delta = -10$, the inner P-atom is represented by a triplet at $\delta = -25$ ppm. The large coupling constant (864 Hz) between the fluorine and the phosphorus nucleus (Fig. 24) is worth mentioning, which is of course found in the ^{31}P NMR spectrum as well as in the ^{19}F NMR spectrum.

2.1.6. Identification and quantification of impurities

There is a long-standing tradition of using TLC and HPLC for the quantification of impurities arising from synthetic processes or degradation of drugs. In the last five years, the pharmacopoeia committees have required the structures of impurities to be displayed in a transparency statement at the end of each drug substance monograph. This HPLC analysis is more reliable for separation and identification of impurities. Meantime, many new manufacturers (often from China and India) provide drugs on the market which have been synthesised in other ways than registered. Consequently, the pattern of impurities has changed and the HPLC method used in the pharmacopoeiae may no longer be suitable. In this context, a NMR method can be superior to HPLC methods because a new impurity can be easily found and identified in NMR spectra.

The impurity pattern (about 15 percent!) of dequalinium chloride varies depending on synthesis conditions. The drug is obtained by conversion of 4-aminoquinaldine with 1,10-dihalodecane.
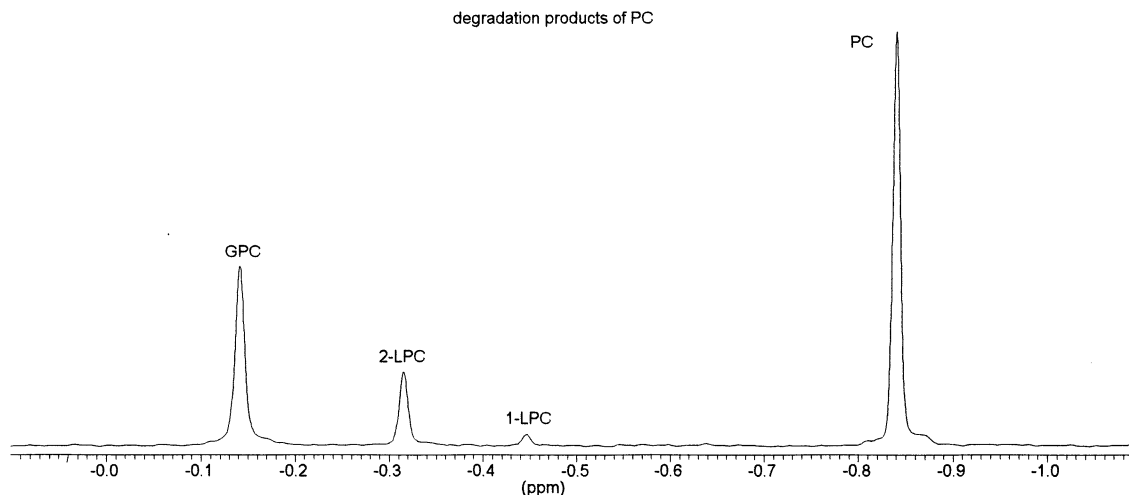
Fig. 18. ^{31}P NMR spectrum of phospholipid degradation products: 1-LPC, 2-LPC and GPC, glycerophosphatidylcholine; 121 MHz, solvent CDCl$_3$/methanol (2:1).

Apart from residual aminoquinaldine and a product consisting of three quinaldine molecules connected with two decanes, as well as another overalkylation product, which were found by HPLC, the 400 MHz ^{1}H NMR analysis exhibits a further impurity, 4-amino-1-[10-[(2-methylquino-lin-4-yl)amino]decyl]-2-methylquinolinium chloride (see Fig. 26) [23], which has a defined pharmacological activity. It is used in practice to treat trypanosomiasis in cattle. Thus, this impurity has to be quantified and limited in a pharmacopoeia monograph [24]. The ^{1}H NMR is an effective method because the examination of the methyl region (2.6–2.9 ppm) reveals distinct signals for at least one of the methyl groups of each compound. Accurate integration of these methyl signals allows determination of the (molar or weight) composition of the samples. Some commercially available samples were found to contain 75.7% dequalinium chloride, 18.2% overalkylation impurity, 5.8% of the new impurity and 0.3% 4-aminoquinaldine [22].

A further analysis of a dequalinium chloride was made by Spectral Service [11] (see Fig. 25). The methyl group as well as the aromatic CH-signals (6.3–7.12 ppm) show the same impurity signals. The molar content of the impurities was calculated from the methyl signal region as well as from the aromatic signal region. The results are displayed in Table 1.

Captopril, an antihypertensive agent, is known to contain the disulfide analogue, the *RS*-diastereomer (epicaptopril) and methylmercapto-propionic acid as impurities [25]. Although the 400 MHz ^{1}H NMR spectrum recorded in DMSO-d$_6$ exhibits two amido conformers, the diastereomers and the disulfide can be distinguished by signals between 4.2 and 4.7 ppm, representing the methine hydrogen attached to C-2 of the pyrrolidine ring [25]. These signals can be also used for quantification (Fig. 27).

Vitamin E, + -,α-tocopherol as free phenol, acetate or succinate ester, contains small amounts of β-, γ-, and/or δ-tocopherol. Owing to partially poor resolution and the requirement of authentic reference standard, HPLC and GC analysis turned out to be unsatisfactory. Baker and Myers [26] could prove that both methods, ^{1}H and ^{13}C NMR, are suitable to quantify the composition of vitamin E by integration of corresponding signals.

There are countless examples which describe the structure elucidation of drug impurities by means of NMR spectroscopy. Mostly, the impurities were collected, purified and an NMR spectrum recorded and assigned, e.g. the recently reported isomers of betamethasone [27]. Since no quantitative analyses have been performed, those examples are outside the scope of this review.
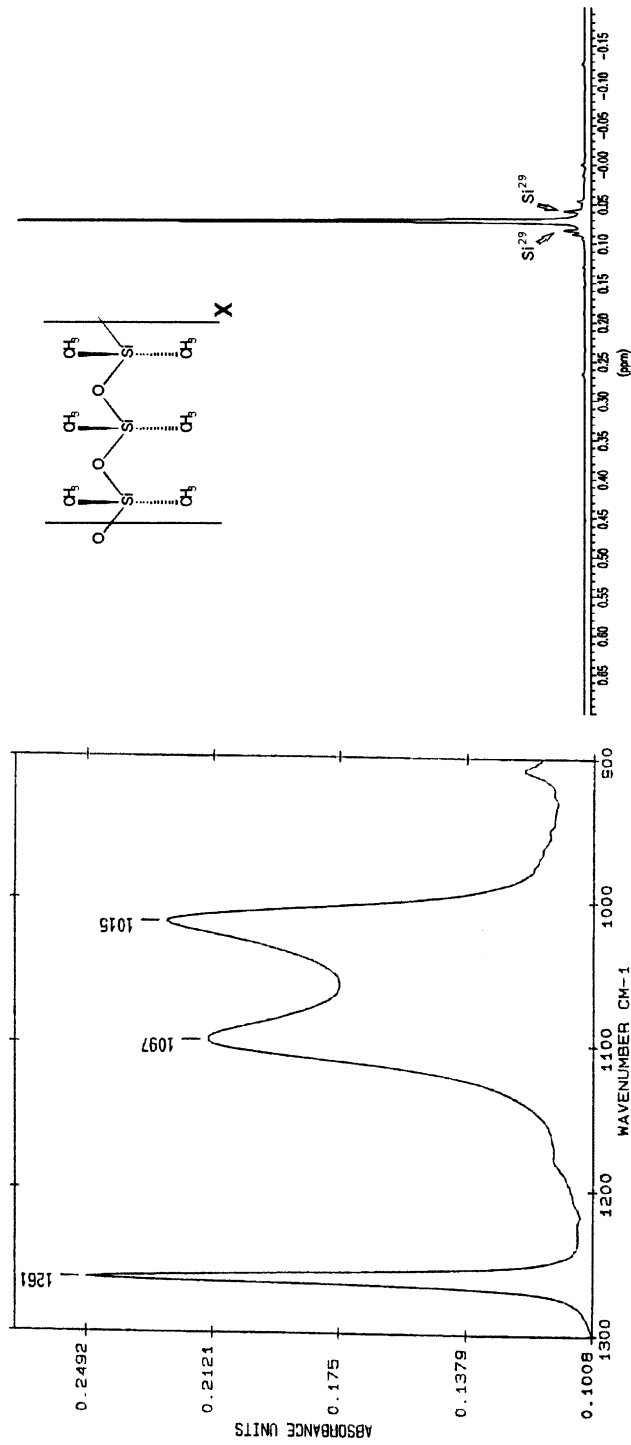
Fig. 19. IR spectrum (film) and ^{1}H NMR spectrum, (300 MHz, solvent CDCl$_3$) of simethicone.
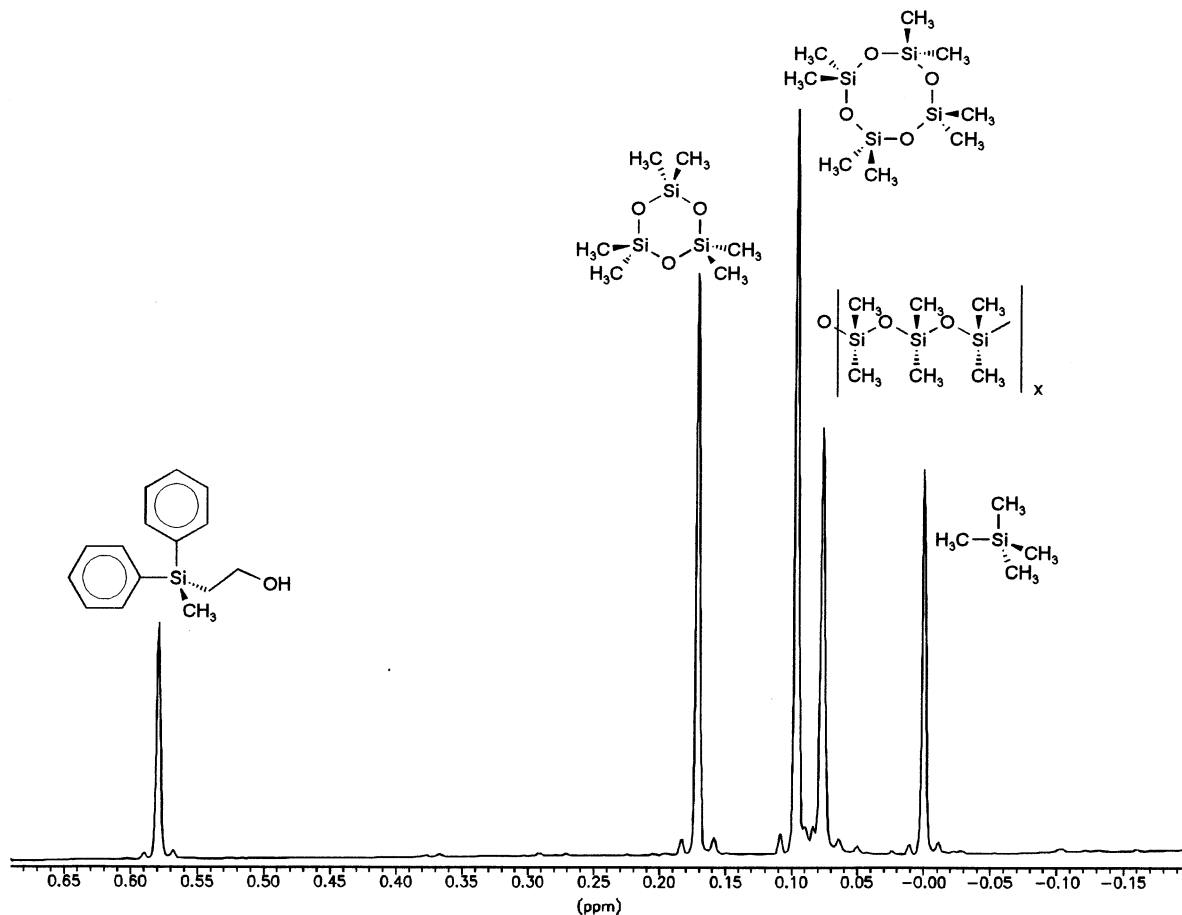
Fig. 20. ^{1}H NMR spectrum of a mixture of siloxanes, 300 MHz, solvent CDCl$_3$.

2.1.7. Decomposition reactions

Benzodiazepines, which attract interest as anxiolytics and sedatives, are known to hydrolyse in physiological medium, giving benzophenone derivatives by ring-opening (Fig. 28). Dawson et al. tried to observe the degradation process of flurazepam dihydrochloride in different media at various temperatures, using ^{1}H-, ^{13}C- and ^{19}F NMR spectroscopy [28,29].

Since ^{19}F NMR allowed detection of initial trace amount (< 1%) of the ring-opened product, it also afforded the best means of detection and quantifying the various entities in solution. Over a period of 24 h the equilibrium between flurazepam and the ring-opened form in D$_2$O was found to amount to 44:56 percent ratio. The equilibrium did not influence the degradation re-

action resulting in the benzophenone. Some aged samples were investigated; the percentage of benzophenone ranged from 5–10, no further degradation was observed in solution over a period of 24 h at 0 and 27°C. Similar studies have been described for several drugs, e.g. the alkaline hydrolysis of cefotaxime which was elucidated and the kinetic of the reaction observed by means of ^{1}H NMR spectroscopy [30], or the decomposition process of bispyridinium aldoximes, acetylcholine esterase reactivators, at different pH values and various temperatures [31].

The process of photodecomposition of the calcium channel blocker nifedipine can also be observed by ^{1}H NMR spectroscopy [32]. The singulets between 2.3 and 2.6 ppm representing the methyl groups and 3.3 and 3.7 belonging to
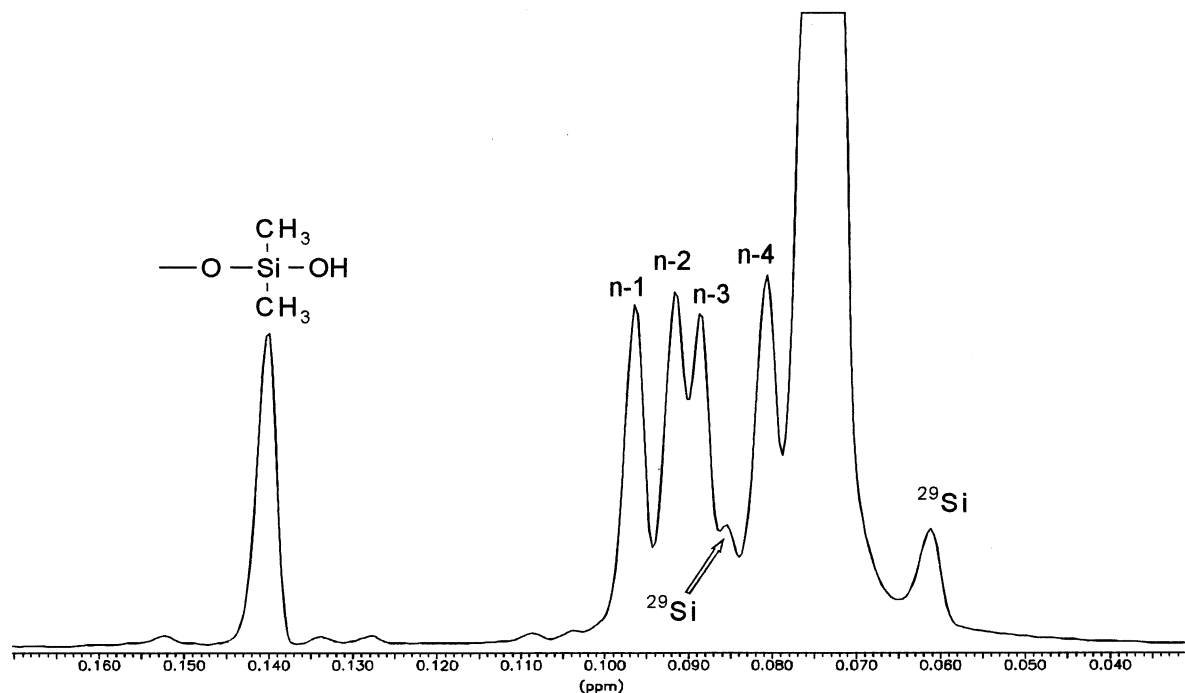
Fig. 21. Expansion of an ^{1}H NMR spectrum of silicone, 300 MHz, solvent CDCl$_3$.

the methoxy groups are well separated for each compound. Nifedipine accurately weighed was taken from a capsule, dissolved in a mixture of 40% CDCl$_3$ in CCl$_4$, and *t*-butanol added as an internal standard. The spectrum was recorded and the content of photodecomposition calculated from the integral of the methoxy signals in comparison with the *t*-butyl signal. Additionally, a spectrum was taken after exposure of the solution in the NMR-tube to diffused sunlight. Nearly 100 percent of the 4-(2′-nitrosophenyl)pyridine compound was found after 8 h. The method was as accurate and precise as the HPLC method described in the USP. In this case, the NMR method is superior to the HPLC procedure with respect to rapidity (about 5 min), simplicity and specificity (Figs. 29–31).

Quantitative analysis could be improved by the introduction of the joint application of HPLC and NMR spectroscopy in drug impurity profiling. This is demonstrated by Görög et al. for some examples, such as the *E*/*Z* isomers of an ethinylestradiol and impurities of enalapril maleate [33]. The details of this technique will be discussed in the section NMR in body fluids.

Some drugs are not stable in aqueous solutions and undergo equilibrium reactions. These equilibration reactions can be observed by NMR spectroscopy. Madopar®, used in treatment of Parkinson's disease, consists of levodopa and benserazide (BZ). Serylhydrazide (SZ) is a degradation product of benserazide always present in tablets. The amount of serylhydrazide increases during storage due to a reaction with water. The same reaction takes place when a sample is prepared for analysis in an aqueous solution [11]. The equilibrium reaction is shown in Fig. 32.

In ^{1}H NMR spectra, all three molecules are detectable. The ratio of the aromatic products can be taken from the integrals of the benzylic protons, since no response factor has to be taken into account. This ^{1}H NMR technique does not need standards and can be used as an ab initio method. Figs. 33–35 show the ^{1}H NMR spectra of a freshly prepared MeOD/D$_2$O/DCl extract of Madopar® and of an extract which is heated for 30 min to 80°C. The equilibrium ratio is about 1:1. It is not possible to isolate the dibenserazide (DBZ) to produce a standard for HPLC analysis.
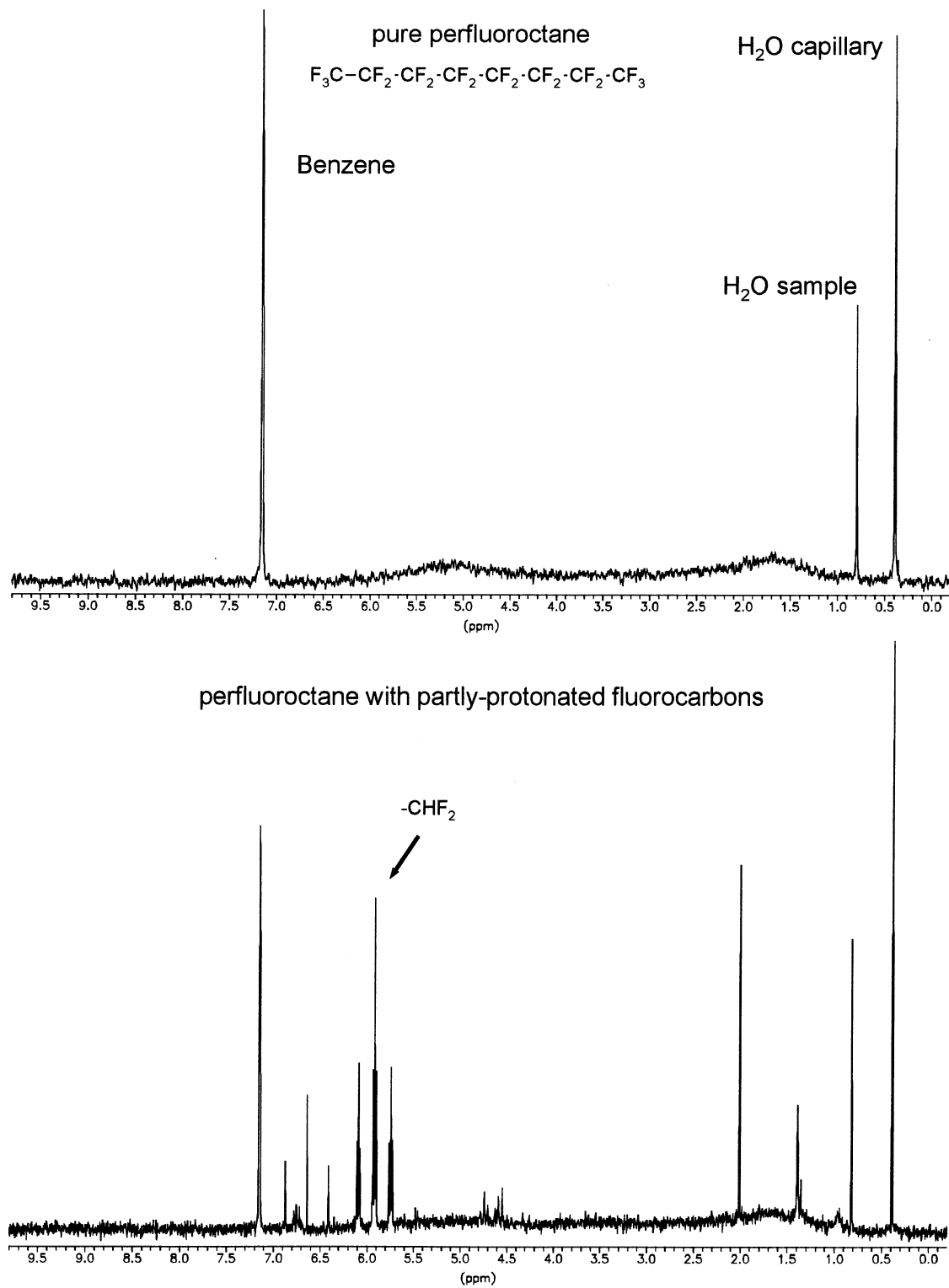
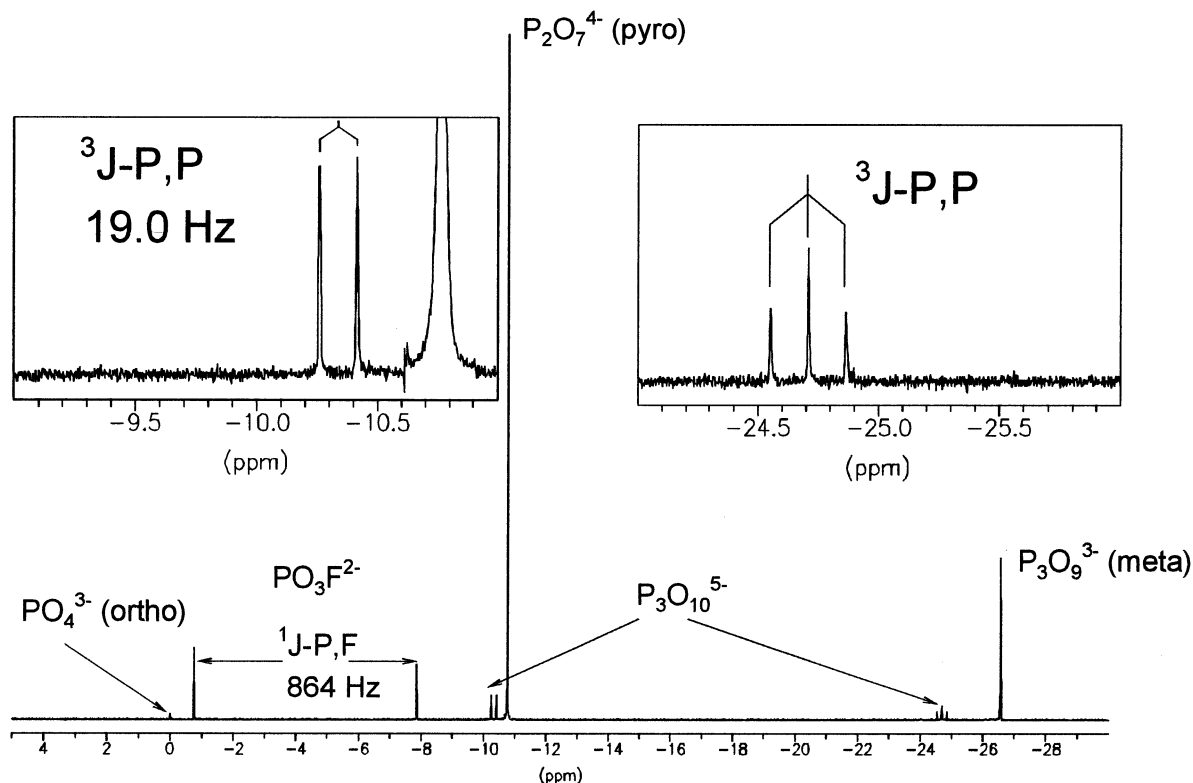Fig. 22. Analysis of fluorocarbons, 300 MHz, neat liquid, external lock.

Fig. 23. ^{31}P NMR spectrum of a tooth paste, 121 MHz, solvent D_2O.

Cyanoacrylates are used to close cuts or wounds, even vessels can be glued together. These materials contain stabilisers like hydroquinone in amounts between 100 and 500 ppm. Due to the high reactivity with respect to polymerisation, chromatographic methods are not practicable and can not be recommended to quantify the amount of hydroquinone. In a $CDCl_3$ solution the acrylate is stable for some hours and quantification is possible by means of ^1H NMR spectroscopy [11] (Fig. 36).

The ^{13}C-satellites of the acrylate signals are used for calibration of the hydroquinone signal. To facilitate integration, the addition of some benzene-d_6 is necessary to shift the hydroquinone signal between the ^{13}C-satellites of the acrylate signal. Due to the natural abundance of the ^{13}C nucleus, the area of a ^{13}C satellite is defined to be 5600 ppm in relation to the main signal. The amount of hydroquinone can be calculated from the expression with MW as molecular weight:

Hydroquinone [ppm]

$$= \frac{5600 * area_{hydro.} * MW_{hydro.}}{2 * (area_{H1} + area_{H2}) * MW_{acrylate}}$$

The method was validated in the range of 30–1000 ppm.

In Fig. 37 the ^1H NMR spectrum of a cyanoacrylate containing 450 ppm hydroquinone is shown.

These examples impressively demonstrate the high potential of NMR spectroscopy in quantitative analysis: On the one hand, a drawback of NMR in comparison to MS is the fact that the sample size required for structure elucidation of an impurity is much higher. On the other hand, there are several advantages:

- In addition to the structural information, an NMR spectrum can simultaneously provide information on the quantity of an impurity. In most cases, the integration of signals used for quantification is more precise and accurate than the HPLC analysis.
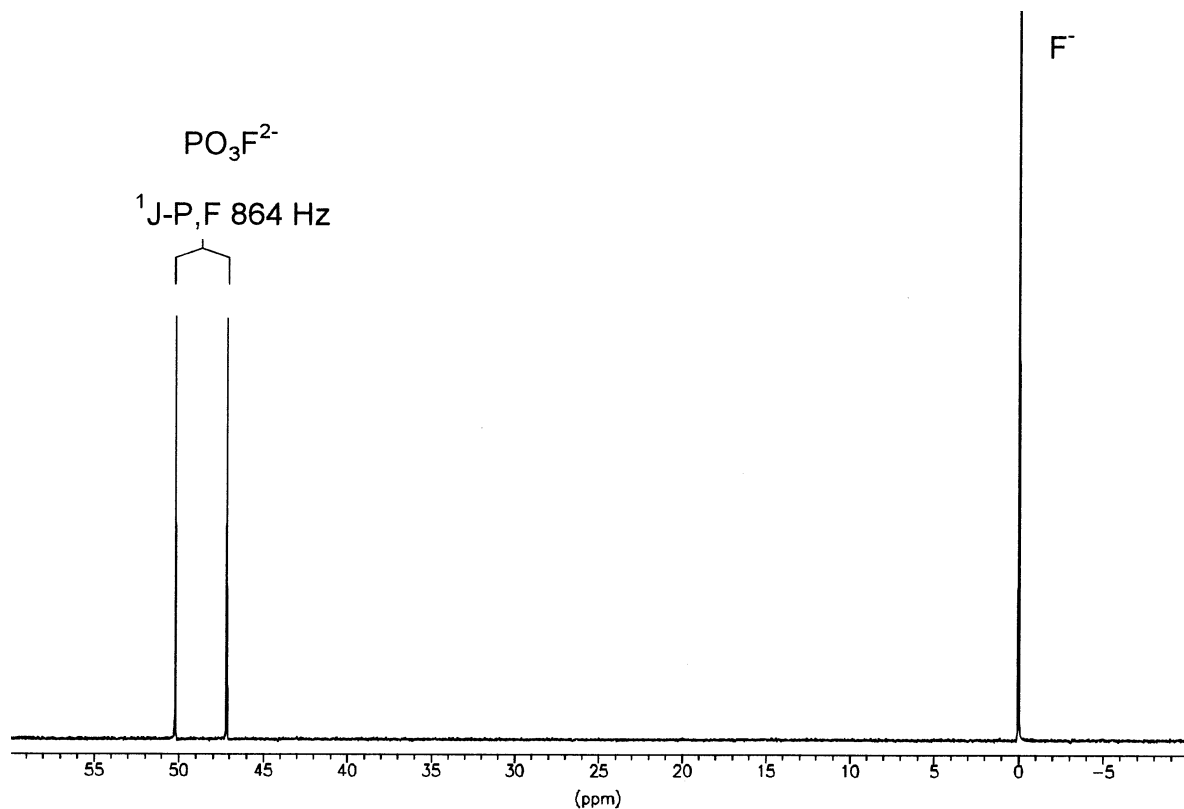
Fig. 24. ^{19}F NMR spectrum of a tooth paste, 297 MHz, solvent D_2O.

- Normally, no isolation of the impurity is necessary.
- No expensive authentic reference samples are necessary.
- In most cases, NMR spectroscopy is quicker (e.g. no equilibration of HPLC column), easy to perform and more specific.

2.2. Determination of the isomeric composition of drugs

Apart from chiral HPLC methods, ^{1}H NMR spectroscopy has been often used to determine the enantiomeric excess (ee [%] = {$(R - S)/(R + S)$} × 100) of an asymmetric synthesis by derivatisation with chiral (enantiomerically pure) reagents, e.g. Mosher's reagent, α-methoxy-α-trifluormethyl-phenylacetic acid (MTPA) [34]. Recently, international authorities, e. g. the European Pharmacopoeia Commission, have

aimed for the introduction of NMR spectroscopy for chiral analysis.

Derivatisation methods yielding diastereomeric substances depend sensitively on the quantitative conversion of both enantiomers with chiral reagent. In addition, it is necessary to prove that no isomerization occurs during the derivatisation reaction. Thus, the subsequent analysis may be falsified by this reaction [35]. Moreover, the additional step is time-consuming, which is undesirable in routine procedures. Chiral lanthanide shift reagents (LSR), such as tris[3-(trifluoromethylhydroxymethylene)-d-camphorato]ytterbium [Yb(tfc)$_3$] or tris(((heptafluoropropyl)hydroxymethylene)-d-camphorato)-europium [Eu(hfc)$_3$], also have a long-standing tradition in chiral discrimination, especially for low-field NMR instruments [36]. Due to the formation of diastereomeric complexes between the enantiopure shift reagent and the enantiomers analysed, a double set of more or less shifted signals can be
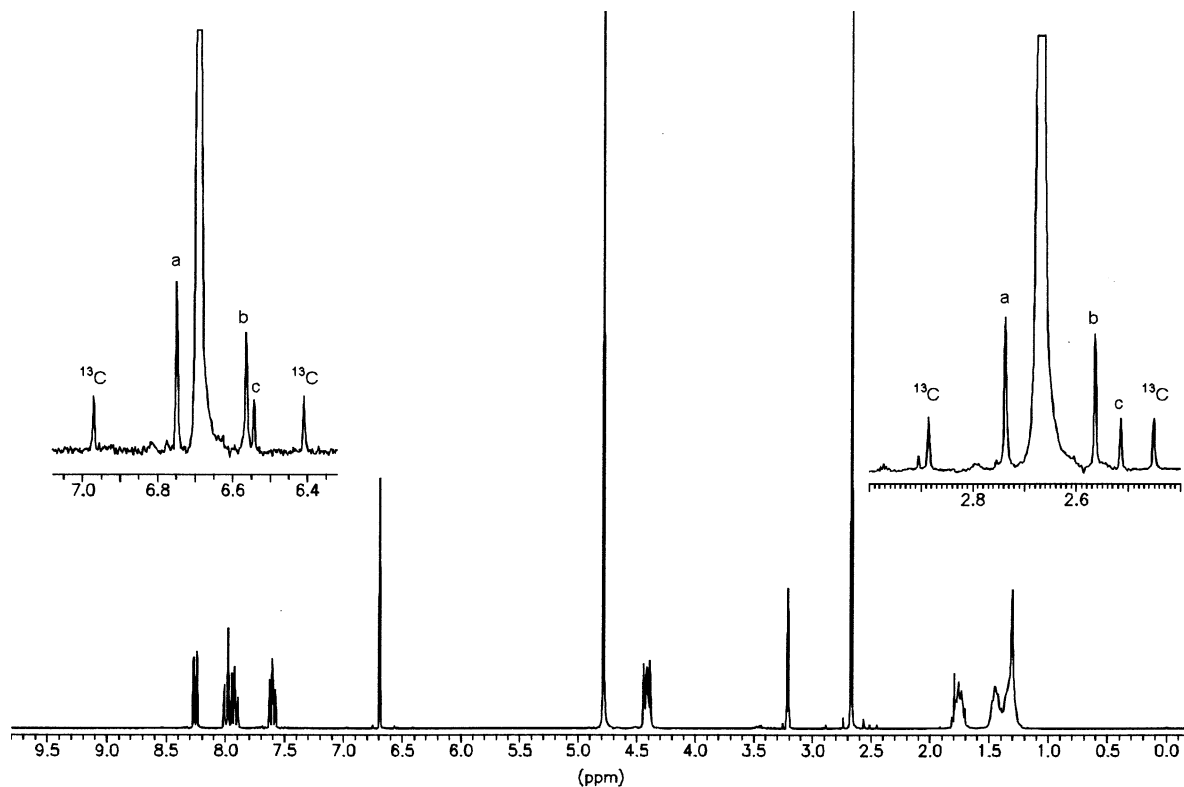
Fig. 25. ^{1}H NMR spectrum of dequalinium chloride, 300 MHz, solvent D_2O.

seen for the diastereomers. The integrals of a corresponding set of hydrogens can be used to determine the optical purity of a sample. However, on high-field machines the CSRs tend not to work well due to severe line-broadening. In addition, impurities in the sample measured as well as in the solvent may create problems, e.g. the presence of water, result in the formation of a precipitate of europium oxide [37,38]. Therefore, most analyses under development use chiral solvating agents (CSA) for chiral discrimination, because they do not suffer from these disadvantages [39]. Again, chiral recognition is created by formation of diastereomeric complexes. Hence, CSA and solute should have common features of complementary functional groups, which are in general hydrogen bond donors or acceptors. Thus, acids, amines, alcohols or cyclic compounds, e.g. cyclodextrins, crown ethers or peptides are suitable CSAs (see Fig. 38) [40].

Dawson et al. presented a study using different reagents, such as α-, β-, and γ-cyclodextrins, S-α-methylbenzylamine, (R) - N - 3,5 - dinitrobenzoyl -- methylbenzylamine, (S) - 1 - (1 - naphthyl) - ethyl - amine and (R)-2,2,2-trifluoro-1-(9-anthrylethanol) (R-TFAE), for enantiomeric evaluation of the dissolution of ketoprofen (Fig. 39) formulations [41].

Samples of known amounts of drugs were dissolved in $CDCl_3$ containing the various CSAs as well as a known amount of internal standard (o-dimethoxybenzene) and ^{1}H NMR spectra recorded on a 400 MHz instrument. It was checked whether the CSAs produced well resolved peaks for both compounds, whether the CSAs were stable and had no resonances close to the signals of interest in ketoprofen, and whether the signals of the enantiomers of ketoprofen were well separated at reasonably low concentrations of the CSAs. With the exception of the naphthylethyl-
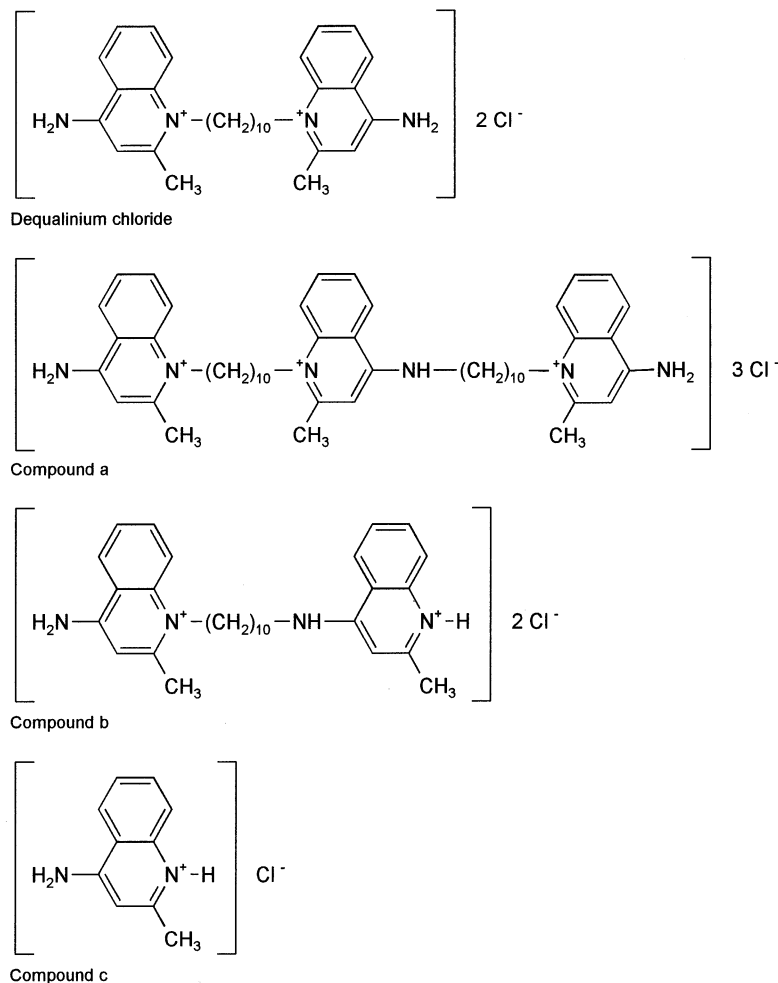
Fig. 26. Dequalinium chloride and by-products a, b, c.

amine, all CSAs produced good separations of optical isomer resonances, and so can be used to determine the isomeric ratio upon dissolution and metabolism.

Dauwe and Buddrus [42] tested a series of chiral amines for their ability to discriminate between the enantiomers of several acidic compounds. Strong basic amidines, as shown in Fig. 38, turned out to be suitable for resolution of even weak acidic compounds, such as phenols, barbiturates and alcohols. The same group [43] developed chiral palladium complexes with diamines for discrimination of α-amino acids and determination of *ee* resulting from asymmetric synthesis.

Methylphenidate, an antipsychotic drug, has two centres of chirality (Fig. 39): A comparative study of the central and peripheral stimulant effects of the four isomers has indicated that the *threo*-isomers are more active than the *erythro*-forms, and that the $(2R,2'R)$-*threo* enantiomer is more active than the corresponding antipode [44]. Thus, it is pivotal to know the isomeric composition of the drug in clinical use. Hanna and Lau-Cam first used a chiral Eu(III) shift reagent for the determination of the enantiomeric excess [45]. Although the method suffered from line-broadening, they were able to determine 98.8–100.5% of the added enantiomer ($99.5 \pm 0.7\%$ of added). (R)-

Table 1
Molar distribution of dequalinium chloride and by-products

	Calculated from	
	Methyl signal region	Aromatic signal region
Dequalinium chloride	96.20	96.42
Compound a	1.90	1.77
Compound b	1.43	1.38
Compound c	0.48	0.43

TFAE was found to be superior to the CSR in a 90 MHz spectrum when using a TFAE/substrate molar ratio of 4.8 [46]. Looking at the methyl resonances of methylphenidate the mean ± S.D. recovery value for the $(2S,2'S)$-threo-isomer amounted to 99.9 ± 0.6% of added, which is in good agreement with the CSR study. However, the TFAE approach is simpler, because it does not require reagents and solvents of high purity and anaerobic working conditions. In addition, with (R)-TFAE it was possible to determine the absolute configuration of the enantiomers.

Lacroix et al. compared an HPLC with a ^{1}H NMR method for quantification of the *R*-enantiomer in *S*-timolol maleate, a β-blocking substance (Fig. 39) [47]. For satisfactory resolution, the HPLC required the expensive cellulose tris-3,5-dimethylphenylcarbamate column (Chiracel OD-H) and a mobile phase of 0.2% dimethylamine and 4% isopropanol in hexane and ^{1}H NMR (400 MHz) R-TFAE as a CSA in CDCl$_3$. The resonance of the *t*-butyl group between 1.0 and 1.1 ppm was used to measure the *ee*. With both methods, 0.2–4.0% of the *R*-enantiomer in *S*-timolol could be determined precisely. Additionally, both methods, HPLC and NMR, were found to be superior to specific rotation, which is used in the USP XXIII for determination of optical purity. This superiority of NMR and HPLC methods over optical rotation has often been reported [34].

Diltiazem hydrochloride, a coronary vasodilatator, contains two centres of chirality in the benzothiazepine skeleton, which results in four isomers (Fig. 39). Only the 2,3-*cis*-(+)-isomer is pharmacologically active. From a 400 MHz [1]H
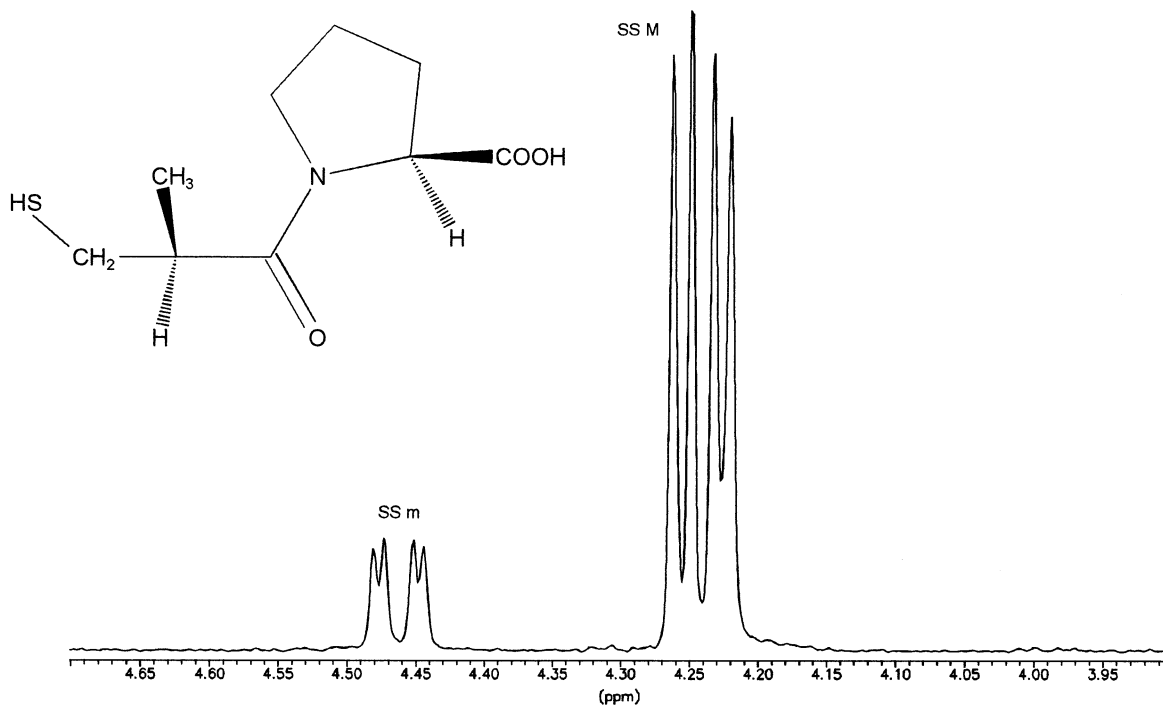

Fig. 27. ^{1}H NMR spectra of captopril, 300 MHz, solvent DMSO-d$_6$; m = minor, M = major.
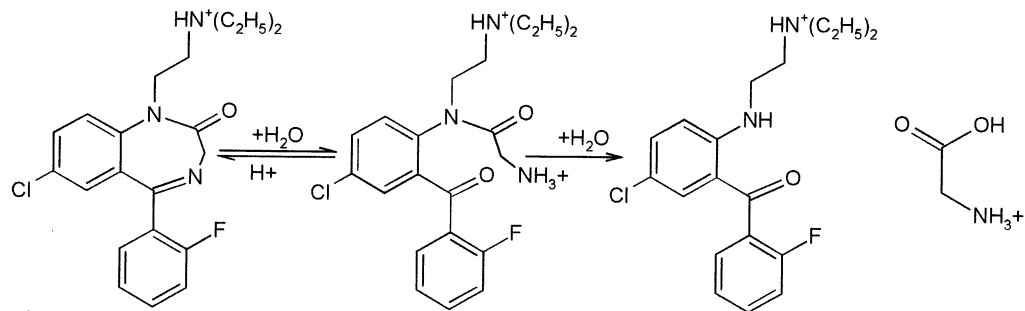
Fig. 28. The degradation pathway of flurazepam.

NMR spectrum of raw material in CDCl$_3$, minimum amounts of the *trans*-diastereomer could be easily detected over a range of 0.5–5.0% [48]. After addition of 10 mg (R)-TFAE to 2 mg (*cis*)-diltiazem in 500 μl CDCl$_3$, the amount of the antipode could be determined using the integration of the acetyl-signal. The enantiomeric impurity could be quantified down to levels of about 0.2%. The limits for the method could easily be lowered by increasing the number of scans (here 128 scans). It is noteworthy that the method is also superior to the specific rotation applied in the USP, which allows about 2.6% of the antipode to be present (+ 110 to + 116°).

Nadolol, another β-blocker applied in treatment of angina pectoris and hypertension, consists of two additional centres of chirality in the 1,2,3,4-tetrahydro-naphthalenediol moiety beside the propanol chain (Fig. 39). Since the ring hydroxyl groups are in *cis*-position, four isomers, two racemic diastereomers, are possible. The racemic ratio was determined after derivatisation with benzoic anhydride/dimethylamino-pyridine to give a tribenzoate derivative [49]. Using the relative height of the signals of the *t*-butyl groups, the racemic composition was found not to meet the USP limits. Thus, a further determination of enantiomeric composition made no sense. However, the use of CSAs, such as (R)-1,1′-bi-2-naphthol, in order to separate *t*-butyl signals of the four isomers failed.

The S-isomer of nicotine (Fig. 39), originally extracted from *Nicotiana* species, is attracting pharmacological interest for its potential to aid smokers to cope with the cigarette abstinence syndrome. The R-enantiomer is also of pharmacological interest.

Thus, it is important to have a method to determine the ee in inhalators, nasal sprays, skin absorption patches, or chewing gums. Since CSR, such as Eu(tfc)$_3$ and Yt(tfc)$_3$, cause a strong line-broadening of the ^{1}H NMR signal, [50,51] ^{13}C NMR spectroscopy in connection with Yt(tfc)$_3$ was applied in this case [50]. Upon complexation with the chiral lanthanide reagent, signals of all aliphatic carbon atoms were resolved. The signal of the *ipso*-carbon atom C-2′ turned out to be the best for routine analysis. Using INEPT spectra with pulse delays of 0.9 s, at least a 100-fold excess of one of the enantiomers could be determined. For very high *ee*s of one enantiomer an amount of 1–3 mg nicotine and an overnight acquisition was necessary. Larger amounts found in pharmaceutical formulations would be analysed within 1–2 h.

Cyclodextrins of various size (α-, β-, γ-CD) have been often shown to discriminate between drug enantiomers upon complexation [52].

Using β-CD the enantiomers of several drugs, e.g. flurbiprofen [53], ephedrine [54], prostaglandin E1 [55], several chiral H-1 antihistamines [56], gliclazide [57], or thromboxane antagonists [58], could be well resolved. Derivatised CDs often exhibit improved chiral recognition properties [59]. *Heptakis*(2,3-di-*O*-acetyl)-β-CD is able to resolve a series of pharmacologically active phenethylamines with their structures being systematically varied [60–62]. Similar results have been reported for hydroxypropyl, as well as for permethylated β-CD [63] and perphenylcarbamated b-CD to resolve atenolol [64].

The advantages of CDs as CSA are water solubility, no signal broadening effects, and the narrow chemical shift range of the CDs. Thus, they
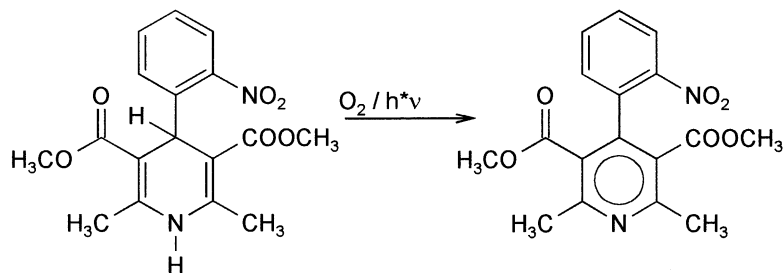
Fig. 29. Nifedipine and its photodecomposition product.

are suitable reagents for quantification of the chiral purity of drugs. The enantiomeric excess of the psychostimulants amphetamine and norephedrine and the antiparkinson drug selegiline (Fig. 39) could be easily determined using *heptakis*(2,3-di-*O*-acetyl)-*β*-CD. For example, many signals of the racemate selegiline are resolved in the presence of the CD, dissolved in aqueous buffer, pH 4.5, see Fig. 40. The signals of the C-methyl group were used to determine the enantiomeric impurities (see Fig. 41) [65].

Taken together with regard to the determination of the isomeric composition of drugs, NMR has several advantages over HPLC:

1. The development and validation, respectively, of an NMR method usually takes a few weeks, while a HPLC methodology may take several months.
2. NMR methods were found to be more robust than HPLC, which is subject to incalculabilities of column reproducibility and mobile phase composition; a suitability test has to be performed to eliminate the vagaries of HPLC.
3. After development an NMR analysis can be completed within an hour, whereas the HPLC analysis may take a day or more, unless the instrument is already prepared for the job. In routine analysis both methods can be automated. Hence, the analysis time will be the same.
4. Mostly, the NMR analysis may take less technician time than an HPLC method. Thus, the cost of running the NMR analysis could be less than that for an HPLC analysis.

3. NMR of body fluids

Since high-field NMR-instruments have been introduced to scientific laboratories, it has been possible to directly detect and quantify drugs, metabolites of drugs, and toxic substances in urine and other body fluids. Since no assumptions have to be made prior to the analysis, more or less no working-up procedure is necessary, no artefacts have to be expected and no recovery experiments have to be performed, NMR spectroscopy is an extremely powerful tool to measure levels of metabolites in various body fluids, e.g. urine, bile, blood, or plasma, as well as to elucidate unknown metabolites and metabolism pathways. However, the NMR method suffers from the drawback that the analyte must be present at a concentration of $> 50 \ \mu M$ in the sample. Attempts to overcome this problem will be described in the second part of this chapter.

3.1. NMR of urine: studies of metabolism

3.1.1. ^1H NMR spectroscopy

Since ^1H, ^{13}C, ^{14}N, and—with restrictions—^{19}F, and ^{31}P are present in endogenous biomolecules and drugs, these magnetically active nuclei can be used in metabolism studies. In case of ^1H NMR spectra the intense water signal occurring in urine has to be suppressed by a certain pulse sequence, e.g. the application of a homogated secondary irradiation field at the solvent resonance frequency resulting in a selective saturation of the signal, the WEFT (water elimination Fourier Transform) or WATR (water attenuation by T_2 relaxation) method; for summary see Refs. [66,67]. An alternative method to avoid the water
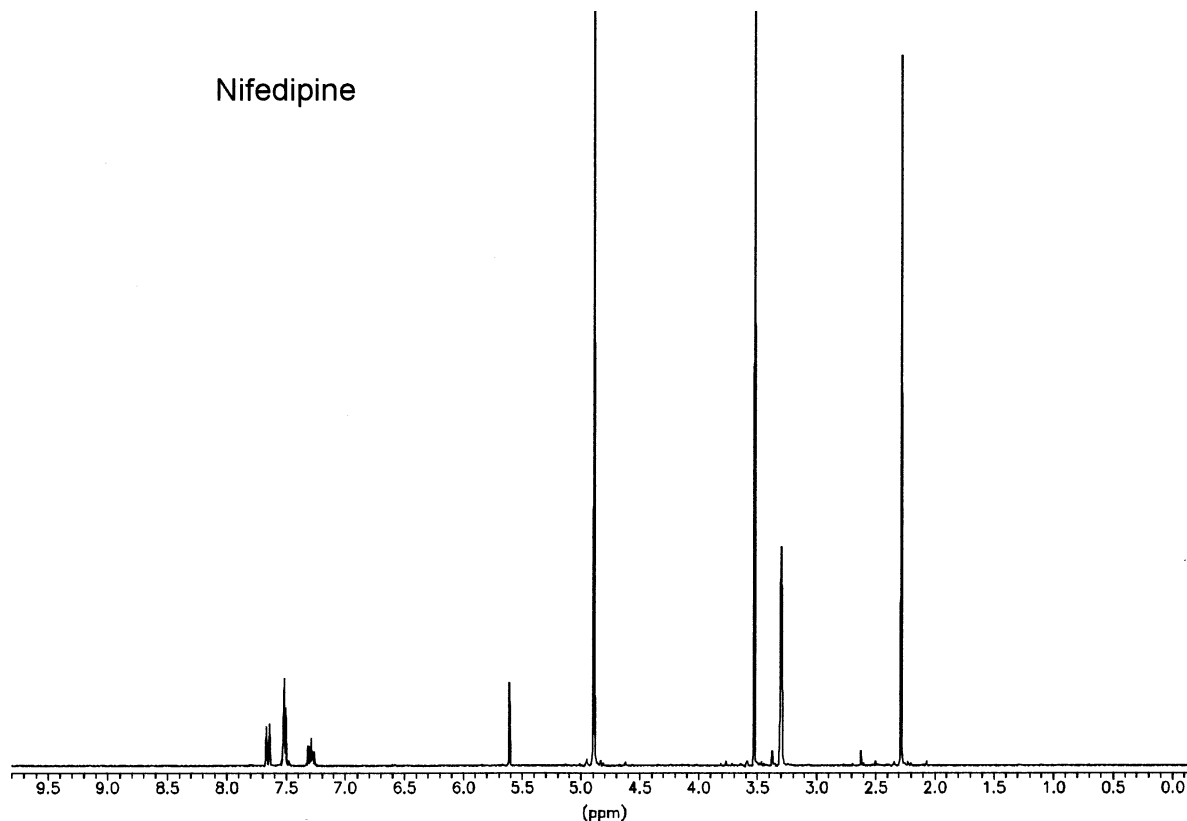
Nifedipine

Fig. 30. ^{1}H NMR spectrum of nifedipine, 300 MHz, solvent methanol-d_4.

problem is provided by the freeze-drying of the urine. After dissolution in D_2O, the NMR spectrum can be recorded without any special pulse technique. In addition, relatively high-molecular weight substances such as proteins and lipids present in most biological samples cause a line-broadening of the signal due to high values of $T2$. The Hahn spin-echo sequence can be used to overcome this problem.

Everett et al. first reported the application of NMR spectroscopy in metabolism studies. In 1984, they studied the metabolites of ampicillin in rats using spin-echo ^{1}H NMR spectroscopy [68]. Apart from the unchanged penicillin, the natural (5R)-isomer of penicilloic acid, the epimerised (5S)-isomer as well as a new metabolite, a dike-topiperazine derivative, were identified in urine. Reinvestigation of ampicillin and corresponding penicillins ten years later confirmed the results; in

case of amoxycillin, a dimeric metabolite was additionally found [69].

In the 1980s and 1990s, it was especially the group of J.K. Nicholson who developed the NMR spectroscopy into a routine method in drug metabolism studies. Some representative examples are discussed below.

In the beginning, for the most part the metabolites of drugs could only be identified in urine when a spectrum of the isolated (or independently synthesised) substance was known. For example, oxpentifylline, extensively used in the treatment of vascular diseases, and its acidic metabolite 1-(4'-carboxybutyl)-3,7-dimethylxanthine can be directly identified and quantified in a freeze-dried urine sample using a 250 MHz spectrometer [70]. The medication of lymphatic filariasis with di-ethylcarbamazepine has to be monitored for several reasons [71]. Since the drug lacks an absorbing chromophore (HPLC/UV-detection)
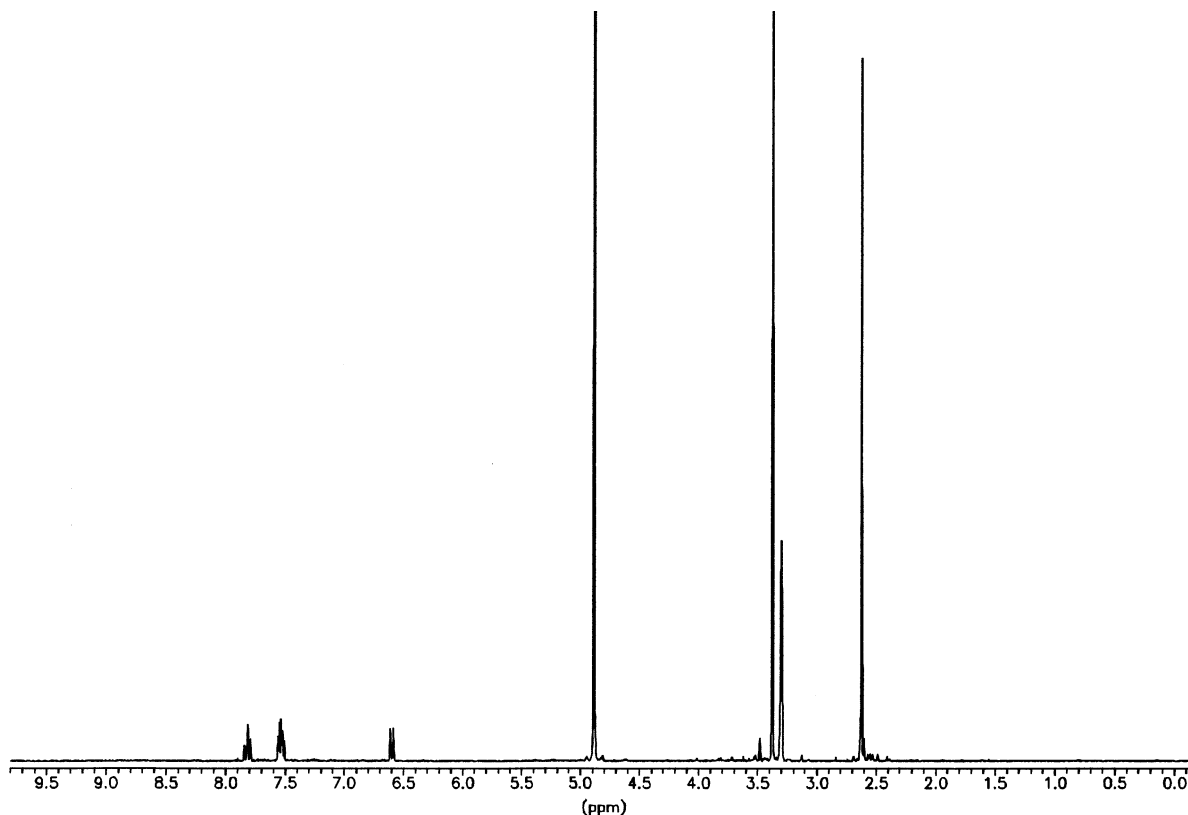
Fig. 31. ^{1}H NMR spectrum of nifedipine after photo-oxidation, 300 MHz, solvent methanol-d$_4$.

and GC needs a special detector and 'troublesome' extraction method, ^{1}H NMR spectroscopy appears to be suitable for the urine analysis: the urine samples were mixed with 10% D$_2$O and directly measured afterwards. The unchanged drug and the corresponding N-oxide, a minor product of metabolism, could be quantified with high precision and accuracy which was better than 15% (obtained from using a calibration graph) [70].

Often solid phase extraction (SPE) was performed before running the ^{1}H NMR spectrum. In this way, the *O*-desmethyl metabolite of naproxen (*d*-2(6-methoxy-2-naphthyl)propionic acid) could be found in human urine [72]. The main metabolites of paracetamol, i.e. sulfate and glucuronide, could be identified and quantified in the urine NMR spectrum without prior purification (Fig. 42) [73].

The main metabolites of ibuprofen, the glucuronides of ibuprofen and of the side-chain hy-droxylated derivative (2-[4-(2-hydroxy-2-methyl-propyl)phenyl]propionic acid) and a side-chain ox-idised compound (2 - [4 - (2 - carboxy - 2 - methyl-propyl)phenyl]-propionic acid) could be detected after SPE using a 250 MHz instrument (Fig. 43) [74].

However, linking NMR spectroscopy to HPLC led to the discovery of further metabolites in both cases (see Section 3.3). Leo and Wu identified the glucuronide of suprofen after HPLC separation [75].

The latter examples clearly show that the ^{1}H NMR inspection of urine suffers from insensitivity due to the detection limit of $\sim$ 10nM (using a 600 MHz instrument) on the one hand, and due to chemical noise caused by considerable signal over-lap on the other hand. These problems can be overcome by prior SPE or by HPLC NMR cou-pling, which will be discussed at the end of this chapter. Increasing the field strength (600–750
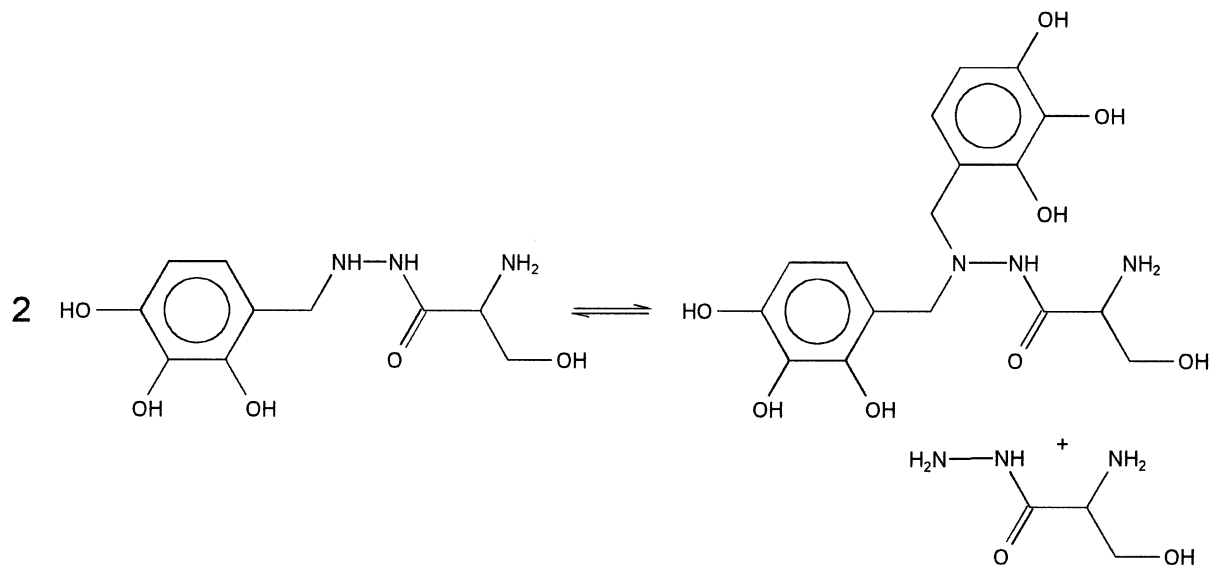
Fig. 32. Equilibrium reaction of benserazide.

MHz) results in an enhanced resolution of the signals of both xenobiotics and natural compounds occurring in the urine. Two-dimensional NMR techniques provide a further possibility to solve the problem of signal overlap. Whereas 2D-COSY experiments require relatively large experimental data arrays and, in addition, high usage of disk storage space and time-consuming data processing, two-dimensional J-resolved ^{1}H NMR spectroscopy (JRES) seems to offer an efficient means of reducing peak overlap [76]. Using this technique, 30 natural components of urine can be identified from the F2 projection and the additional contour plot showing the coupling pattern. In addition, the signals of paracetamol and all its metabolites are well separated. Due to different T_2 relaxation rates, the concentration of the metabolites cannot be directly determined by the measurement of the integrals of the corresponding signals. The spectra have to be carefully calibrated by means of standard addition of compounds. Taken together, the power of the JRES technique is the signal assignment in urine rather than the quantification of a component.

3.1.2. ^{19}F NMR spectroscopy

There are several reasons why ^{19}F NMR spectroscopy is especially suitable for following the pathway of metabolism: firstly, it is almost as sensitive as ^{1}H NMR spectroscopy (83% of ^{1}H) and has a wide range (~ 200 ppm); secondly, it does not have any problem with the huge water signal and thirdly, there are no interfering signals with abundant urinary components of endogenous origin. The drawback that only a single atom in a drug can be monitored in the metabolism studies is partially neutralised by the fact that the chemical shift of fluorine atoms is rather sensitive against structural changes in the molecule which are 6–8 atoms apart from the fluorine. Thus, metabolic conversion of drugs is mostly mirrored in the change of the chemical shift. However, the structural information derived from the spectra is poor in comparison with ^{1}H and ^{13}C NMR spectra. Thus, ^{19}F NMR spectroscopy can only be exploited for monitoring catabolic pathways of fluorinated drugs when the structures and the corresponding spectra of the metabolites are already known.

As early as in 1984, Malet-Martino et al. reported the detection and quantification of five metabolites of 5′-deoxy-5-fluorouridine, an antitumor drug, in plasma, blood and urine without any sample pre-treatment [77]. Unmetabolised 5′-deoxy-5-fluorouridine, 5-fluorouracil, 5,6-dihydrofluorouracil and α-fluoro-β-alanine (Fig. 44),
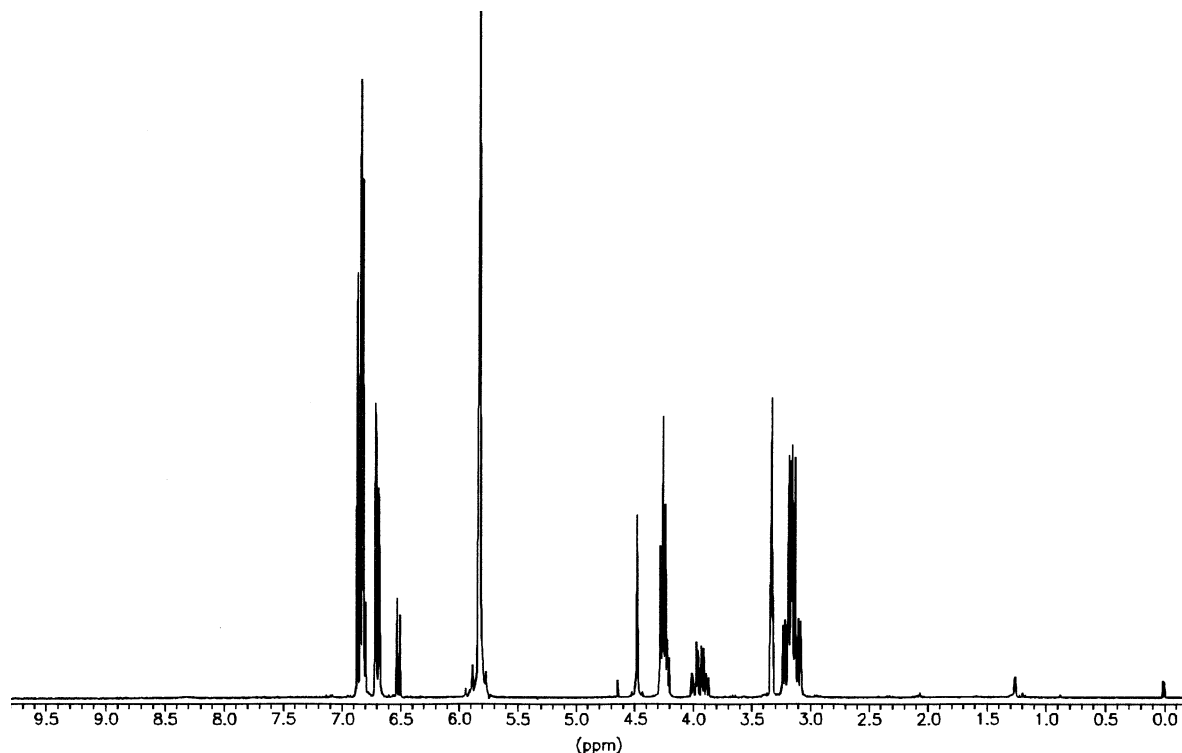
Fig. 33. ^{1}H NMR spectrum of Madopar®, 300 MHz, solvent methanol-d_4.

the latter previously not reported, were found in blood, whereas the unchanged drug and α-fluoro-β-alanine appeared to be the major metabolites in urine. Monitoring of plasma and urine levels of the unchanged drug and each metabolite gave a complete description of the metabolic profile as displayed in Fig. 44 [78].

As expected, the metabolism of 5-fluorouracil, the first metabolite of 5′-deoxy-5-fluorouridine, turned out to be similar [79]. Interestingly, the intermediate, α-fluoro-β-guanidinopropanoic acid, could not be detected in this study, which utilised ^{19}F NMR and HPLC methods. Martino et al. also developed a ^{19}F NMR assay method to determine the extent of protein binding of 5′-deoxy-5-fluorouridine, which was as valid as equilibrium dialysis [80].

In addition to the ampicillin studies using ^{1}H NMR spectroscopy, the group of Everett monitored the metabolism of a fluorinated penicillin, flucloxacillin, by means of ^{19}F NMR spectra [81]. Since the chemical shifts in 19F NMR spectra are extremely dependent on the pH value of the solution, flucloxacillin and its metabolites, the corresponding 5S- and 5R-penicilloic acid as well as 5′-hydroxymethylflucloxacillin, could be detected and quantified upon spiking the urine with the authentic sample. ^{1}H,^{19}F heteronuclear shift correlated 2D spectra confirmed the findings [82]. In addition, the results obtained from the direct ^{19}F NMR spectroscopy were comparable to those obtained from HPLC and microbiological studies.

In a pilot scheme for xenobiotics, the metabolism of ortho-, meta- and para-trifluoromethylbenzoic acid (TFMBA) was studied in rats [83]. In ^{19}F NMR spectra recorded after addition of D$_2$O to the post-dose urine, an ester glucuronide and a transacylated ester glucuronide could be easily detected in case of o-TFMBA, an ester glucuronide and a glycine conjugate in case of m-TFMBA as well as transacylated ester glucuronide in case of p-TFMBA. For structure elucidation of the metabolites, the urine samples were subjected to a solid-phase extraction chromatography followed
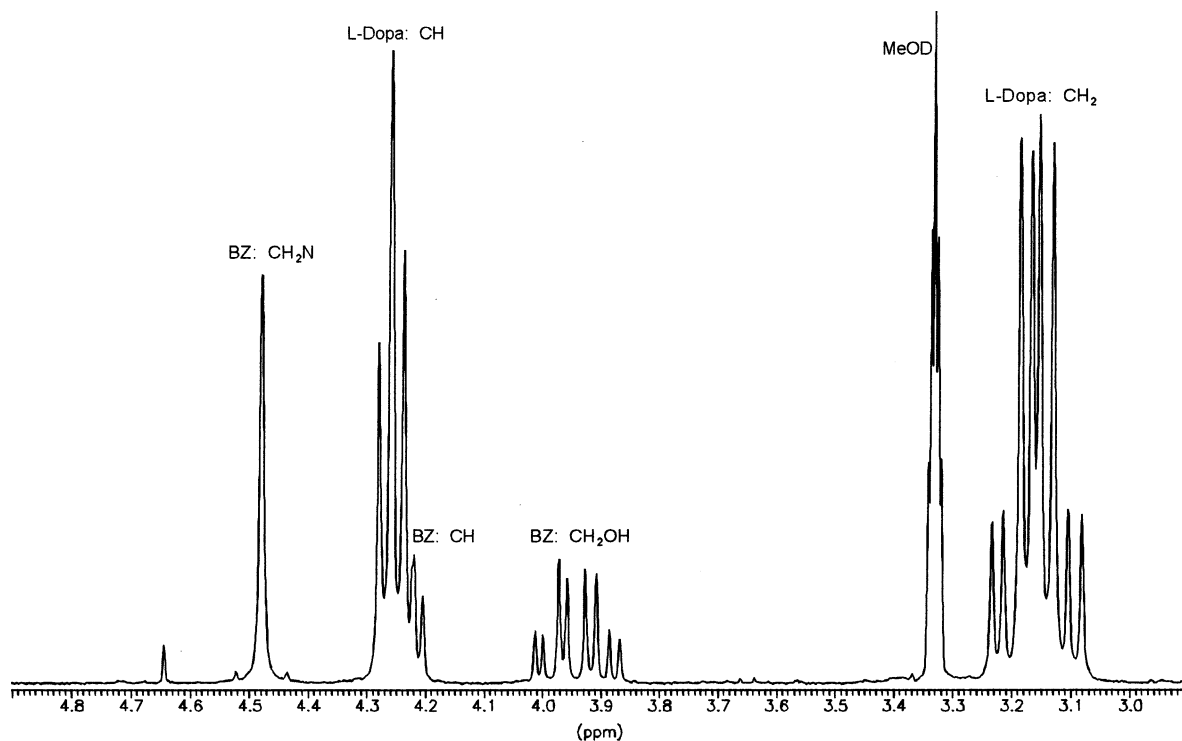
Fig. 34. ^{1}H NMR spectrum of Madopar®, CH_2OH region, 300 MHz, solvent methanol-d_4.

by ^{1}H NMR detection. In a subsequent extended study [84], quantitative ^{19}F NMR spectroscopy was used to derive quantitative structure-metabolism relationships for xenobiotics.

The metabolism of the entire class of fluoroquinolones, bactericide gyrase inhibitors, is suitable for monitoring by ^{19}F NMR spectroscopy because metabolic conversions occur mainly at the piperazine ring which is closely located to the fluorine substituent. Unexpectedly, the fluorine signals of the fluoroquinolones were found to be broad. Upon addition of EDTA, the shape of the signals became narrow indicating that Ca^{2+} and/or Mg^{2+} caused the broadening by chelation [85]. Since it is rather difficult to dissolve the isolated metabolites in aqueous solutions, it is almost impossible to spike the urine in order to assign the different fluorine signals in the spectrum. However, signals for pefloxacin and its metabolites, norfloxacin, pefloxacin-*N*-oxide and oxo-pefloxacin, could be identified in human urine upon addition of a small amount of NaOD [86].

The metabolism of the anti-inflammatory drug flurbiprofen is characterised by hydroxylation of the second phenyl ring in *para*- and *meta*-position. ^{19}F NMR using continuous broad-band ^{1}H irradiation to enhance the sensitivity and ^{19}F-^{1}H 2D shift correlated spectra revealed a total of ten metabolites (two major and eight minor), which were assumed to be glucuronide and sulfate conjugates of flurbiprofen and its hydroxy metabolites [87]. In a second study the urine of a volunteer treated with 200 mg flurbiprofen was subjected to a HPLC NMR analysis [88]. The two major metabolites, namely the glucuronides of flurbiprofen and 4′-hydroxy-flurbiprofen, were found to be diastereomers which were formed by in vivo conjugation of the racemic drug and its metabolite with D-glucuronic acid. The diastereomeric proportion could be evaluated.

3.1.3. ^{15}N NMR spectroscopy

The metabolism of hydrazine, a starting product of several important industrial chemicals
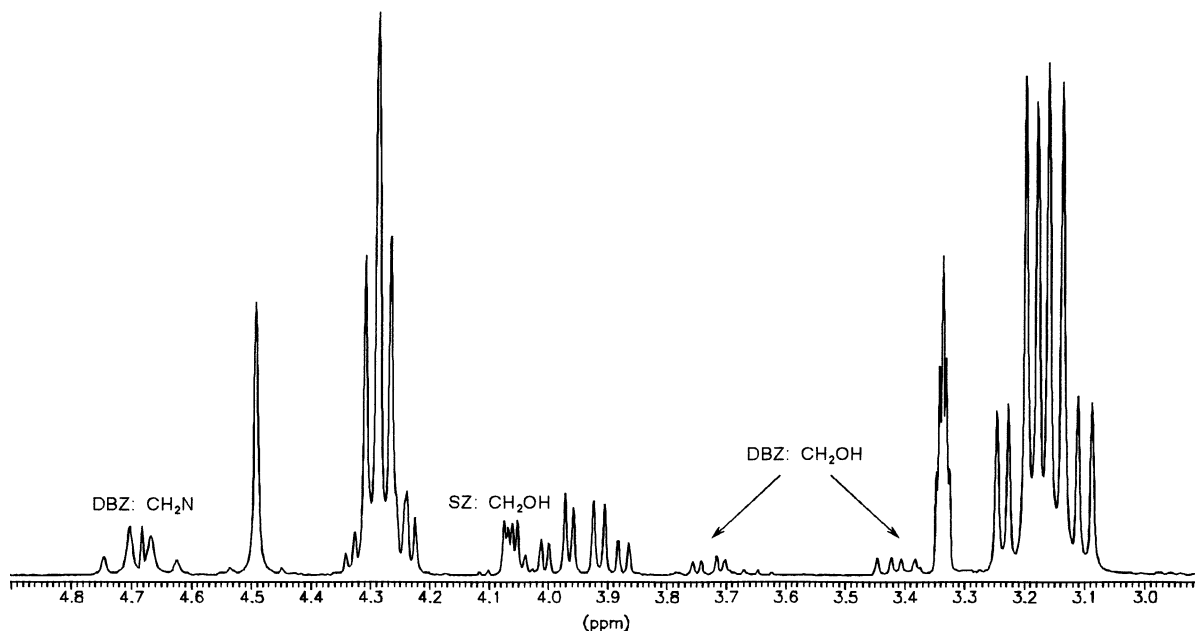
Fig. 35. ^{1}H NMR spectrum of Madopar® after 30 min at 80°C, CH$_2$OH region, 300 MHz, solvent methanol-d$_4$.

as well as a metabolite of drugs, e.g. isoniazide and hydralazine, is important to know. Even though the sensitivity of ^{15}N NMR spectroscopy has proved to be poor, a number of metabolites could be identified in lyophilised urine, which was reconstituted in a reduced volume of water [89]. By spiking the urine with authentic samples, the signals of carbazic acid, urea, acetyl- and diacetylhydrazine could be assigned. In addition, a doublet centred at 150 ppm and a singulet at 294 ppm were assigned to the cyclisation product of hydrazine and oxoglutarate and a singulet detected at 316 ppm, to a pyruvate hydrazone. Although this study using ^{15}N NMR spectroscopy was rather successful, this NMR technique has only a small chance of becoming a routine method because a huge number of accumulations (> 10 000 scans) and the optimisation of the relaxation time, both time-consuming processes, are necessary in order to be able to observe ^{15}N signals of a drug and its metabolites.

3.1.4. ^{31}P NMR spectroscopy

The bioactivation of cyclophosphamide, a highly potent, alkylating chemotherapeutic agent, can easily be observed by ^{31}P NMR spectroscopy.

The phosphorus signals of the different metabolites, e.g. phosphoramide mustard (the active metabolite), dechloroethylcyclophosphamide, ketocyclo-phosphamide and carboxyphosphamide, are clearly separated, when measuring the buffered urine of patients. The comparison of the ^{31}P NMR spectra of urine samples (2 ml of a 0–8 h fraction) obtained from a patient with breast cancer during a conventional-dose (500 mg m^2 body surface) and high-dose (100 mg kg^{-1} body weight) adjuvant chemotherapy with cyclophosphamide revealed the change in the relative contribution of the two primary metabolic steps, e.g. formation of 4-hydroxy-cyclophosphamide, an intermediate in the bioactivation, and the side-chain oxidation. During dose escalation the inactivating pathways are favoured, thereby indicating the saturation of bioactivating enzymes [90].

3.2. NMR of bile, blood plasma, cerebrospinal and seminal fluids

Apart from the appearance of drugs and their metabolites in urine, the concentration levels in other body fluids are often important to know in order to titrate the exact dosage necessary for a
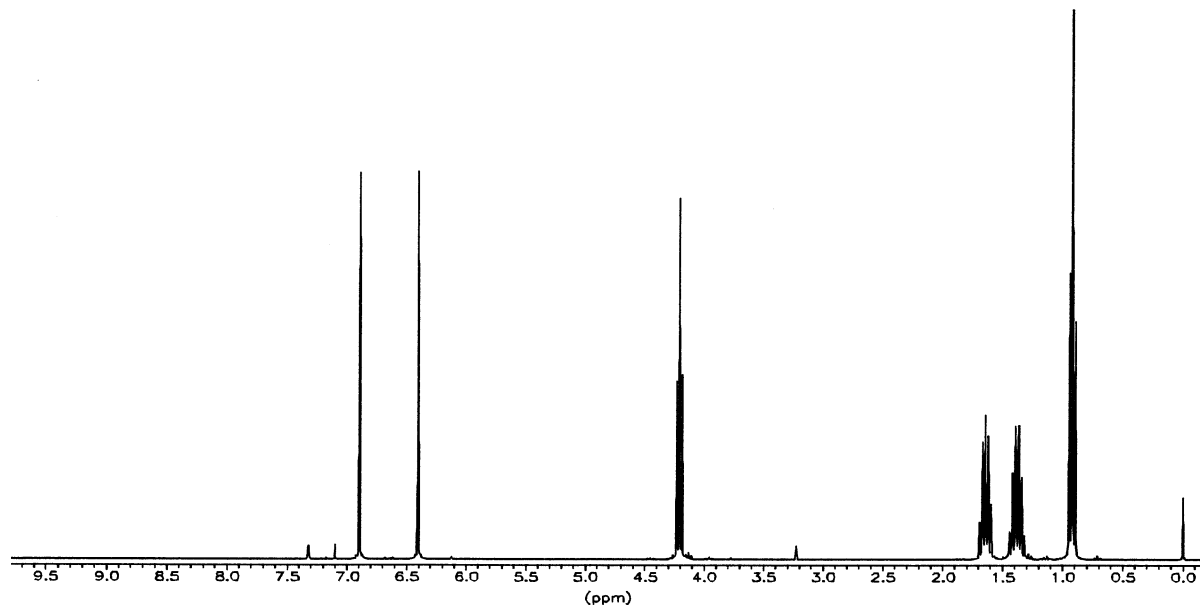
Fig. 36. ^{1}H NMR spectrum of cyanoacrylate and hydroquinone as antioxidant, 300 MHz, solvent $CDCl_3$/benzene-d_6 (5:1).

pharmacological or antimicrobial effect, or in order to find routes of excretion of a drug other than urine. In this context, rat bile was examined for a catecholic cephalosporin and its metabolites using ^{1}H NMR spectroscopy [91]. As expected from the experiments performed with urine, the cephalosporin could easily be identified in the bile; in addition, a methoxylated metabolite was found whose structure was elucidated after purification. The antibiotic cefoperazone is not metabolised by the liver. Therefore, the biliary excretion was monitored by means of ^{1}H NMR spectroscopy [92]: 43% of the drug were found to be unchanged. The biliary excretion of several other xenobiotics, such as benzyl chloride [92] and 4-cyano-N,N-dimethyl aniline [93], were investigated after complete assignment of the natural components of the bile.

^{1}H NMR spectra of blood plasma suffer from the same problem of signal overlap as the spectra of urine. Again, the JRES technique can help to simplify the spectra in order to assign the intact fluid. This is especially necessary because broad resonances caused by macromolecules such as lipids and proteins (in particular albumin and immunoglobulins) can hide minor metabolites of

drugs. Using this technique, Foxall et al. [76] were able to fully assign the complex spectral region between 3 and 4 ppm consisting of signals of α- and β-glucose, several amino acids, glycerol, trimethylamine-N-oxide, choline and phosphorylcholine. The increase of the field frequency from 600 to 750 MHz results in an increase of the sensitivity now approaching 1 nM ml^{-1}. This high frequency enabled Foxall et al. to detect, e.g. abnormal metabolites associated with chronic renal failure, 'uraemic toxins' as methylamine, dimethylamine and the already reported trimethylamine-N-oxide, which are believed to cause nausea, itching, headache etc. [94].

In an extended study, Nicholson et al. [95] assigned the components of the complex blood plasma by means of several pulse sequences, COSY-45, WEFT, JRES, [1]H-^{13}C HMQC, NOESYPRESAT, and TOCSY [96,97] using a 750 MHz instrument. Thus, the prerequisite to recognise a changed pattern of metabolites, and in line with this, a disease, is fulfilled.

Apart from monitoring blood plasma, bile, and urine, it is also possible to characterise the natural composition of a fluid such as amniotic fluid. Deviations from the normal pattern may give a hint for a certain disease, herein diabetes,
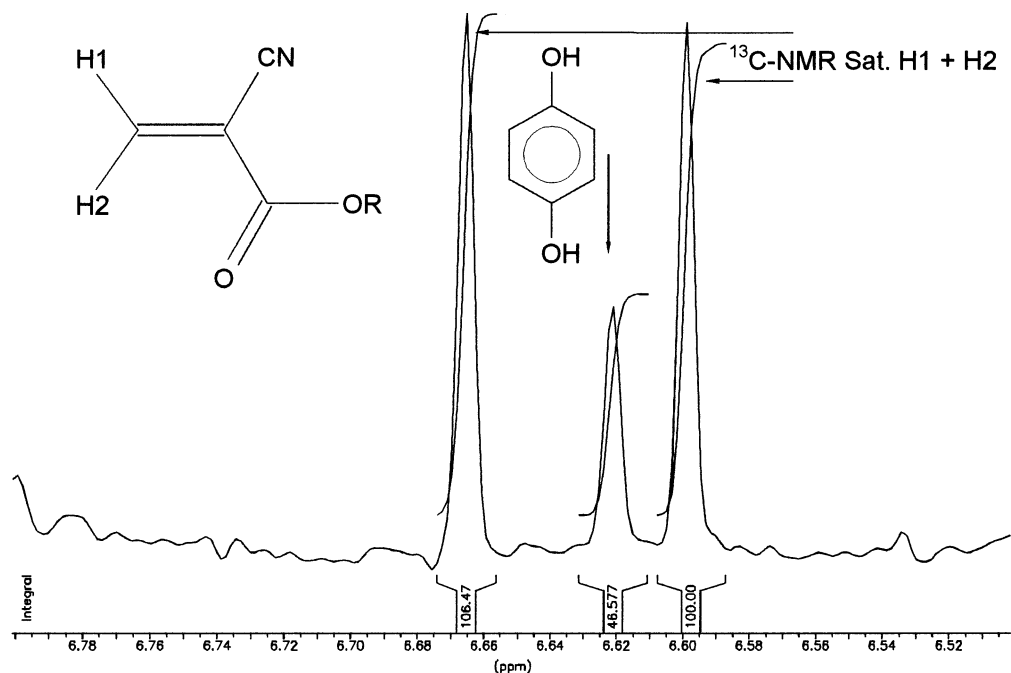
Fig. 37. ^{1}H NMR spectrum (detail) of cyanoacrylate stabilised with hydroquinone, 300 MHz, solvent CDCl$_3$/benzene-d$_6$ (5:1).

preclampsia or foetal malfunction [98]. The amniotic fluid was centrifuged to remove cells etc. and freeze-dried for concentration. After resuspension of the lyophilised sample with D$_2$O, the components of the fluid were identified and quantified upon addition of authentic samples. Citrate, valine, alanine, glucose, lactate and acetate as well as indoxylsulphate, histidine and formate could be easily identified in a concentration range of 16–40 μM. With exception of alanine and valine (due to signal overlap with HDL, VDL and LDL), the quantitative analysis by means of NMR is in good agreement with values reported from other methods. Cerebrospinal fluid [99] and seminal (Fig. 45) as well as prostatic fluids [100] were also subjected to similar investigations, which are characterised firstly by full assignment of spectra measured from samples of healthy persons by means of several one and two dimensional techniques (see above) and, secondly, by identification of abnormal metabolites or abnormal levels of natural metabolites, both indicating malfunctions or diseases.

Taking the studies of various body fluids together, it can be stated that it is possible to identify and partially quantify the components of the fluids as well as of drugs and their metabolites. Thus, the NMR spectroscopy provides a new method for the diagnosis of diseases associated with changes in the composition of body fluids. A major advantage of using NMR methods in this area is that the measurement can be performed with minimal sample preparation. In addition, a whole analytical profile of the biological fluid can be obtained. However, it has to be stressed that very high resonance frequencies, 600–750 MHz, are necessary for this purpose. Additionally, special pulse sequences have to be applied in order to assign all signals to the components. Since such high field instruments are only very rarely available in clinical laboratories at the moment, NMR spectroscopy is not a matter of routine examinations.

3.3. HPLC NMR coupling

As described in Section 3.2, the monitoring of natural components as well as of drugs and their corresponding metabolites in body fluids suffer from the signal overlap due to the complexity of the fluids studied and low sensitivity. Typically,
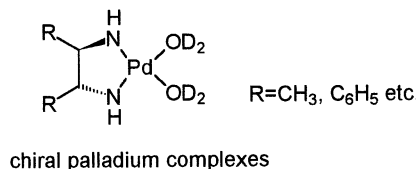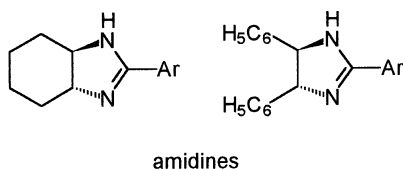
CSAs

α-methylbenzylamine

1-(1-naphthyl)-ethylamine

2,2,2-trifluoro-1-
(9-anthranyl)-ethanol

n=6, α-cyclodextrin
n=7, β-cyclodextrin
n=8, γ-cyclodextrin

amidines
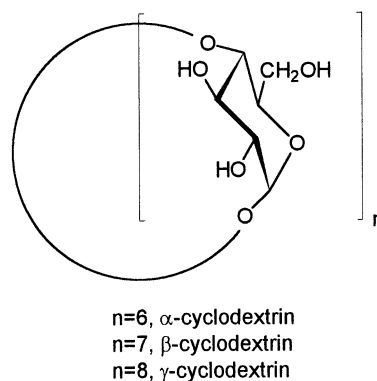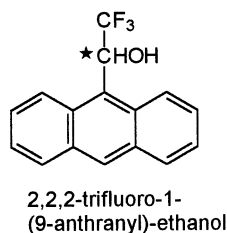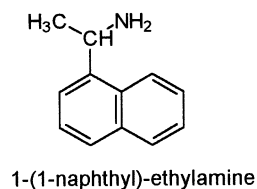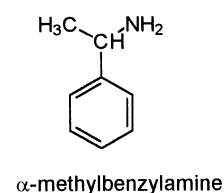
chiral palladium complexes

R=CH$_3$, C$_6$H$_5$ etc

Fig. 38. Structural formulae of the chiral solvating agents discussed.

freeze-drying of the sample or solid-phase extractions were performed prior to NMR spectroscopy to overcome these problems. In this context, the hyphenation of the highly potent HPLC separation technique to NMR spectroscopy, the best technique for structure elucidation, was often discussed but had several drawbacks owing to the need of expensive deuterated solvents, inadequate solvent suppression techniques and low sensitivity. The development of high field strength instruments (500–750 MHz) in the last 10 years provided greater sensitivity, new probe designs and better solvent suppression methods. Three different modes of operation are used today: firstly, a continuous-flow HPLC NMR, providing a 2D plot with chemical shifts on the horizontal axis and chromatographic retention times on the vertical axis; from the 2D plot it is possible to identify characteristic chemical groups such as aromatics. Secondly, the stopped-flow mode, giving complete ^{1}H NMR spectra of each peak and/or various parts of each peak and, thirdly, peak parking. Herein, the peaks are taken out of the eluent stream into loop in order to measure NMR spectra ([101], for technical details see Refs. [102–104]). Especially the ^{1}H NMR metabolism studies considering urine, bile and blood plasma took advantage of the linking of the HPLC to NMR spectroscopy.

Racemic Compounds

ketoprofen

methylphenidate

S-timolol

nicotine

diltiazem

selegiline (R= CH₃ , R'= CH₂–C≡CH, R"= H)

norephedrine (R= R'= H, R"= OH)

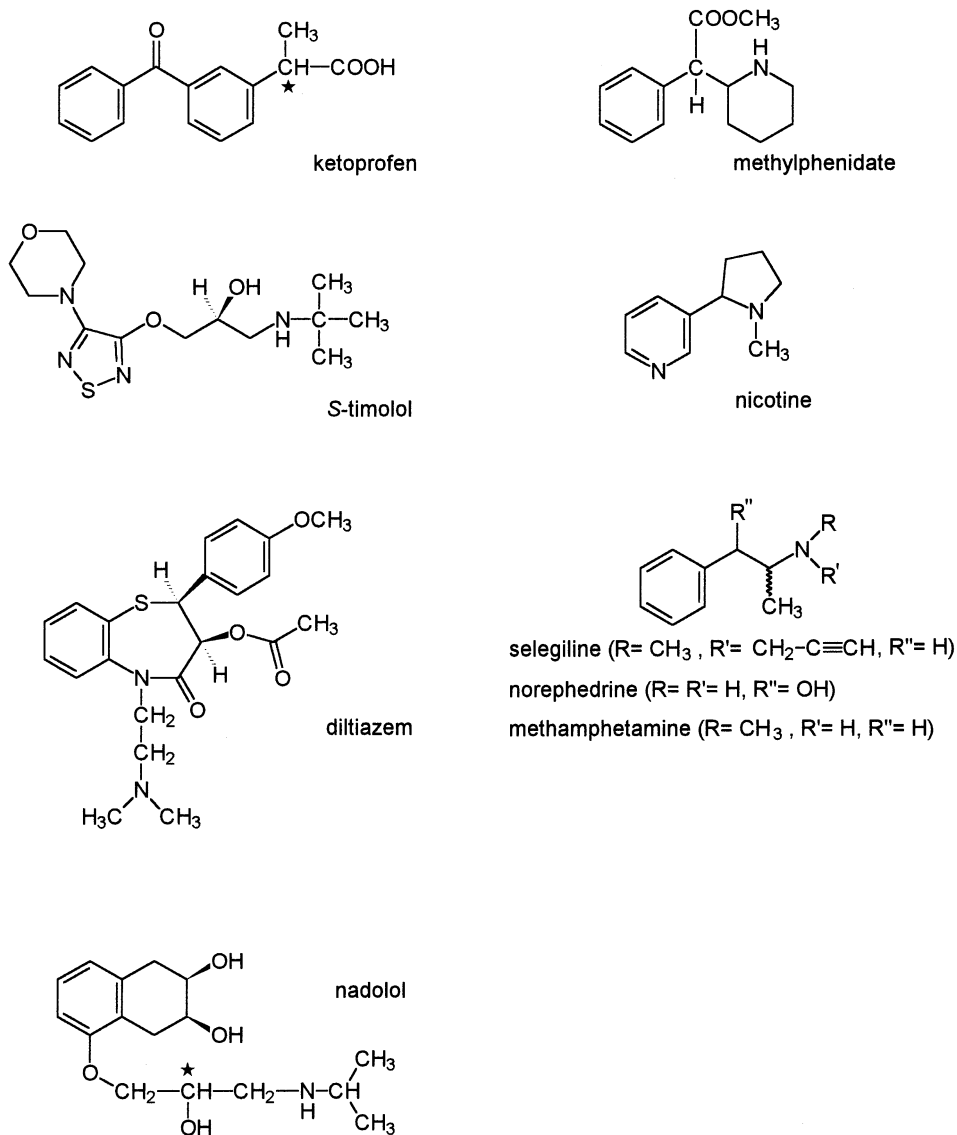methamphetamine (R= CH₃ , R'= H, R"= H)

nadolol

Fig. 39. Structural formula of the racemic compounds discussed.

In order to prove the advantages of HPLC NMR hyphenation, urine samples of rats and man containing the drugs and their corresponding metabolites, e.g. ibuprofen, flurbiprofen, antipyrine or paracetamol (Section 3.1.1), were reinvestigated. Using both, continuous-flow and the stopped-flow techniques (Fig. 46), apart from the already reported metabolism pattern [73], the non-conjugated side-chain oxidised diacid metabolite of ibuprofen could be identified [105].

HPLC NMR measurements of flurbiprofen metabolites in human urine [101] confirmed the previous study [88]. In case of antipyrine and its metabolites excreted in human urine, gradient
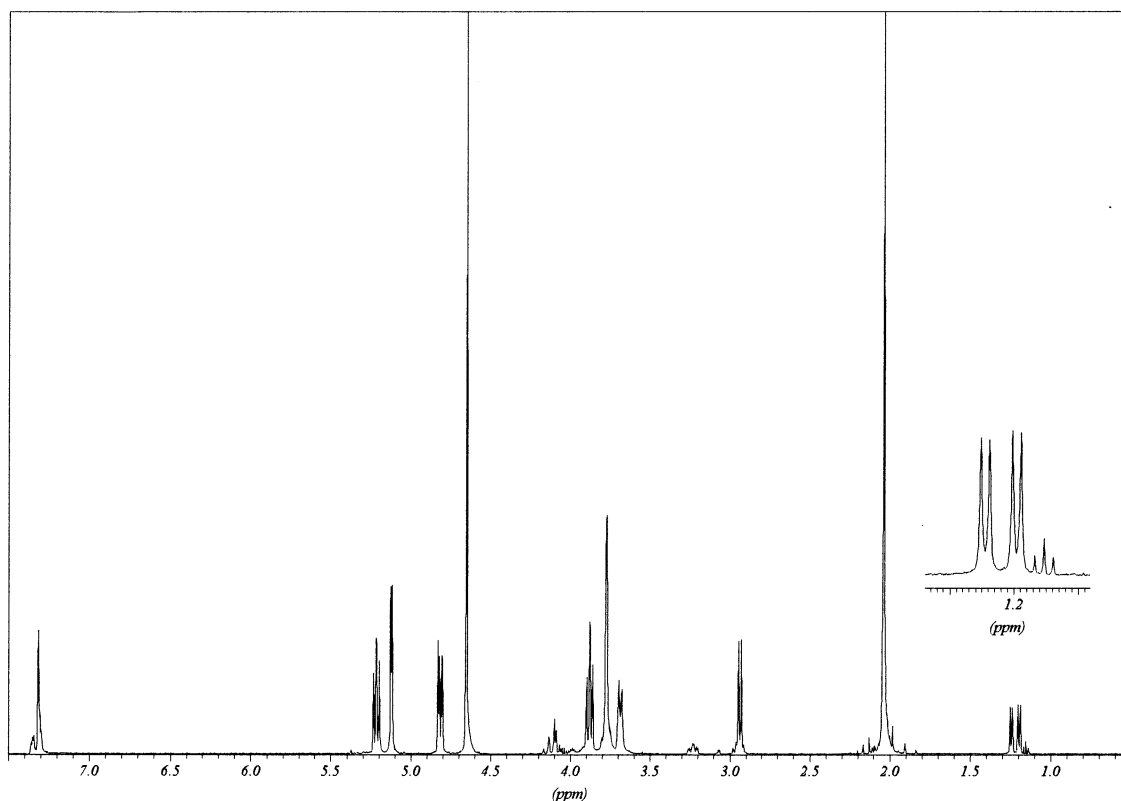
Fig. 40. ^{1}H NMR spectrum of racemic selegiline in presence of *heptakis*(2,3-di-*O*-acetyl)-β-CD.

RP-HPLC with stopped-flow ^{1}H NMR gave the following pattern: the 4-hydroxylated antipyrine and the corresponding ether glucuronide, the nor-antipyrine and a small amount of the 3-hydroxy-antipyrine glucuronide [106]. Utilising the stopped-flow operation again, an additional minor metabolite of paracetamol, the *N*-acetylcysteinyl paracetamol, could be identified in the urine of male rats apart from the major metabolites, the sulfate and glucuronide [107,108]. HPLC NMR, especially the continuous-flow technique in connection with a 750 MHz instrument, was used to observe the acyl migration of fluorobenzoyl-glu-curonides, resulting in a mixture of 1-, 2-, 3- and 4-*O*-acylglucuronides and corresponding α- and β-anomers [109,110]. Similar studies concerning the acyl migration were performed with the non-steroidal antiinflammatory drug 6,11-dihydro-11-oxodibenzo[*b,e*]oxepin-2-acetic acid [111,112]

because the positional isomers of related drugs are believed to cause allergic reactions.

Apart from metabolism studies, the linking of HPLC to NMR spectroscopy provides a powerful tool for the identification of impurities in drugs. Using the stopped-flow technique, Roberts and Smith [113] were able to find minor components in a mixture of chromane compounds at a 3% level. Albert et al. [114] took advantage of separation and identification by HPLC NMR in order to charac-terise the mixture of vitamin A isomers (Fig. 47).

Even though the sensitivity of HPLC NMR hyphenation is about 10 µg on-column with the on-flow mode and 1 µg with the stopped-flow mode [101], the linking is shown to be rather effective. As soon as it is possible to detect components in mixtures in a nanomolar range [115], the NMR detection will surpass the MS technique because NMR detection automatically provides detailed structural information according to the direct and
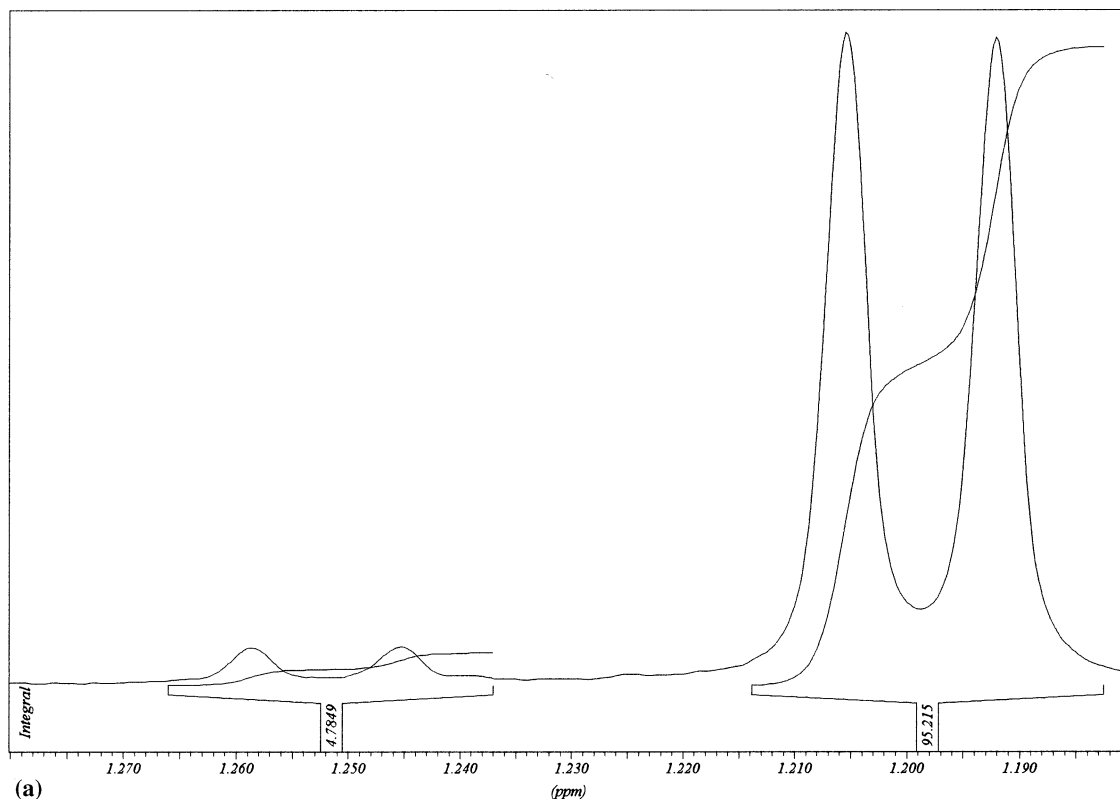
Fig. 41. ^{1}H NMR expansions of the C-methyl region of 95% (a), 97.5% (b) and 98.75% (c) *R*-selegiline in presence of *heptakis*(2,3-di-*O*-acetyl)-β-CD.

well understood relationship between molecular structure and NMR chemical shifts, coupling constants and relaxation times.

At the moment, the hyphenation of HPLC to NMR has to compete with LC/MS/MS systems with regard to sensitivity, structure determination and the amount of samples required. Mutlib et al. [116], however, combined both methods to elucidate the metabolism of the new antipsychotic drug iloperidone, which is metabolised extensively for renal and biliary elimination. Thus, the identity of known metabolites could be established rather quickly by means of LC/MS and, in addition, the structures of minor unknown metabolites could be elucidated using semi-preparative HPLC coupled with both MS and NMR. Using the hyphenation of HPLC NMR MS, Shockcor et al. [117] found phenylacetylglutamine, an endogenous metabolite, in addition to the metabolites of

paracetamol in urine. The two HPLC-MS NMR applications recently reported in the literature clearly demonstrate the power of this method.

Since the sensitivity of the NMR spectroscopy could be enhanced, supercritical fluid chromatography [118–120] and capillary electrosphoresis [120] were recently coupled to the NMR and successfully applied in food analysis [121] and agricultural chemistry [122].

Taking this chapter together, it can be stated that the linking of NMR spectroscopy to various separation (600 and 750 MHz) techniques developed into rather a powerful tool. Since a very high field strength is necessary for the reasons described above, the methods will be reserved for special problems, e.g. stereochemical problems in metabolism studies. However, the method detects all compounds present above the detection threshold, irrespective of chemical class as well as
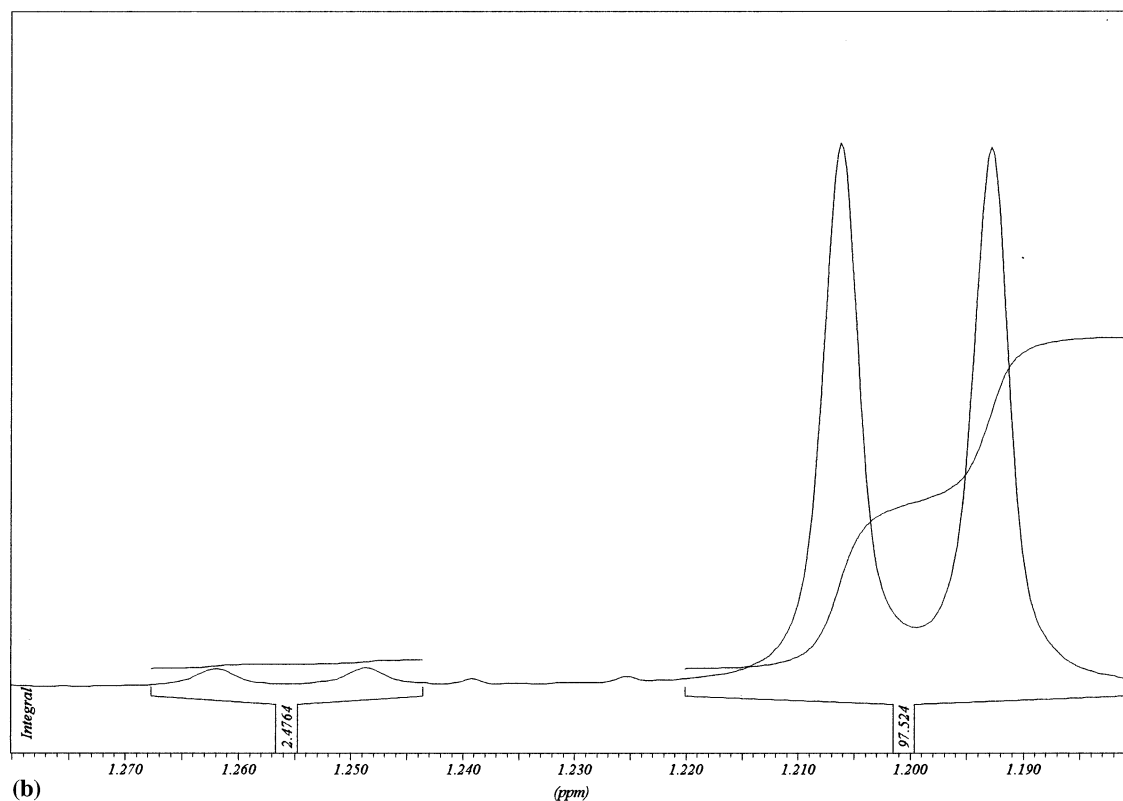
Fig. 41. (*Continued*)

4. Solid state NMR

It is somewhat surprising that solid-state NMR is used so rarely for the characterisation of drugs, taking into account that the majority of pharmaceutical products exists in the solid form. The aim of this chapter is to show its advantages and applications in pharmaceutical research.

NMR spectra cannot be measured in solids in the same way in which they are routinely obtained in solutions because NMR lines from solids are too broad. Whereas in solutions, dipolar interactions of the nuclei are averaged to zero by thermal motions of molecules, in solids, this is not the case, and the interactions depend on the kind of nuclei i and j, their mutual orientation $(3 \cos^2 \theta - 1)$ and the distance r_{ij}^3. The dipolar interactions vanish for $\cos^2 \theta = 1/3$, thus, if the sample is rotated around an axis inclined at an angle $\theta = 54° 44''$ (the magic angle) to the magnetic field, the line broadening is significantly suppressed. The question arises of how fast the sample should rotate.

Unfortunately, the ^1H–^1H interactions are of the order 10–50 kHz, and it is not possible to rotate the sample fast enough to remove them. The solid-state ^1H NMR spectrum of an ureido sugar obtained with a rotation speed of 16 kHz, illustrated in Fig. 48, shows a broad line covering the whole region of 10 ppm; high resolution ^1H spectra cannot be obtained with the magic angle spinning (MAS) technique alone and, therefore, are of little practical use for the characterisation of pharmaceutical solids.

Solid-state ^{13}C spectra are obtained by a combination of MAS, high-power decoupling (to re-
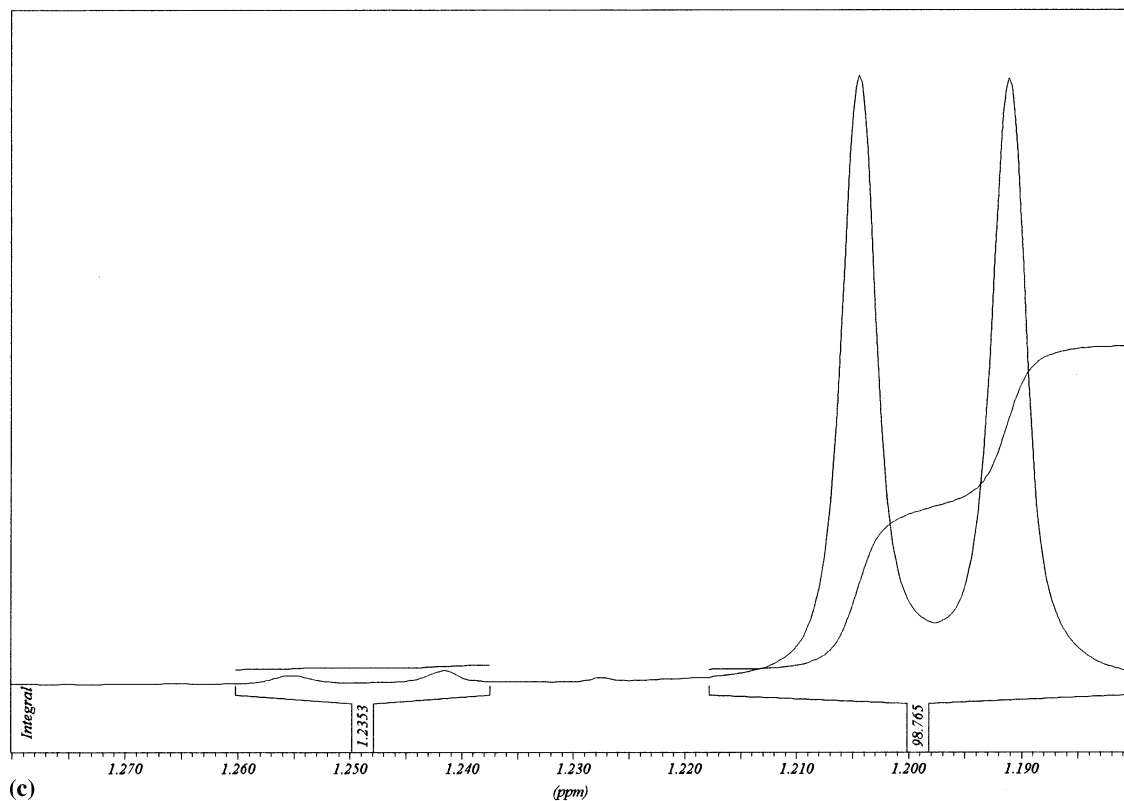
Fig. 41. (*Continued*)

move dipolar interaction with ^1H) and cross-polarisation (the sequence of pulses irradiated at ^1H and ^{13}C frequency increases the polarisation of ^{13}C nuclei, giving large improvement in sensitivity with short recycle time). The ^{13}C cross-polarisation (CP) MAS NMR spectrum of acetylated ureido sugar with dipeptide (Ala-Gly) residue is shown in Fig. 49. When spinning slowly (approximately 3.3 kHz), one observes a set of lines spaced at the rotation frequency at the left and right side of the carbonyl carbon signals. Chemical shift anisotropies for aliphatic carbons are 15–50 ppm, but as much as 120–200 ppm for aromatic and carbonyl carbons and thus, rotational side-bands are mainly observed for these carbon signals. The rotational lines decrease in intensity and move away from the centre as the spinning rate increases; rotation at about 6–10 kHz is sufficient for obtaining good quality spectra of most organic solids.

Solid-state ^{13}C NMR spectra of nitrogen containing compounds recorded under CP MAS conditions often show asymmetric doublets or broadening arising from the unaveraged, residual coupling to quadrupolar ^{14}N nuclei [123]. It is obvious that the effect of ^{14}N nuclei on adjacent ^{13}C allows easy assignment of carbons directly bonded to nitrogen (see the signals of NCON, NCH and NCH$_2$ carbons in Fig. 49).

The rate of cross-polarisation of particular carbons is, in a first approximation, proportional to the number of directly bonded protons, and the following relative rates are usually observed: CH$_3$ (static) > CH$_2$ > CH > CH$_3$ (rotating) > C (non-protonated) [124]. In the spectra recorded with short cross-polarisation time (or contact time, t_{CP}), only signals of carbons bearing hydrogens appear because quaternary carbons take longer to cross-polarise. Introducing a short (50 µs) delay just before acquisition results in a loss of intensity
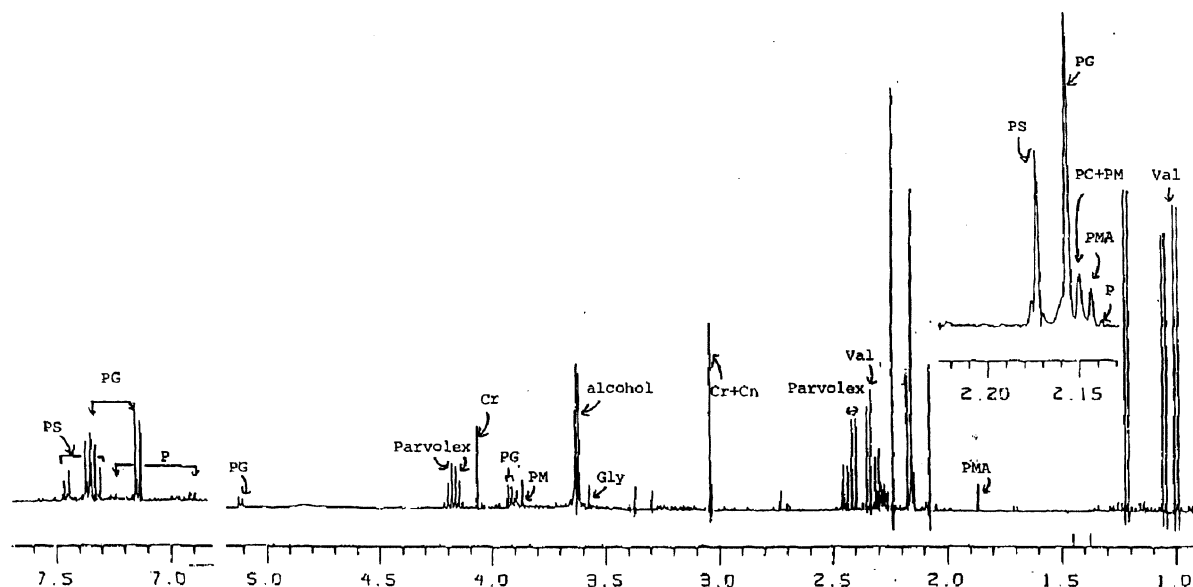
Fig. 42. 400 MHz ^{1}H NMR spectrum of a urine sample from a patient who had taken paracetamol overdose and had been treated with parvolex (N-acetylcysteine). P, paracetamol; PG, paracetamol glucuronide; PS, paracetamol sulfate; PMA, paracetamol mercapturic acid; PC, paracetamol L-cystein; PM, methoxy-paracetamol (taken with permission from the thesis of M.L. Sitanggang, Bath, UK, 1988).

(dephasing) of signals of carbons linked to hydrogens because of very effective proton relaxation, whereas the resonances of non-protonated carbons remain intense. These two techniques, 'short contact' and 'delayed decoupling', allow selective observation of protonated and non-protonated carbons, respectively; both are helpful for the assignment of signals in solid-state ^{13}C NMR spectra.

In the following, the spectra recorded for solid-state are compared with those obtained for solutions. For solution spectra, conformational information is lost by averaging of chemical shifts through fast intramolecular motions whereas, in solids, conformations are locked, revealing specific chemical environments with different chemical shifts. Therefore, similar chemical shifts are usually observed for rigid molecular fragments, and significant differences between solid and solution state spectra are indicative of conformational equilibration. These differences, for example, reached 9.2 ppm for carbons *ortho* to the OCH_3 or OH group in methoxy (hydroxy)-benzenes and made possible the assignment of resonances of the synthetic estrogen, *meso*-hexestrol [125].

It must be stressed that ^{13}C resonance intensities of CP MAS NMR spectra are governed by cross-polarisation kinetics and relaxation processes (mainly proton relaxations) and, therefore, the intensities cannot be used for quantitative analysis. In order to obtain quantitatively reliable results, a series of spectra should be recorded as a function of varied cross-polarisation and repetition times (to determine proton spin lattice relaxation times in laboratory frame T_{lp}). The details of quantitative ^{13}C CP MAS NMR have been reported [126–128]. Solid-state ^{13}C NMR was used to determine the amounts of carbamazepine anhydrate and dihydrate [129] and, recently [130], to quantify relative amounts of delavirdine mesylate polymorphs and pseudopolymorphs; these investigations demonstrated that unambiguous identification of the polymorphic forms was possible with empirical detection limits of about 2–3%. The greatest advantage of the NMR method lies in the assignment of the minor polymorphic form in the dominant environment of the other form. Additionally, the analysis is carried out in a non-destructive manner. The majority of applications of solid-state NMR reviewed by Brittain et al.
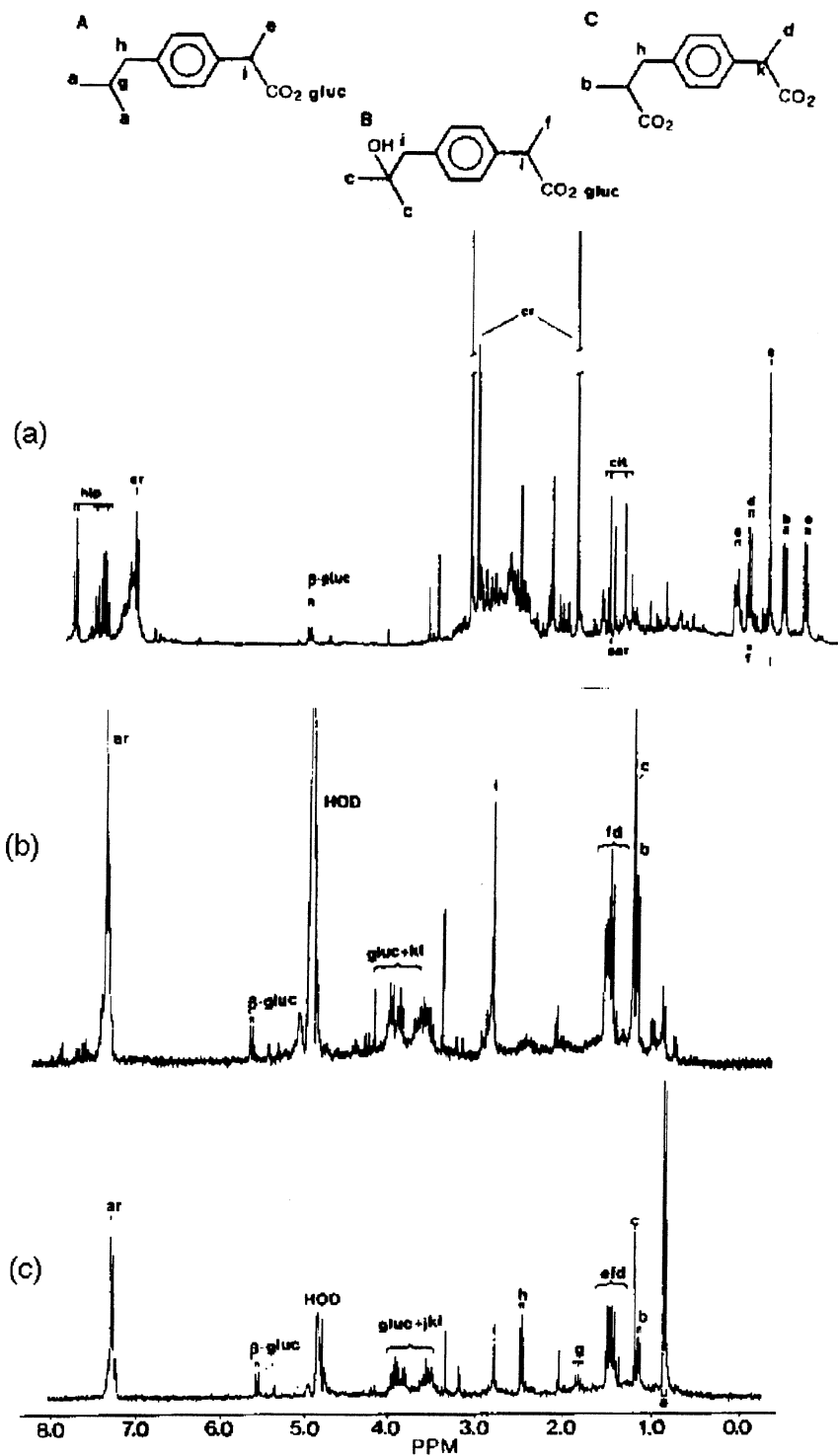
Fig. 43. (a) 250 MHz ^{1}H NMR spectrum of a sample of freeze-dried urine (2 ml) obtained from a subject following oral dosing with 400 mg of ibuprofen. The sample was redissolved in D_2O for spectroscopy. (b) 200 MHz ^{1}H NMR spectra showing the eluates obtained with 40:60 methanol/water (248 scans) and (c) 60:40 methanol/water (66 scans) from a C18 SPE column on which 2 ml of human urine from a sample obtained for the 2–4 h after a 400-mg dose of ibuprofen (Reprinted with permission from Ref. [74], page 2831, Copyright 1997 ACS).
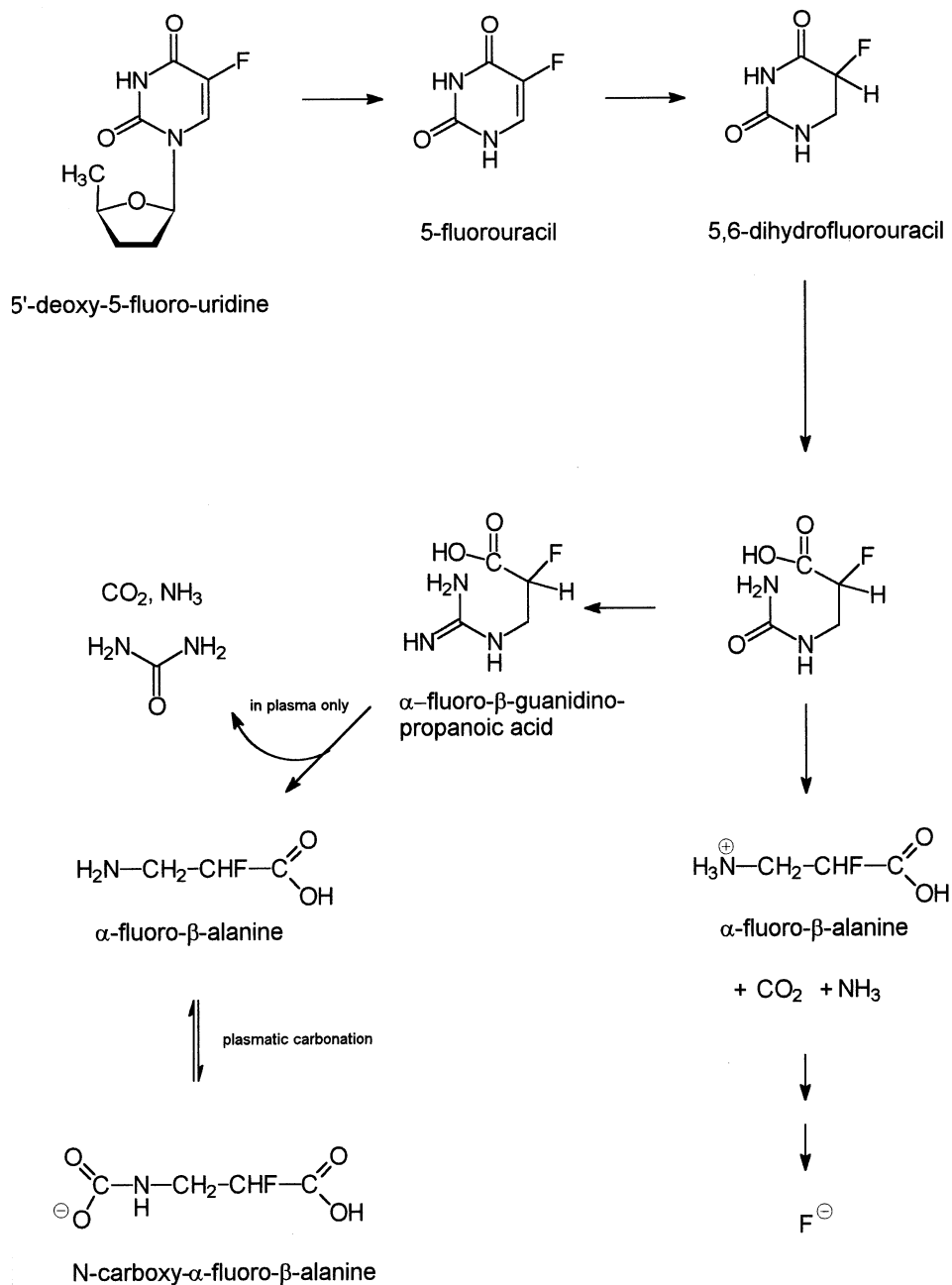
Fig. 44. Metabolic pathway of 5'-deoxy-5-fluoro-uridine and 5-fluorouracil, respectively.

[131] and later by Bugay [132] were the investigations of pharmaceutical polymorphs. Different polymorphic forms of solid benoxaprofen, nabilione and pseudopolymorphic (e.g. including solvent) crystal forms of cefazolin have been reported in 1985 by Byrn et al. [133]. Various polymorphs of steroids were found: three pseudopolymorphic forms of testosterone [134], anhydrous and monohydrate forms of androstanolone [135], six crystal forms of cortisone acetate [136]

and five forms of prednisolone *t*-butylacetate [137].

Structural diversity was detected by crystallography (X-ray diffraction, XRD) and solid-state NMR evidenced the effects of crystal packing, which were mainly due to various hydrogen bonding patterns. Solid-state ^{13}C NMR is a complementary technique to crystallography for obtaining structural information about solids, as the number of resonances in the spectrum is equal to the number on non-equivalent carbons in an asymmetric structural unit. Thus, NMR provides immediate indication of the presence of any configurational or conformational multiplicity that may exist in the solid-state. Since NMR uses powdered compounds, one can avoid the difficulties of growing good quality single crystals suitable for XRD analysis. The method is particularly useful in those cases where crystallographic examination is not possible because the sample is neither crystalline nor does it form a complex polymorph. In numerous studies, together with crystallography, IR spectroscopy and differential scanning calorimetry (DCS), solid-state NMR has been used as an additional source of structural information. The characterisation of solid-state structures of losartan [138], frusemide (furosemide) [139], cyclopenthiazide [140], chiral leukotriene antagonists [141,142], fosinopril sodium [143,144] and the antibiotics cefaclor (dihydrate) [145] and penicillin [146] was possible.

It is of interest to the pharmaceutical industry to be able to characterise drugs directly in their final dosage forms. Two of the first tablets studied were aspirin [147,148] and acetaminophen [149].

^{13}C CP MAS NMR spectra of tablets of non-steroidal antiinflammatory agents (ibuprofen, flurbiprofen, mefenamic acid, indomethacin, diflunisal, sulindac and piroxicam) prednisolone, enalapril maleate, lovastatin, simvastatin, showed [150] that solid-state NMR can detect the drug in low dose tablets, e.g. differentiate between tablets containing drug or placebo even in presence of large amounts of excipients.

Polymers of amorphous solids used as pharmaceutical excipients have also been studied by means of solid-state NMR. The investigations included celluloses [151], amyloses and starches [152,153], hydrated and anhydrous lactose [131] as well as cyclodextrins [154], which are readily available natural host compounds and increasingly popular for the encapsulation of drugs.

Solid-state NMR studies of biomolecules, D,L-methionine [155], natural and synthetic melanins [156], aminoacids and peptides [157,158], and flavonoids [159] should also be mentioned.

5. NMR imaging

5.1. ^{1}H NMR imaging of tablets

NMR signals for solids not subjected to magic angle spinning usually exhibit broad signals with low intensity, and the solid substances cannot be visualised with spin-echo or gradient-echo methods usually applied in Magnetic Resonance Imaging (MRI, or tomography) for taking images. However, if a liquid such as water or oil, producing intense signals, is introduced into the cavities of a solid, the inner structure can be displayed. The method was utilised for the first time [160] to study the porosity of tablets filled with silicone oil. The images of the tablets were obtained with a Bruker AM 300 NMR spectrometer, wide bore magnet and probe head specially designed for micro-imaging allowing sample diameters from 1–25 mm. The cross sectional signals through the tablet were transformed by computer into contour plots and colour images. Spatial resolution was about 95 μm for an edge length of the cube, and as the resolution at microscopic level was achieved, the method is also called NMR microscopy. Two-dimensional images of tablets can be obtained in about 30 min; for three-dimensional ones, 4 h are necessary, depending on the required resolution and signal-to-noise ratio. Porosity distribution within tablets, cracks and cavities were seen in physically intact tablets. The different states of densification during compaction of powders and different compaction mechanisms with plastic-type or brittle-type tablets could be observed.

NMR imaging was applied [161] for studying the formation of the gel layer in hydrating hydroxypropylmethylcellulose (HPMC) tablets. The
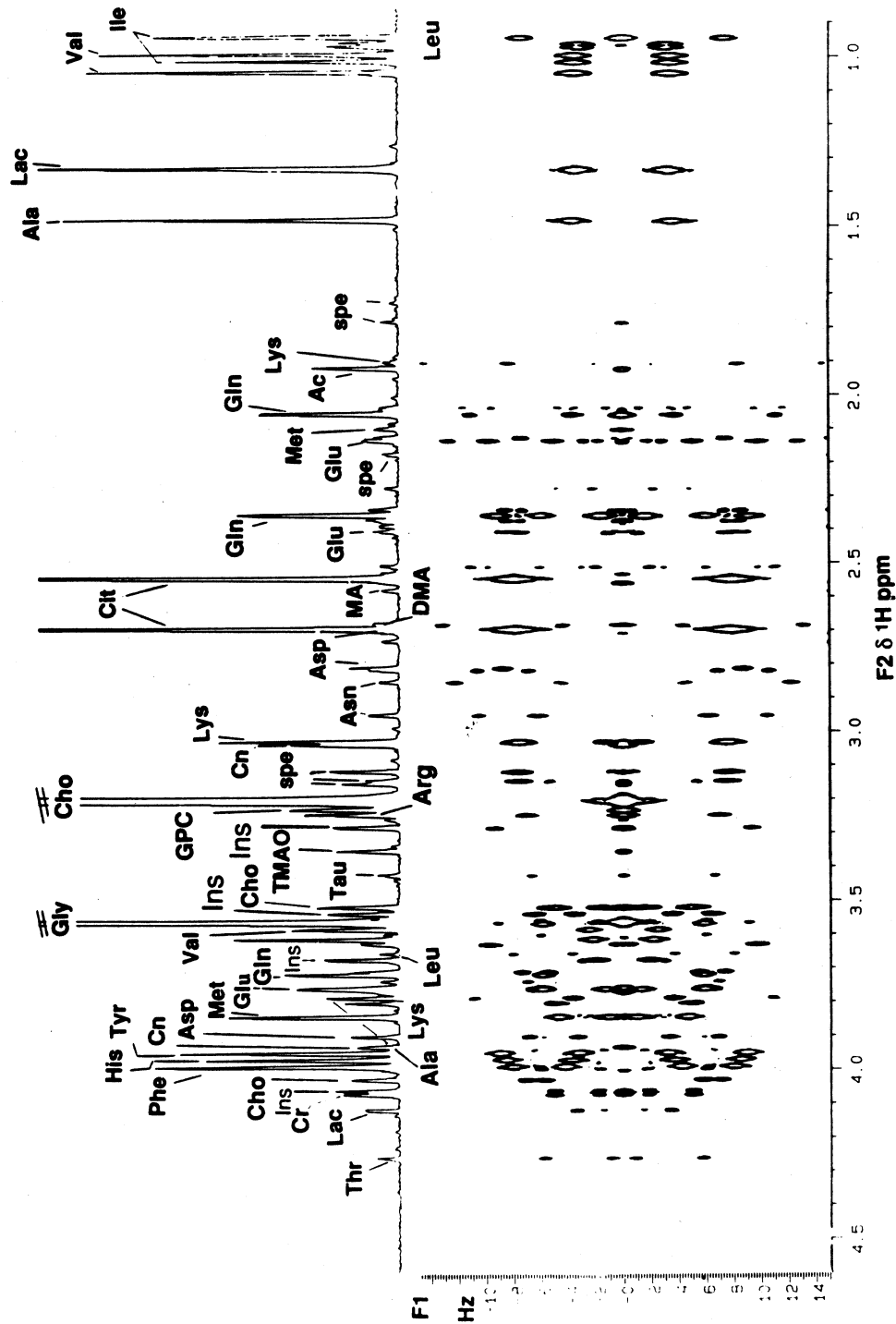
Fig. 45. 600 MHz ^{1}H NMR spectrum JRES of the aliphatic region of control human seminal fluid from δ 0.9–4.6 ppm, showing contour plot and skyline F2 projection. Key: Ac, acetate; Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartate; Cho, choline; Cit, citrate; Cr, creatine; DMA, dimethylamine; Gln, glutamine; Glu, glutamate; Gly, glycine; GPC, glycerophosphorylcholine; His, histidine; Ile, isoleucine; Ins, *myo*-inositol; Lac, lactate; Leu, leucine; Lys, lysine; MA, methylamine; Met, methionine; Phe, phenylalanine; Spe, spermine; Tau, taurine; Thr, threonine; TMAO, trimethylamine-*N*-oxide; Trp, tryptophan; Tyr, tyrosine; Uri, uridine; Val, valine (Reprinted from Ref. [100], page 10, Copyright 1997) with kind permission of Elsevier Science NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands).
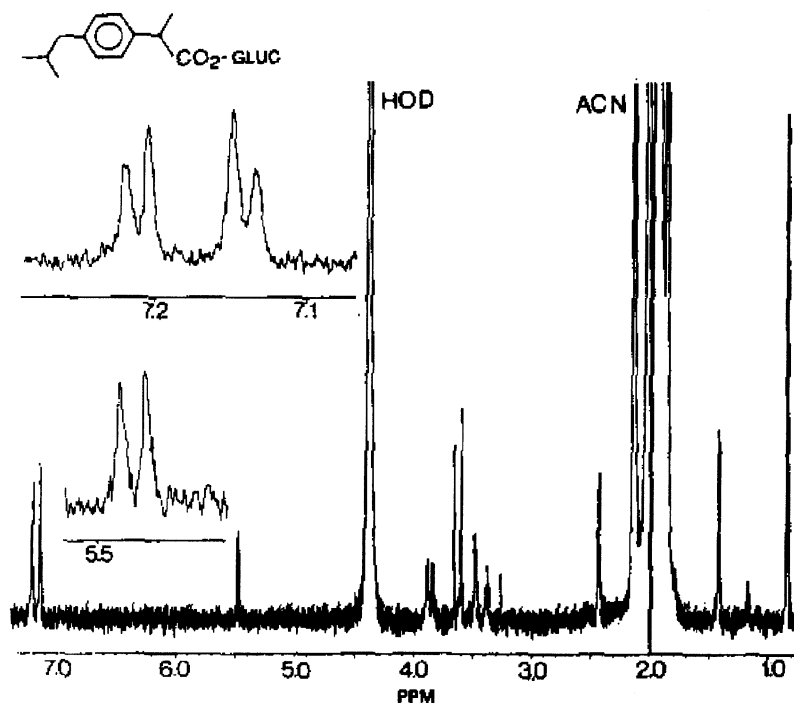
Fig. 46. 500 MHz stopped-flow ^1H NMR spectrum obtained for ibuprofen glucuronide; 128 scans, NOESY pulse sequence with presaturation, 1 Hz line boradening. (Reprinted with permission from Ref. [105], page 329, Copyright 1997, ACS).

tablets were mounted and hydrated for up to 3 h. The in-plane resolution was 100 μm and each image was acquired in about 2 min. Diffusion weight images showed different degrees of polymer hydration at different depths within the gel, and revealed additionally that HPMC matrices expand on hydration in the axial rather than in the radial direction. This work demonstrated that NMR imaging could be used to non-invasively measure the diffusion of water, thus obtaining valuable information on dynamics within various dosage forms.

For the studies of fast water displacement, the FLASH (Fast Low Angle Shot) technique may be used [162], which allows to obtain good quality images within a fraction of a second, with minutes or hours being necessary for conventional spin-echo methods. The images shown in Fig. 50a, b were obtained in 0.4 s, using the home-built NMR spectrometer (^1H frequency 271.3 MHz, 6.4 T magnet) in the Institute of Nuclear Physics, Cracow, Poland. The time of the experiment was short enough to follow the dissolution of the tablet in water (with 0.1 M HCl added to imitate the environment of gastric juice).

The tablet, at the bottom of the glass NMR tube, was placed inside the probe head, and the first image was obtained after 3 min (Fig. 50a). The place of the cross-section is marked with a white line, and at the top of the picture, there is the respective plot of water signal intensity. The tablet dissolves fast and after 20 min part of the coating was separated, seen as a black shape on the right side (Fig. 50b). The intensity of the water signal in time can be considered as the measure of the drug release rate, which may give rise to different dosage form characteristics.

The fast imaging method opens a new field of investigation of drugs dissolution and release from various types of tablets now tested in vitro, but in the near future also in vivo.

5.2. Multinuclear NMR imaging in vivo

NMR systems for investigations in vivo use horizontal-axis superconducting magnets (1.5–9.4
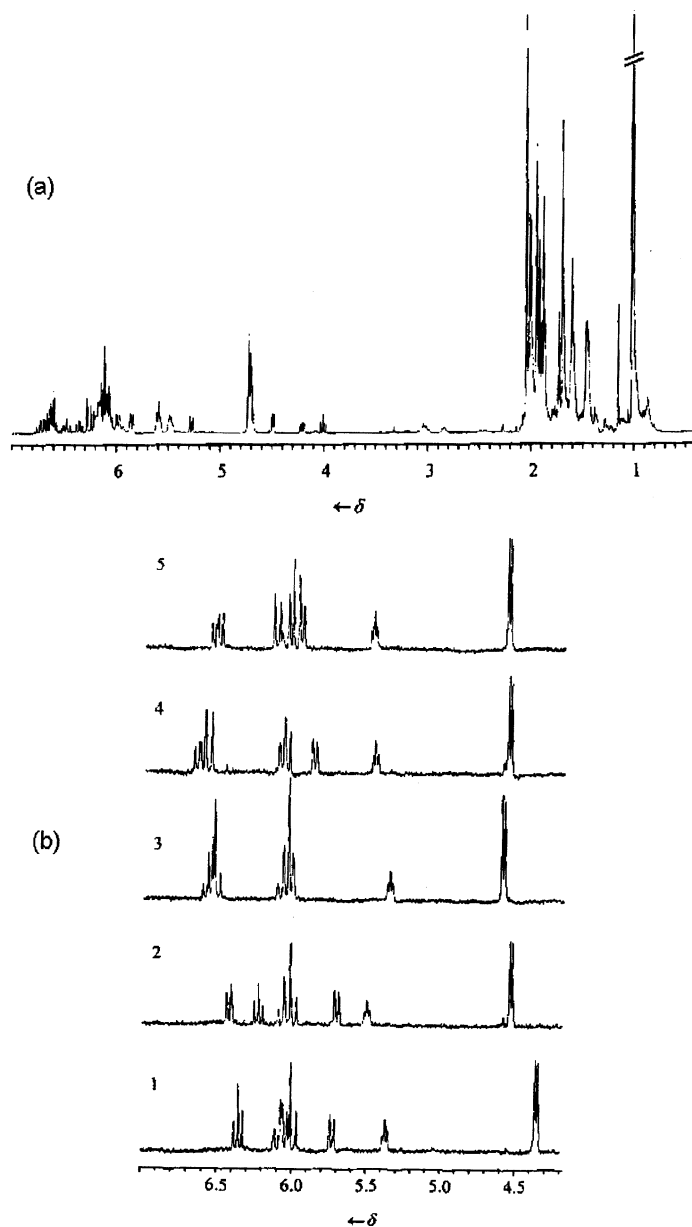
Fig. 47. (a) 400 MHz ^{1}H NMR spectrum of the isomeric mixture of vitamin-A-acetate and (b) continuous-flow 400 MHz ^{1}H NMR spectra of each isomer: 1: 11,13-di-*cis*-, 2: 11-*cis*, 3: 13-*cis*, 4: 9-*cis*, 5: all-*trans*-vitamin A-acetate (Reprinted with permission from Ref. [114], page 1103, Copyright 1997, VCH Verlagsgesellschaft, Weinheim, and the author K. Albert).

T) with bore diameters of 15–90 cm; probes for sample diameters of 7–20 cm are designed for small animals whereas larger bores and magnetic field strength lower than 4 T are applied to humans. The imaging technique is capable of giving images of cross sections through the human/animal body with good resolution in all three dimensions. Depending on the dimensionality of the region from which the signal is observed, one can obtain information by point-, line-, plane- or volume-
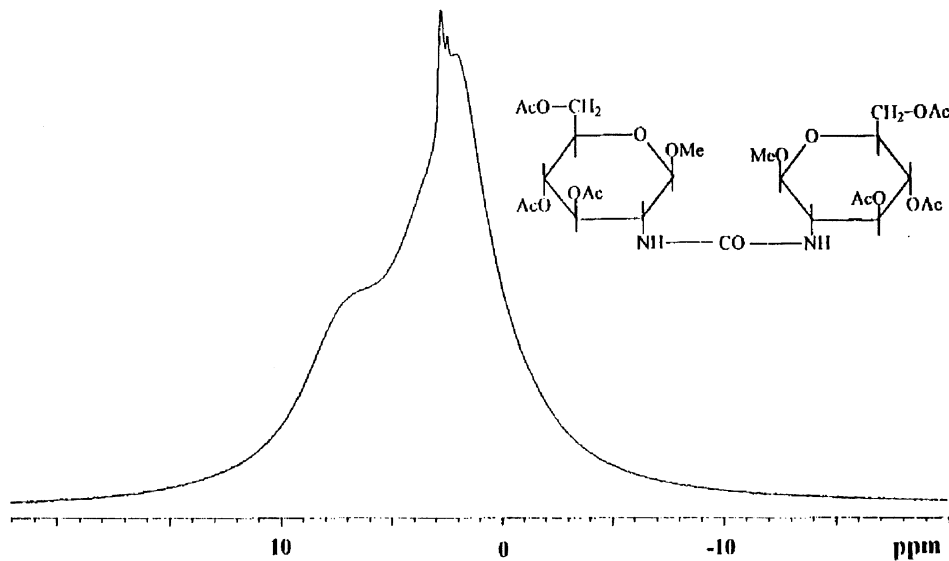
Fig. 48. [1]H MAS spectrum (at 400 MHz) of acetylated ureido sugar, rotation speed 16 kHz.

selective techniques. The point-selective technique can be used to obtain a spectrum from a chosen place in order to examine chemical shifts (in vivo Magnetic Resonance Spectroscopy, MRS). The two-dimensional technique with slice selection is most frequently used for imaging (in vivo Mag-

netic Resonance Imaging, MRI). MRI yields anatomical information (section through the head of one of the authors showing the brain, obtained by ^{1}H MRI is illustrated in Fig. 51) and is mainly applied in clinical diagnostics. Research applications involve ^{1}H, ^{19}F, ^{31}P and also ^{13}C and [15]N

Fig. 49. [13]C CP MAS spectrum (at 75.5 MHz of acetylated ureido sugar with amino acid (Ala-Gly) residues; rotation speed 3.29 kHz, rotational side-bands are marked with asterisk.

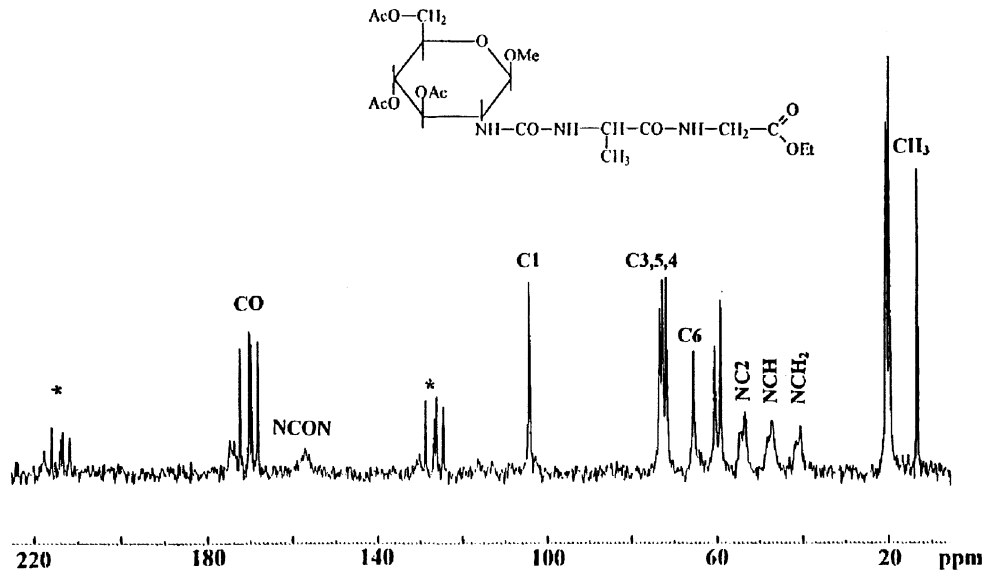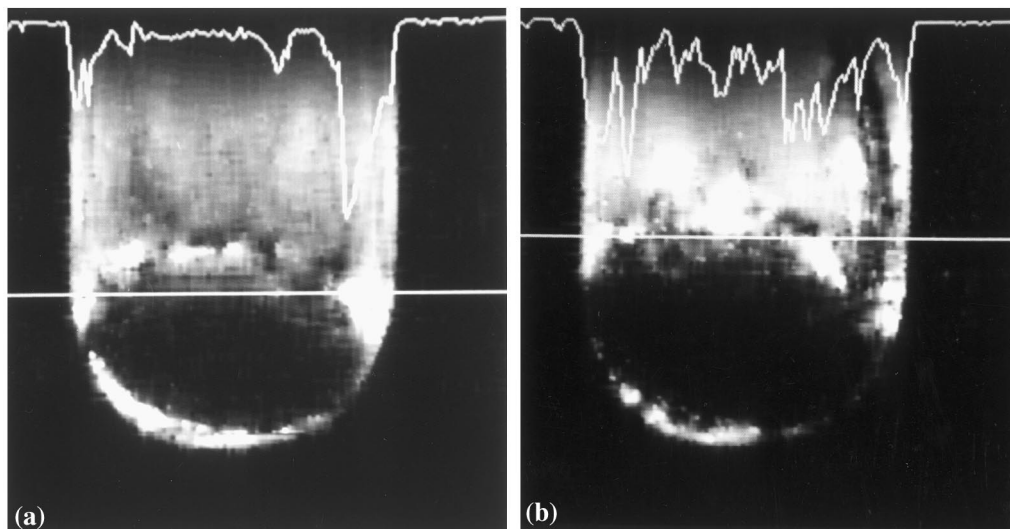Fig. 50. The images of a tablet (Fenactil) in water obtained by FLASH ^{1}H NMR imaging, (a) after 3 min, (b) after 20 min. The penetration of water inside the tablet is illustrated (at the top) as a plot of signal intensity at the place marked with a white line.

(with isotopically enriched substances) and provide new possibilities in many areas of medicine and pharmacology, as endogenous and exogenous compounds as well as the reaction of an organism to the drug can be monitored (either by imaging or by localised spectroscopy).

^{1}H MRS and MRI studies were performed to differentiate in vivo the normal ageing human brain from age-associated memory impairment and dementia of the Alzheimer type. ^{1}H MRS showed significantly lower N-acetylaspartate concentration and higher inositol concentration in pathological conditions [163]. ^{1}H MRI monitored the progression of adjuvant arthritis in rat leg joints and its response to indomethacin treatment; the authors proposed MRI monitoring of the therapy of arthritis in humans [164]. The Multi-slice technique of MRI was used to study regional effects of the central stimulant amphetamine on the rat brain [165]; amphetamine caused significant increases in perfusion in many areas of the brain.

Interesting investigations of drug distribution in vivo were possible using ^{19}F NMR imaging. With ^{19}F MRI/MRS, the uptake, distribution and elimination of sevoflurane (a fluorinated anaesthetic) in the rat brain was observed [166]. An assessment of quantitative imaging of cerebral blood flow in a cat, using trifluoromethane, has been reported [167]. A

fluorinated representative of antifolates (which are being evaluated for the treatment of human cancer) was followed in vivo in mice after intravenous injection; the drug was found in gall and urinary bladders [168]. Fast ^{19}F MRI with FLASH technique was applied in vivo and the imaging time amounted to only 470 ms [169]; the half-life of perfluorooctylbromide in inner organs of rats was determined [170].

^{13}C MRI was used to observe the glucose metabolism in the human brain [171]; the spectra were obtained with a resolution time of 9 min using intravenous infusions of 1-^{13}C-glucose. At an isotopic enrichment level of 20%, the signals of α- and β-glucose at 92.7 and 96.6 ppm, respectively, were detected; increasing the enrichment level to 99%, the metabolic breakdown products of 1-^{13}C-glucose, glutamate, glutamine and lactate, could be recorded in the human brain.

The in vivo activity of phosphate-activated glutaminase was measured in the brain of rats by ^{15}N NMR [172].

Brain glutamine was enriched in ^{15}N by intravenous infusion of ^{15}NH$_4^+$, further glutamine synthesis was stopped by injection of methionine-DL-sulphoxime and the infusate was changed to ^{14}NH$_4^+$. The decrease of ^{15}N-glutamine, the production of ^{15}NH$_4^+$ and its assimilation into glutamate were monitored in the brain.

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MAGNETOM 1.5 T   UK BENJAMIN FRANKLIN FUB
IWONNA                        D2 FB    2SE
2264                UP                  H-SP
26-NOV-94
11:49:48
STORE>33
                                        B
                                        A
                                        C
                                        K

                                        | 5 CM
                                        |

                     BRAIN
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MAGNETOM 1.5 T   UK BENJAMIN FRANKLIN FUB
IWONNA                        D2 FB    2SE
2264              FRONT           CR-H-SP
26-NOV-94
11:39:12
STORE>14
                                        L
                                        E
                                        F
                                        T

                                        | 5 CM
                                        |

                     BRAIN
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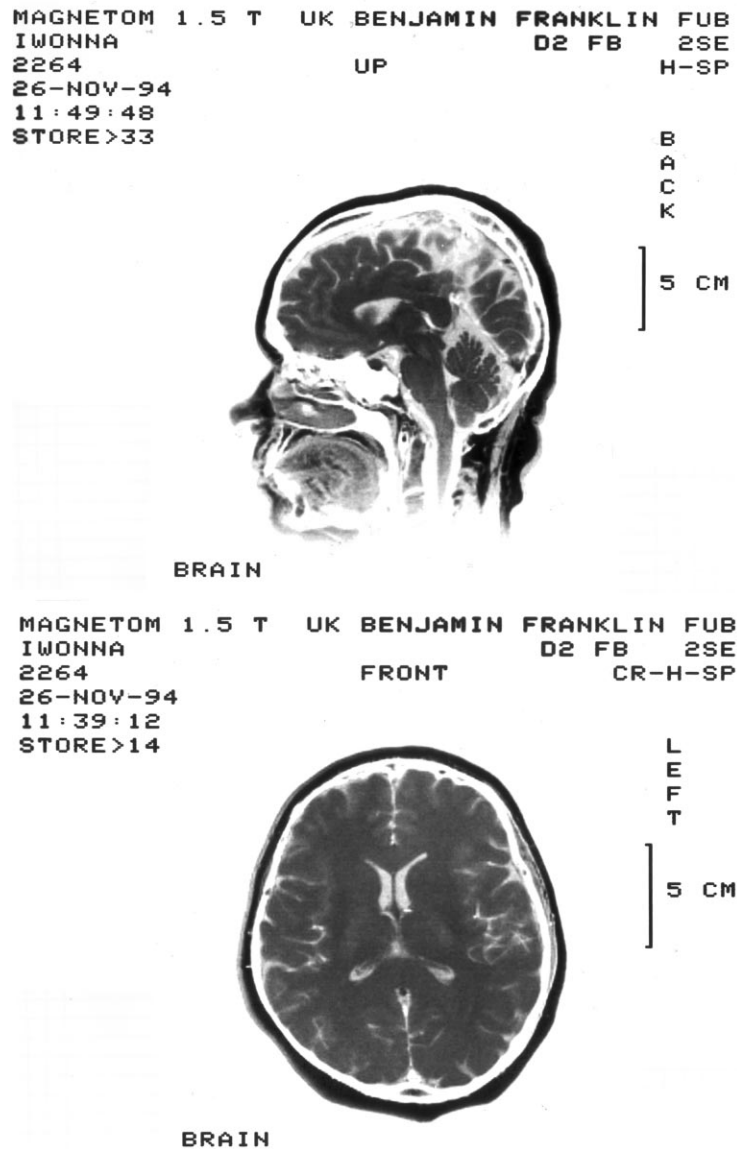
Fig. 51. Sections through the human head showing anatomical details of the brain; obtained by MRI at 40 MHz, 1.5 wide-bore magnet.

Several examples of clinical applications of ^{31}P and ^{1}H MRS (spectra of liver and brain) were reviewed in 1996 by Bell [173]. Since the studies of dose-response relationships carried out in vivo are of particular interest, the effect of L-alanine infusion on the concentrations of gluconeogenic intermediates in normal human liver [174] studied by ^{31}P spectroscopy should be mentioned.

Pharmacological applications of multinuclear MRS/MRI are in accordance with the new trend towards non-invasive techniques that reduce the number of tested animals. Usually, numerous animals must be sacrificed for one pharmacokinetic study, and the results contain uncertainties because of the individual variability of living organisms. The authors are of the opinion that the

techniques of magnetic resonance in vivo will be employed increasingly and are particularly important for testing new and/or potent drugs in preclinical research in the pharmaceutical industry.

6. Concluding remarks

NMR spectroscopy has a long-standing tradition in the elucidation and confirmation of the structure of synthetic products as well as compounds of natural origin. In this review we have attempted to illustrate the power and versatility of modern high-field NMR techniques in the characterisation of drugs with regard to purity, stability and isomeric composition. A rather new field is the monitoring of the pharmacokinetics, e.g. metabolism, by means of NMR spectroscopy, especially in combination/hyphenation with HPLC and other chromatographic or electrophoretic methods. As soon as 600 and 750 MHz instruments become more general equipment in analytical laboratories, the application of those techniques will increase rapidly. The importance of non-invasive techniques of NMR imaging will enhance in different fields of medicine, e.g. in monitoring the distribution and fate of drugs or naturally occurring compounds. The power of solid state measurement has not yet been fully recognised by the scientific community of pharmaceutics. NMR spectroscopy can be equal to powder diffraction spectroscopy and corresponding techniques. Since the application of NMR in the elucidation of protein-ligand complexes and the correlation between X-ray structures and structures of drugs in solution has recently been reviewed [176], we have not touched this area. However, the importance of NMR spectroscopy in drug development and drug analysis should not be underestimated. On the contrary, it will increase in the future.

References

[1] K. Wüthrich, Acta Cryst D51 (1995) 249–270.
[2] G.A. Neville, T.J. Racey, R.N. Rej, A.S. Perlin, J. Pharm. Sci. 79 (1990) 425–427.
[3] G.A. Neville, F. Mori, T.J. Racey, P. Rochon, K.R. Holme, A.S. Perlin, J. Pharm. Sci. 79 (1990) 339–343.
[4] G.A. Neville, F. Mori, K.R. Holme, A.S. Perlin, J. Pharm. Sci. 78 (1989) 101–104.
[5] U.R. Desai, R.J. Linhardt, J. Pharm. Sci. 84 (1995) 212–215.
[6] S. Tachibana, S. Nishiura, S. Ishida, K. Kakehi, S. Honda, Chem. Pharm. Bull. 38 (1990) 2503–2506.
[7] B. Casu, M. Guerrini, A. Naggi, G. Torri, L. De-Ambrosi, G. Boveri, S. Gonella, A. Cedro, L. Ferro, E. Lanzarotti, M. Paterno, M. Attolini, M.G. Valle, Arzneim.-Forsch./Drug. Res. 46 (1996) 472–477.
[8] F. Nachtmann, G. Atzl, W.D. Roth, Anal. Profiles Drug Subst. 12 (1983) 215–276.
[9] K.G. Ludwig-Baxter, R.N. Rej, A.S. Perlin, G.A. Neville, J. Pharm. Sci. 80 (1992) 655–660.
[10] G.A. Neville, P. Rochon, R.N. Rej, A.S. Perlin, J. Pharm. Sci. 80 (1991) 239–244.
[11] (SSL), Method developed by Spectral Service GmbH, Cologne, Germany, not be published previously.
[12] G.L. Silvey, J. Pharm. Sci. 81 (1992) 471–474.
[13] A.E. Tonelli, The NMR spectra and the microstructures of polymers, in: Allan H. Fawcett (Ed.), Polymer Spectroscopy, Wiley, Chichester, 1996, pp. 55-95.
[14] P.P. Lankhorst, M.M. Poot, M.P.A. de Lange, Pharmacopeial Forum 22 (3) (1996) 2414–2422.
[15] G.M. Hanna, C.A. Lau-Cam, J. Pharm. Biomed. Anal. 11 (1993) 855–859.
[16] T.A. Schmedake, L.E. Welch, J. Chem. Educ. 73 (1996) 1045–1048.
[17] B.W.K Diehl, W. Ockels, Quantitative Analysis of Phospholipids, in: G. Cevc, F. Paltauf (Eds.), Proc. 6th Int. Colloq. Phospholipids. Characterization, Metabolism and Novel Biological Applications, AOCS Press, Champaign, 1995, pp. 29-32. Also B.W.K. Diehl, W. Ockels,

H. Herling, R. Unger, S. Winkler, Quantitative Determination of Egg-Lecithine in a Liposome-Preparation, Validation of the ^{31}P NMR Method, 7th Int. Cong. Phospholipids, Brussels, Belgium, September 1996

[18] J. De Kock, The European Analytical Subgroup of I.L.P.S.: A Joint Effort to Clarify Lecithin and Phospholipid Analysis, Fat Sci. Technol. 95 (9) (1993) 352–355.

[19] J. De Kock, The European Analytical Subgroup of I.L.P.S., in: G. Cevc, F. Paltauf (Eds.), Characterization, Metabolism and Novel Biological Applications, AOCS Press, Champaign, pp. 22-28.

[20] L.L. Olson, A.P. Cheung, J. Pharm. Biomed. Anal. 8 (1990) 725–728.

[21] Pharmeuropa 8 (1986) 232-234.

[22] C. Boschetti, G. Fronza, C. Fuganti, P. Grasselli, A.G. Magnone, A. Mele, C. Pellegatta, Arzneim.-Forsch./Drug Res. 45 (1995) 1217–1221.

[23] Pharmeuropa 8 (1996) 521-523.

[24] Pharmeuropa 6 (1994) 291.

[25] A.F. Casy, G. H Dewar, J. Pharm. Biomed. Anal. 12 (1994) 855–861.

[26] J.K. Baker, C.W. Myers, Pharm. Res. 8 (1991) 761–770.

[27] A. TM Chan, K.A. Evans, E.A. Belsky, D.J.S. Ditnfeld, A.T. Tsai, A. McPhail, Magn. Res. Chem. 34 (1996) 1025–1030.

[28] B.A. Dawson, D.B. Black, G.A. Neville, J. Pharm. Biomed. Anal. 13 (1995) 395–407.

[29] G.A. Neville, H.D. Beckstead, D.B. Black, B.A. Dawson, H.F. Shurvell, J. Pharm. Sci. 83 (1994) 1274–1279.

[30] B. Vilanova, F. Munoz, J. Donoso, J. Frau, F.G. Blanco, J. Pharm. Sci. 83 (1994) 322–327.

[31] E. Inkmann, K.F. Hesse, U. Holzgrabe, Pharmazie 52 (1997) 764–774.

[32] G.S. Sadane, A.B. Ghogare, J. Pharm. Sci. 80 (1991) 895–898.

[33] S. Görög, G. Balogh, M. Gazdag, J. Pharm. Biomed. Anal. 9 (1991) 829–833.

[34] J.A. Dale, D.L. Dull, H.S. Mosher, J. Org. Chem. 34 (1969) 2543–2649.

[35] D. Parker, Chem. Rev. 91 (1991) 1441–1457.

[36] H.Y. Aboul-Enein, Anal. Lett. 21 (1988) 2155–2163.

[37] L.M. Sweeting, D.C. Crans, G.M. Whiteside, J. Org. Chem. 52 (1987) 2273–2276.

[38] P.E. Peterson, M. Stepanian, J. Org. Chem. 53 (1988) 1907–1911.

[39] G.R. Weisman, in: J.D. Morrison (Ed.), Asymmetric Synthesis, vol. 1, ch. 8, Academic Press, New York, 1983, p. 153-171

[40] W.H. Pirkle, D.J. Hoover, Top. Stereochem. 13 (1982) 264–331.

[41] B.A. Dawson, S.A. Qureshi, D.B. Black, 33rd Experimental Nuclear Magnetic Resonance Conference, Pacific Grove, CA, 1992

[42] C. Dauwe, J. Buddrus, GIT Fachz. Lab. 5 (1994) 517–518.

[43] B. Staubach, J. Buddrus, Angew. Chem. 108 (1996) 1443–1445.

[44] V. Dinnendahl, U. Fricke, Arzneistoff-Profile, Govi-Verlag, Frankfurt a. M. 1982-1996.

[45] G.M. Hanna, C.A. Lau-Cam, Pharm. Res. 7 (1990) 726–729.

[46] G.M. Hanna, C.A. Lau-Cam, J. Pharm. Biomed. Anal. 11 (1993) 665–670.

[47] P.M. Lacroix, B.A. Dawson, R.W. Sears, D.B. Black, Chirality 6 (1994) 484–491.

[48] B.A. Dawson, D.B. Black, 37th Experimental Nuclear Magnetic Resonance Conference, Pacific Grove, CA, 1996

[49] B.A. Dawson, D.B. Black, J. Pharm. Biomed. Anal. 13 (1995) 39–44.

[50] C.G. Chavdarian, E.B. Sanders, R.L. Bassfield, J. Org. Chem. 47 (1982) 1069–1073.

[51] J.W. Jaroszewski, A. Olsson, J. Pharm. Biomed. Anal. 12 (1994) 295–299.

[52] A.F. Casy, TRAC 12 (1993) 185–189.

[53] C.A. Marchant, S.K. Branch, J. Pharm. Pharmacol. 42 (1990) P68.

[54] T.T. Ndou, S. Mukundan Jr, I.M. Warner, J. Incl. Phenom. 15 (1993) 9–15.

[55] M. Wiese, H.-P. Cordes, H. Chi, J.K. Seydel, T. Backensfeld, B.W. Müller, J. Pharm. Sci. 80 (1991) 153–156.

[56] A.F. Casy, A.F. Drake, C.R. Ganellin, A.D. Mercer, C. Upton, Chirality 4 (1992) 356–366.

[57] J.R. Moyano, M.J. Arias-Blanco, J.M. Gines, A.M. Rabasco, J.I. Perez-Martinez, M. Mor, F. Giordano, J. Pharm. Sci. 86 (1997) 72–75.

[58] A.F. Casy, A.D. Cooper, T.M. Jefferies, R.M. Gaskell, D. Greatbanks, R. Pickford, J. Pharm. Biomed. Anal. 9 (1991) 787–792.

[59] C.J. Easton, S.F. Lincoln, Chem. Soc. Rev. 1 (1996) 163–170.

[60] S.K. Branch, U. Holzgrabe, T.M. Jefferies, H. Mallwitz, M. Matchett, J. Pharm. Biomed. Anal. 12 (1994) 1507–1517.

[61] S.K. Branch, U. Holzgrabe, T.M. Jefferies, H. Mallwitz, F.J.R. Oxley, J. Chromatogr. A 758 (1997) 277–292.

[62] U. Holzgrabe, H. Mallwitz, S.K. Branch, T.M. Jefferies, M. Wiese, Chirality 9 (1997) 211–219.

[63] A. Taylor, D.A.R. Williams, I.D. Wilson, J. Pharm. Biomed. Anal. 9 (1991) 493–496.

[64] Y. Kuroda, Y. Suzuki, J. He, T. Kawabata, A. Shibukawa, H. Wada, H. Fujima, Y. Go-oh, E. Imai, T. Nakagawa, J. Chem. Soc., Perkin Trans. 2 (1995) 1749–1759.

[65] U. Holzgrabe, M. Thunhorst, Magn. Res. Chem. 36 (1998) 211–216.

[66] J.K. Nicholson, I.D. Wilson, Prog. Drug Res. 31 (1987) 428–479.

[67] S.K. Branch, A.F. Casy, Prog. Med. Chem. 26 (1989) 355–436.

[68] J.R. Everett, K.R. Jennings, G. Woodnutt, M.J. Buckingham, J. Chem. Soc. Chem. Commun. 1 (1984) 894–895.

[69] S.C. Connor, J.R. Everett, K.R. Jennings, J.K. Nicholson, G. Woodnutt, J. Pharm. Pharmacol. 46 (1994) 128–134.

[70] I.D. Wilson, J. Fromson, I.M. Ismail, J.K. Nicholson, J. Pharm. Biomed. Anal. 5 (1987) 157–163.

[71] J.W. Jaroszewski, D. Berenstein, F.A. Slok, P.E. Simonsen, M.K. Agger, J. Pharm. Biomed. Anal. 14 (1996) 543–549.

[72] I.D. Wilson, I.M. Ismail, J. Pharm. Biomed. Anal. 4 (1986) 663–665.

[73] J.R. Bales, D.P. Higham, I. Howe, J.K. Nicholson, P.J. Sadler, Clin. Chem. 30 (1984) 1631-1636; S.K. Branch, L.J. Notorianni, M.L. Sitanggang, J. Pharm. Pharmacol. 38(Suppl.) 115P (1986); M.L. Sitanggang, Thesis, Bath, UK, 1988; T.D. Spurway, K.P.R. Gartland, A. Warrander, R. Pickford, J.K. Nicholson, I.D. Wilson, J. Pharm. Biomed. Anal. 8(Suppl) (1990) 969-973.

[74] I.D. Wilson, J.K. Nicholson, Anal. Chem. 59 (1987) 2830–2832.

[75] G.C. Leo, W.-N. Wu, J. Pharm. Biomed. Anal. 10 (1992) 607–613.

[76] P.J.D. Foxall, J.A. Parkinson, I.H. Sadler, J.C. Lindon, J.K. Nicholson, J. Pharm. Biomed. Anal. 11 (1993) 21–31.

[77] M.-C. Malet-Martino, R. Martino, A. Lopez, J.-P. Beteille, M. Bon, J. Bernadou, J.-P. Armand, Cancer Chemoth. Pharmacol. 13 (1984) 31–35.

[78] M.-C. Malet-Martino, J.-P. Armand, A. Lopez, J. Bernadou, J.-P. Beteille, M. Bon, R. Martino, Cancer Res. 46 (1986) 2105–2112.

[79] W.E. Hull, R.E. Port, R. Herrmann, B. Britsch, W. Kunz, Cancer Res. 48 (1988) 1680–1688.

[80] D. Meynial, A. Lopez, M.-C. Malet-Martino, L.S. Hoffmann, R. Martino, J. Pharm. Biomed. Anal. 6 (1988) 47–59.

[81] J.R. Everett, K. Jennings, G. Woodnutt, J. Pharm. Pharmacol. 37 (1985) 869–873.

[82] J.R. Everett, K. Jennings, G. Woodnutt, J. Pharm. Biomed. Anal. 7 (1989) 397–403.

[83] F.Y.K. Ghauri, C.A. Blackledge, I.D. Wilson, J.K. Nicholson, J. Pharm. Biomed. Anal. 8 (1990) 939–944.

[84] E. Holmes, B.C. Sweatman, M.E. Bollard, C.A. Blackledge, C.R. Beddell, I.D. Wilson, J.C. Lindon, J.K. Nicholson, Xenobiotics 25 (1995) 1269–1281.

[85] M. Tugnait, F.Y. Ghauri, J.K. Nicholson, K. Borner, I.D. Wilson, Bioanalytical Approaches, vol. 22, ch. 48, 1992, pp. 291-296.

[86] U. Holzgrabe, S.K. Branch, Magn. Res,. Chem. 31 (1994) 192–196.

[87] K.E. Wade, I.D. Wilson, J.A. Troke, J.K. Nicholson, J. Pharm. Biomed. Anal. 8 (1990) 401–410.

[88] M. Spraul, M. Hofmann, I.D. Wilson, E. Lenz, J.K. Nicholson, J.C. Lindon, J. Pharm. Biomed. Anal. 11 (1993) 1009–1015.

[89] N.E. Preece, J.K. Nicholson, J.A. Timbrell, Biochem. Pharmacol. 41 (1991) 1319–1324.

[90] D. Busse, F.W. Busch, F. Bohnenstengel, M. Eichbaum, P. Fischer, J. Opalinska, K. Schumacher, E. Schweizer, H.K. Kroemer, J. Clin. Oncol. 15 (1997) 1885–1896.

[91] M.J. Basker, S.C. Finch, J.W. Tyler, J. Pharm. Biomed. Anal. 8 (1990) 573–576.

[92] D.A. Ryan, J.K.M. Sanders, G.C. Curtis, H. Hughes, J. Pharm. Biomed. Anal. 13 (1995) 723–734.

[93] D.A. Ryan, J.K.M. Sanders, G.C. Curtis, H. Hughes, J. Pharm. Biomed. Anal. 13 (1995) 735–745.

[94] P.J.D. Foxall, M. Spraul, R.D. Farrant, J.C. Lindon, G.H. Neild, J.K. Nicholson, J. Pharm. Biomed. Anal. 11 (1993) 267–276.

[95] J.K. Nicholson, P.J.D. Foxall, M. Spraul, R.D. Farrant, J.C. Lindon, Anal. Chem. 67 (1995) 793–811.

[96] M. Spraul, J.K. Nicholson, M.J. Lynch, J.C. Lindon, J. Pharm. Biomed. Anal. 12 (1994) 613–618.

[97] M. Liu, J.K. Nicholson, J.C. Lindon, Anal. Chem. 68 (1996) 3370–3376.

[98] P.E. McGowan, J. Reglinski, R. Wilson, J.J. Walker, S. Wisdom, J.H. McKillop, J. Pharm. Biomed. Anal. 11 (1993) 629–632.

[99] B.C. Sweatman, R.D. Farrant, E. Holmes, F.Y. Ghauri, J.K. Nicholson, J.C. Lindon, J. Pharm. Biomed. Anal. 11 (1993) 651–664.

[100] M.J. Lynch, J. Masters, J.P. Pryor, J.C. Lindon, M. Spraul, P.J.D. Foxall, J.K. Nicholson, J. Pharm. Biomed. Anal. 12 (1994) 5–19.

[101] M. Spraul, M. Hofmann, J.C. Lindon, J.K. Nicholson, I.D. Wilson, in: E. Reid, H.M. Hill, I.D. Wilson (Eds.), Biofluid and Tissue Analysis for Drugs, Including Hypolipidaemics: Methodology Surveys in Bioanalysis of Drug, vol. 23, Royal Society of Chemistry, 1994, pp. 21-32.

[102] M. Hofmann, M. Spraul, R. Streck, I.D. Wilson, A. Rapp, Laborpraxis no. 10, 1993, p. 1/2; M. Thiele, M. Biernacki, M. Spraul, M. Hofmann, Bruker brochure LC NMR

[103] J.C. Lindon, J.K. Nicholson, I.D. Wilson, Adv. Chromatogr. 36 (1996) 315–382.

[104] L. Griffiths, Anal. Chem. 67 (1995) 4091–4095.

[105] M. Spraul, M. Hofmann, P. Dvortsak, J.K. Nicholson, I.D. Wilson, Anal. Chem. 65 (1993) 327–330.

[106] I.D. Wilson, J.K. Nicholson, M. Hofmann, M. Spraul, J.C. Lindon, J. Chromatogr. 617 (1993) 324–328.

[107] M. Spraul, M. Hofmann, J.C. Lindon, R.D. Farrant, M.J. Seddon, J.K. Nicholson, I.D. Wilson, NMR Biomed. 7 (1994) 295–303.

[108] M. Spraul, M. Hofmann, J.C. Lindon, J.K. Nicholson, I.D. Wilson, Anal. Proc. 30 (1993) 390–392.

[109] U.G. Sidelmann, C. Gavaghan, H.A.J. Carless, R.D. Farrant, J.C. Lindon, I.D. Wilson, J.K. Nicholson, Anal. Chem. 67 (1995) 3401–3404.

[110] U.G. Sidelmann, C. Gavaghan, H.A.J. Carless, M. Spraul, M. Hofmann, J.C. Lindon, I.D. Wilson, J.K. Nicholson, Anal. Chem. 67 (1995) 4441–4445.

[111] U.G. Sidelmann, E.M. Lenz, M. Spraul, M. Hofmann, J. Troke, P.N. Sanderson, J.C. Lindon, I.D. Wilson, J.K. Nicholson, Anal. Chem. 68 (1996) 106–110.

[112] E.M. Lenz, D. Greatbanks, I.D. Wilson, M. Spraul, M. Hofmann, J. Troke, J.C. Lindon, J.K. Nicholson, Anal. Chem. 68 (1996) 2832–2837.

[113] J.K. Roberts, R.J. Smith, J. Chromatogr. A 677 (1994) 385–389.

[114] K. Albert, G. Schlotterbeck, U. Braumann, H. Händel, M. Spraul, G. Krack, Angew. Chem. 107 (1995) 1102–1104.

[115] B. Behnke, G. Schlotterbeck, U. Tallarek, S. Strohschein, L.-H. Tseng, T. Keller, K. Albert, E. Bayer, Bayer, Anal. Chem. 68 (1996) 1110–1115.

[116] A.E. Mutlib, J.T. Strupczewski, S.M. Chesson, Drug Metab. Dispos. 23 (1995) 951–964.

[117] J.P. Shockcor, S.E. Unger, I.D. Wilson, P.J.D. Foxall, J.K. Nicholson, J.C. Lindon, Anal. Chem. 68 (1996) 4431–4435.

[118] K. Albert, U. Braumann, L.-H. Tseng, G. Nicholson, E. Bayer, M. Spraul, M. Hofmann, C. Dowle, M. Chippendale, Anal. Chem. 66 (1994) 3042–3046.

[119] U. Braumann, L.-H. Tseng, K. Albert, M. Spraul, GIT 38 (1994) 77–79.

[120] S.A. Korhammer, A. Bernreuther, Fresenius J. Anal. Chem. 35 (1996) 131–135.

[121] U. Braumann, H. Händel, K. Albert, R. Ecker, M. Spraul, Anal. Chem. 34 (1995) 930–935.

[122] S. Johnson, E.D. Morgan, I.D. Wilson, M. Spraul, M. Hofmann, J. Chem. Soc. Perkin Trans. 1 (1994) 1499–1502.

[123] A.C. Olivieri, L. Frydman, L.E. Diaz, J. Magn. Reson. 75 (1987) 50–62.

[124] L.B. Alemany, D.M. Grant, R.J. Pugmire, T.D. Alger, K.W. Zilm, J. Am. Chem. Soc. 105 (1983) 2133–2142.

[125] H. Saito, M. Yokoi, M. Aida, M. Kodama, T. Oda, Y. Sato, Magn. Reson. Chem. 26 (1988) 155–161.

[126] R.K. Harris, Analyst 110 (1985) 649–655.

[127] D.G. Retwitsch, M.A. Jacintha, C.R. Dybowski, Anal. Chim. Acta 283 (1993) 1033–1049.

[128] A. Jurkiewicz, G.E. Maciel, Anal. Chem. 67 (1995) 2188–2194.

[129] R. Suryanarayanan, T.S. Wiedmann, Pharm. Res. 7 (1990) 184–187.

[130] P. Gao, Pharm. Res. 13 (1996) 1095–1104.

[131] H.G. Brittain, S.J. Bogdanowich, D.E. Bugay, J. DeVincentis, G. Lewen, A.W. Newman, Pharm. Res 8 (1991) 963–973.

[132] D.E. Bugay, Pharm. Res. 10 (1993) 317–327.

[133] S.R. Byrn, G. Gray, R.R. Pfeiffer, J. Frye, J. Pharm. Sci. 74 (1985) 565–568.

[134] R.A. Fletton, R.K. Harris, A.M. Kenwright, R.W. Lancaster, K.J. Packer, N. Sheppard, Spectrochim. Acta 43A (1987) 1111–1120.

[135] R.K. Harris, B.J. Say, R.R. Yeung, R.A. Fletton, R.W. Lancaster, Spectrochim. Acta 45A (1989) 465–469.

[136] R.K. Harris, A.M. Kenwright, B.J. Say, R.R. Yeung, R.A. Fletton, R.W. Lancaster, G.L. Hardgrove, Spectrochim. Acta 46A (1990) 927–935.

[137] S.R. Byrn, P.A. Sutton, B. Tobias, J. Frye, P. Main, J. Am. Chem. Soc. 110 (1988) 1609–1614.

[138] K. Raghavan, A. Dwivedi, G.C. Campbell, E. Johnston, D. Levorse, J. McCauley, M. Hussain, Pharm. Res. 10 (1993) 900–904.

[139] C. Doherty, P. York, Int. J. Pharm. 47 (1988) 141–155.

[140] J.J. Gerber, J.G. vanderWatt, A.P. Lötter, Int. J. Pharm. 73 (1991) 137–145.

[141] R.G. Ball, M.W. Baum, J. Org. Chem. 57 (1992) 801–803.

[142] E.B. Vadas, P. Toma, G. Zografi, Pharm. Res. 8 (1991) 148–155.

[143] H.G. Brittain, K.R. Morris, D.E. Bugay, A.B. Thakur, A.T.M. Serajuddin, J. Pharm. Biomed. Anal. 11 (1993) 1063–1069.

[144] A.B. Thakur, K. Morris, J.A. Grosso, K. Himes, J.K. Thottathil, R.L. Jerzewski, D.A. Wadke, J.T. Cartensen, Pharm. Res. 10 (1993) 800–809.

[145] H. Martinez, S.R. Byrn, R.R. Pfeiffer, Pharm. Res. 7 (1990) 147–153.

[146] J. Fattah, J.M. Twyman, S.J. Heyes, D.J. Watkin, A.J. Edwards, K. Proust, Ch.M. Dobson, J. Am. Chem. Soc. 115 (1993) 5636–5650.

[147] C. Chang, L.E. Diaz, F. Morin, D.M. Grant, Magn. Res. Chem. 24 (1986) 768–771.

[148] L.E. Diaz, L. Frydman, A.C. Olivieri, B. Frydman, Anal. Lett. 20 (1987) 1657–1666.

[149] N.R. Jagannathan, Current Science 56 (1987) 827–830.

[150] P.J. Saindon, N.S. Cauchon, P.A. Sutton, C. Chang, G.E. Peck, S.R. Byrn, Pharm. Res. 10 (1993) 197–203.

[151] G.E. Maciel, W. Kolodziejski, M.S. Bertran, B.E. Dale, Macromolecules 15 (1982) 686–687.

[152] M.J. Gidley, S.M. Bociek, J. Am. Chem. Soc. 110 (1988) 3820–3829.

[153] K.R. Morgan, R.H. Furneaux, R.A. Stanley, Carbohydr. Res. 235 (1992) 15–22.

[154] S.J. Heyes, N.J. Clayden, Ch.M. Dobson, Carbohydr. Res. 233 (1992) 1–14.

[155] L.E. Diaz, F. Morin, C.L. Mayne, D.M. Grant, C. Chang, Magn. Reson. Chem. 24 (1986) 167–170.

[156] M. Herve, J. Hirschinger, P. Granger, P. Gilard, A. Deflandre, N. Goetz, Biochim. Biophys. Acta 1204 (1994) 19–27.

[157] S. Ando, I. Ando, A. Shoji, T. Ozaki, J. Am. Chem. Soc. 110 (1988) 3380–3386.

[158] A. Shoji, S. Ando, S. Kuroki, I. Ando, G.A. Webb, in: G.A. Webb (Ed.), Annual Reports on NMR Spectroscopy, vol. 28, Academic Press, New York, 1993, pp. 55-98

[159] I. Wawer, A, Zielinska, Solid-State NMR, in press

[160] G. Nebgen, D. Gross, V. Lehmann, F. Müller, J. Pharm. Sci. 84 (1995) 283–291.

[161] A.R. Rajabi-Siahboomi, R.W. Bowtell, P. Mansfield, M.C. Davies, C.D. Melia, Pharm. Res. 13 (1996) 376–380.

[162] A. Haase, MAGMA 2 (1994) 157–160.

[163] L. Parnetti, D.T. Lowenthal, O. Presciutti, G.P. Pelliccioli, R. Palumbo, G. Gobbi, P. Chiarini, B. Palumbo, R. Tarducci, U. Senin, J. Am. Geriatr. Soc. 44 (1996) 133–138.

[164] B. Borah, M.D. Francis, K. Hovancik, J.T. Boyce, N.M. Szeverenyi, J. Rheumatol. 22 (1995) 855–862.

[165] A.C. Silva, W. Zhang, D.S. Williams, A.P. Koretsky, Magn. Reson. Med. 33 (1995) 209–214.

[166] Y. Xu, P. Tang, W. Zhang, L. Firestone, P.M. Winter, Anestesiology 83 (1995) 766–774.

[167] C.A. Bramch, J.A. Helpern, J.R. Ewing, K.M. Welch, Magn. Reson. Med. 20 (1991) 151–157.

[168] R.J. Maxwell, T.A. Frenkiel, D.R. Newell, C. Bauer, J.R. Griffiths, Magn. Reson. Med. 17 (1991) 189–196.

[169] U. Noth, L.J. Jager, J. Lutz, A. Haase, Magn. Reson. Imag. 12 (1994) 149–153.

[170] L.J. Jager, U. Noth, A. Haase, J. Lutz, Adv. Exp. Med. Biol. 361 (1994) 129–134.

[171] N. Beckmann, I. Turkay, J. Seelig, U. Keller, Biochemistry 30 (1991) 6362–6366.

[172] K. Kanamori, B.D. Ross, Biochem. J. 305 (1995) 329–336.

[173] J.D. Bell, Spectroscopy Europe 8 (1996) 18–24.

[174] P.C. Dagnelie, D.K. Menon, I.J. Cox, J.D. Bell, J. Sargentoni, G.A. Coutts, J. Urenjak, R.A. Iles, Clin. Sci. Colch. 83 (1992) 183–190.

[176] D.J. Craik, K.J. Nielsen, K.A. Higgins, Annu. Rev. Spectrosc. 32 (1996) 143–213.