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The role of solvents in the signal separation for quantitative ^{1}H NMR spectroscopy

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ABSTRACT

Compared to conventional means of quantitative drug analysis, NMR spectroscopy only provides a limited set of adjustable parameters to enhance the quality of the analysis such as pH value, temperature, auxiliary reagents, magnetic field strength or properties of the solvent. In this work we investigate the influence of the kind of solvent on the signal separation of decisive resonances in the NMR spectra of codergocrine mesilate and flupentixol dihydrochloride. Polarity and aromaticity of the solvent play a crucial role in the optimization of signal separation. However, the set of applicable solvents is usually limited due to certain boundary conditions like solubility of the agent. Therefore the effects of solubilizers as well as mixtures solvents on the chemical shift also have to be taken into account. The quantitative results obtained by means of ^{1}H NMR spectroscopy were found to be in good agreement with the results carried out using official HPLC methods of International Pharmacopoeias.

1. Introduction

Besides the well-known application of NMR spectroscopy in structural elucidation especially of newly synthesized, natural and semi-synthesized products, nowadays this technique is of growing importance in the field of quantitative analysis. qNMR is particularly applicable for the determination of the content of the active component [1–5] and its potential impurities [6–11] using different nuclei, especially ^{1}H, ^{19}F, and ^{31}P, which are preferred for quantitative experiments. In spite of the nuclear overhauser effect (NOE) also ^{13}C NMR spectroscopy is qualified for quantitative purposes when using techniques such as inverse-gated decoupling or polarization transfer (DEPT) pulse sequences [12,13]. Several examples are described in the literature where NMR spectroscopy is comparable and even more accurate and precise than conventional methods like HPLC and GC [2,4,9,14]. However, there is a lack of acceptance in International Pharmacopoeias, where NMR spectroscopy is mostly utilized for identification purposes, but only in a few monographs. Compared to chromatographic methods, quantitative NMR spectroscopy offers a variety of advantages for quantitative analysis. For sample preparation the analyte has to be weighed and dissolved in a deuterated solvent only, and can be measured immediately in a relatively short analysis time. Even biofluids, cell or tissue extracts can be measured directly without complex sample preparation [5]. Due to the fact that the intensity of a signal is proportional to the number of nuclei being observed (under well-defined experimental conditions, e.g. relaxation delay, signal-to-noise ratio, integrated region, phase, and dead time between the pulse and the first acquired data point), the amount of the analyzed components can be calculated directly using the ratio of the signal intensities of analyte and internal reference standard [15]. Thus, in order to determine the absolute amount no expensive chemical reference substances are necessary [16]. qNMR is applicable for the simultaneous determination of the major component(s) and related substances, e.g. impurities. Qualitative and quantitative information can be provided in a single measurement, and additional structural information of previously unknown impurities is available. Therefore, this primary method is a powerful tool for impurity profiling, as for example described for gentamicin and unfractionated heparin [17,18].

The prerequisites for quantitative NMR spectroscopy are clearly separated signals which can be reliably assigned. Using supplemental 1D and 2D experiments it has to be ensured that the signals to be integrated do not interfere with those of either the analyzed component or an impurity. It is often sufficient to find one baseline separated resonance for each analyte of interest. Since NMR spectra contain a variety of signals, there is a much higher chance of finding the needed signal separation compared to conventional techniques like HPLC, GC or CE where one component is represented by one

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peak. To achieve an optimal signal separation, a couple of parameters can be adjusted; however, in contrary to techniques like HPLC and CE, in NMR spectroscopy only a few parameters can be optimized to achieve optimal signal separation.

In order to achieve separation of overlapping resonances, the kind of solvent or the pH value of the solution can be changed [19,20], different sample concentrations can be applied, or auxiliary reagents like cyclodextrins [21–23] or lanthanide shift reagents [21,24] can be added; in some cases the temperature also affects the signal separation [25,26]. However, NMR instruments operating at high magnetic field strength can be advantageous for signal separation [26].

The intention of this paper is to demonstrate the power of solvent exchange for signal separation often making qNMR possible in the first place. Even though it is well known, it is rarely used nowadays. Instead elaborate pulse sequences and line shape fitting techniques are applied in order to extract information from crowded spectral regions.

2. Experimental

2.1. Materials and chemicals

Codergocrine mesilate was provided from Novartis Pharma AG and Shire Deutschland GmbH, flupentixol dihydrochloride was donated from Bayer HealthCare AG. Hexadeuteriodimethyl sulfoxide (DMSO-d_6, 99.9 atom% D), acetonitrile-d_3 (ACN-d_3, 99.8 atom% D), benzene-d_6 (C_6D_6, 99.6 atom% D) and toluene-d_8 (Tol-d_8, 99.6 atom% D) were purchased from Sigma–Aldrich (Steinheim, Germany), deuterated water (D_2O, 99.9 atom% D), methanol-d_4 (CD_3OD, 99.8 atom% D) and chloroform-d ($CDCl_3$, 99.9 atom% D) were purchased from Euriso-top (Saarbrücken, Germany). The internal reference standard ethyl-4-(dimethylamino)benzoate (99.60% (g/g)) was donated from Spectral Service (Köln, Germany). All samples were measured in Wilmad 5 mm o.d., 527-PP-7 NMR tubes (Biebesheim, Germany).

2.2. NMR experiments

All the ^{1}H NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer operating at 400.13 MHz using 5 mm probes. All data were processed using Bruker's XWIN-NMR software (version 3.5, Bruker Analytik, Rheinstetten, Germany). For all ^{1}H NMR measurements carried out for method development, approximately 10 mg of the analyte was dissolved in 700 μl pure solvent or corresponding solvent mixtures and constantly shaken for several minutes. Thereof a standardized sample volume of 650 μl was transferred to a 5 mm NMR tube. For ^{1}H spectra, 128 scans were collected into 64 K data points over a spectral width of 8278.15 Hz (20.69 ppm) with a transmitter offset in the center of the spectrum. The acquisition time was 3.96 s followed by a relaxation delay of 1.0 s. All spectra were recorded at 300 K using a flip angle of 30°. The chemical shifts were referred to the signals of the applied solvents, or, in cases of mixtures, to the resonance of the main solvent expressed relative to TMS or TSP. An exponential line broadening window function of 0.3 Hz was used in the data processing. Baseline corrections were done automatically, phasing was always performed manually.

For quantitative NMR analysis, the relaxation delay was generally set to five times the value of the longest spin-lattice relaxation time T_1 of the integrated resonance signals in order to ensure full relaxation of the corresponding protons. The T_1 relaxation times were obtained using the inversion-recovery pulse sequence; the T_1 relaxation values were determined to be about 1.3 s for the H5′ resonances of codergocrine mesilate and approximately 0.9 s for the H1′ signals of flupentixol dihydrochloride. During quantification of both multi-component drugs, the spectra were acquired using a flip angle of 90° reaching maximum signal intensity, the spectral width was set to 16 ppm (codergocrine mesilate) and 12 ppm (flupentixol dihydrochloride), respectively, having on each end of the sample spectral window an additional region of at least 2 ppm, the digital resolution was set to 0.15 Hz/point, and the number of scans was adjusted in such a manner to achieve a signal-to-noise ratio of at least 250:1, which allows signal integration with a standard deviation of less than 1%. All other parameters were set as outlined above.

2.3. HPLC experiments

The quantitative HPLC analysis of codergocrine mesilate, described in the United States Pharmacopoeia (USP 31), was performed on an Agilent system 1200 LC (Waldbronn, Germany) using a reversed-phase column packed with octadecylsilane chemically bonded phase particles (Hypersil Gold; 150 mm × 4.6 mm, 5 μm; Thermo Hypersil-Keystone, Bellefonte, USA). The mobile phase consisted of water, acetonitrile, and triethylamine (80:20:2.5, v/v/v). The HPLC system operated at a flow rate of 1.0 ml/min, the detection wavelength was set at 280 nm and the temperature at 25 °C. Samples were prepared by dissolving an accurately weighed amount of codergocrine mesilate in a mixture of water and acetonitrile (1:1, v/v), yielding a sample concentration of 1 mg/ml. The content of each alkaloid was calculated using the normalization method.

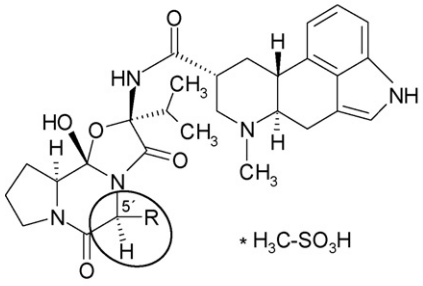
The quantitative determination of flupentixol dihydrochloride was performed on an Agilent system 1200 LC (Waldbronn, Germany) using an unmodified silica gel as stationary phase (Betasil Silica-100; 250 mm × 4.0 mm, 5 μm; Thermo Hypersil-Keystone, Bellefonte, USA). The mobile phase consisted of water, ammonia solution 25%, 2-propanol, and heptane (2:4:150:850, v/v/v/v). The HPLC system operated at a flow rate of 1.5 ml/min, the detection wavelength was set at 254 nm and the temperature at 25 °C. Samples were prepared by dissolving an accurately weighed amount of flupentixol dihydrochloride in the mobile phase, yielding a sample concentration of 0.4 mg/ml. The content of flupentixol was determined using a flupentixol CRS (chemical reference standard).

3. Results and discussion

3.1. Fundamentals of the influence of solvents on the chemical shifts

The choice of the appropriate solvent is a key factor, because it leads to considerable changes in the chemical shift and the coupling pattern; signal dispersion and line shape as well as the overall complexity of the ^{1}H NMR spectrum are also affected, and overlapping resonances may be fully resolved after changing the kind of solvent [27].

Few detailed studies have been presented comparing ^{1}H and ^{13}C NMR chemical shifts of a variety of substances using different solvents. Abraham et al. have given the chemical shifts of 124 compounds containing different functional groups using DMSO-d_6 and $CDCl_3$ as solvent [28]. For protic compounds like primary and secondary amines and alcohols very large differences in the chemical shifts ($\Delta\delta \sim 1$–4 ppm) are found in contrast to non-polar (e.g. alkanes) and polar aprotic (e.g. tertiary amines, esters) groups ($\Delta\delta \leq 0.1$ ppm). Jones et al. presented the ^{1}H and ^{13}C NMR chemical shifts of approximately 60 residual solvents in commonly used solvents such as DMSO-d_6, $CDCl_3$, D_2O and CD_3OD [29], Gottlieb et al. have shown similar data for a multitude of possible impurities in seven solvents [25]. Both studies demonstrate that



Components		-R
Dihydroergocornine	Cor	
Dihydroergocristine	Cr	
α-Dihydroergocryptine	α	
β-Dihydroergocryptine	β	

Fig. 1. Structural formula of codergocrine mesilate.

the chemical shifts can differ extremely from solvent to solvent. Taking a closer look to chloroform, large differences in the chemical shift of the CH-signal are observed ranging from 6.15 ppm in benzene-d_6 to 8.32 ppm in DMSO-d_6; however, for cyclohexane in all seven solvents equal chemical shifts of the CH_2-signal are achieved [28].

Taken together the chemical shift of protons depends strongly on the solvent. The interactions between solute and solvent are responsible for these solvent effects [30] which are due to hydrogen bonding, the anisotropy of the solvent molecules, polar effects, and van der Waals interactions [31].

In most cases hydrogen bonding and interactions between the solute and an aromatic solvent determine the specific solute–solvent interactions of polar substances. Due to hydrogen bonding, for protic hydrogens the chemical shift can vary up to 5 ppm after changing the solvent from a non-polar to a polar one [28]. Large shifting behaviour induced by aromatic solvents (ASIS) of approximately 1 ppm has been observed using non-polar anisotropic solvents such as benzene-d_6. Aromatic solvents tend to produce high-field shifts in the solute due to their large diamagnetic anisotropy. Using the simple example of acetonitrile the CH_3-resonance is shifted upfield after changing the solvent from inert neopentane (absence of solute–solvent complex) to benzene, where the solute protons tend to locate above the plane of the benzene ring and close to the axis [32]. For non-polar, hydrocarbon solutes like ethane, ethylene or naphthalene no shift of the proton resonances can be observed when changing the solvent, demonstrating that the dipole nature of the solute is a necessary condition for the ASIS phenomenon.

Table 1
Results obtained by qNMR and HPLC—for each component of codergocrine mesilate the percentage content was calculated using the normalization method.

	Cor [%]	α [%]	Cr [%]	β [%]
^{1}H NMR	34.9 ± 0.1	22.7 ± 0.1	31.3 ± 0.1	11.1 ± 0.1
HPLC	34.7 ± 0.1	22.9 ± 0.1	31.3 ± 0.1	11.1 ± 0.1

Therefore, a change of solvent is an integral option in order to achieve complete baseline separation of two adjacent signals. However, the solubility of the analyte further determines the selection of suitable solvents, because the fundamental requirement for qNMR is complete solubility. Sometimes mixtures of two solvents have to be used. Thus, the second solvent may be a solubilizer which can also determine the signal separation. Additionally, the location of the solvent signal and the HOD signal (residual water of the solvent) can put a limit to the application of a certain solvent when these signals interfere with signals of interest.

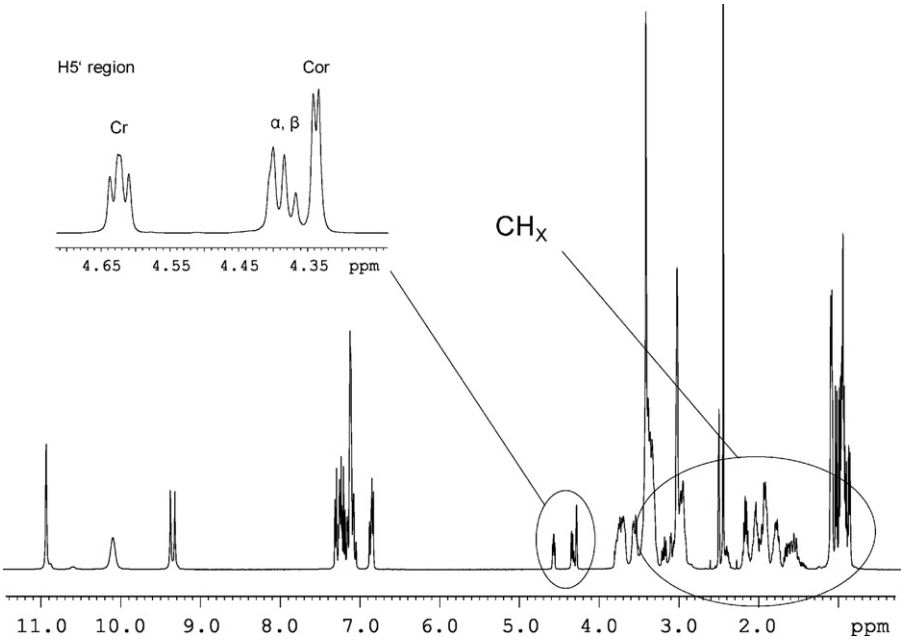
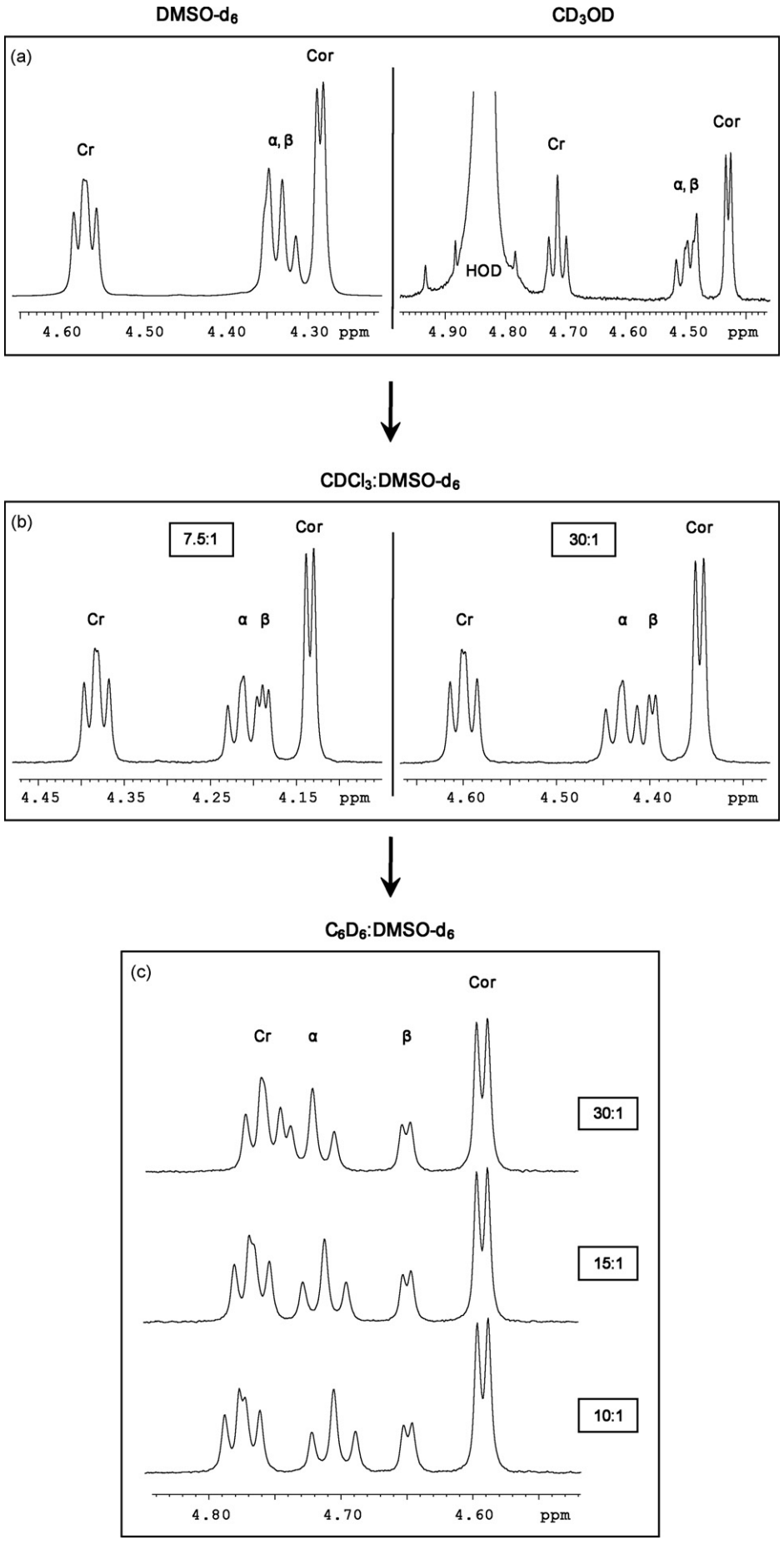


Fig. 2. ^{1}H NMR spectra of codergocrine mesilate in DMSO-d_6 (400 MHz). The inset shows an enlarged view of the H5′ region which contains the ^{1}H signals of dihydroergocornine (Cor), α- and β-dihydroergocryptine (α, β), and dihydroergocristine (Cr).


DMSO-d6
CD3OD
(a)
Cor
Cr
α, β
HOD
4.60 4.50 4.40 4.30 ppm
4.90 4.80 4.70 4.60 4.50 ppm
CDCl3:DMSO-d6
(b)
7.5:1
30:1
Cr
α β
4.45 4.35 4.25 4.15 ppm
4.60 4.50 4.40 ppm
C6D6:DMSO-d6
(c)
30:1
15:1
10:1
4.80 4.70 4.60 ppm

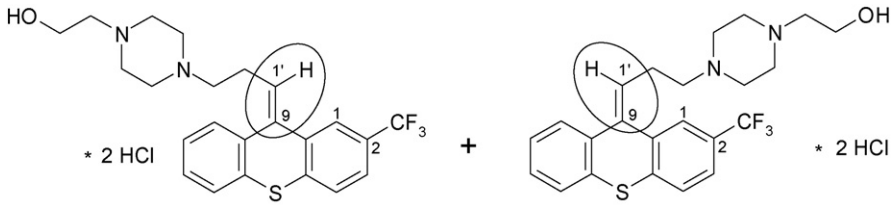


Fig. 4. Structural formula of E-flupentixol dihydrochloride (left) and Z-flupentixol dihydrochloride (right).

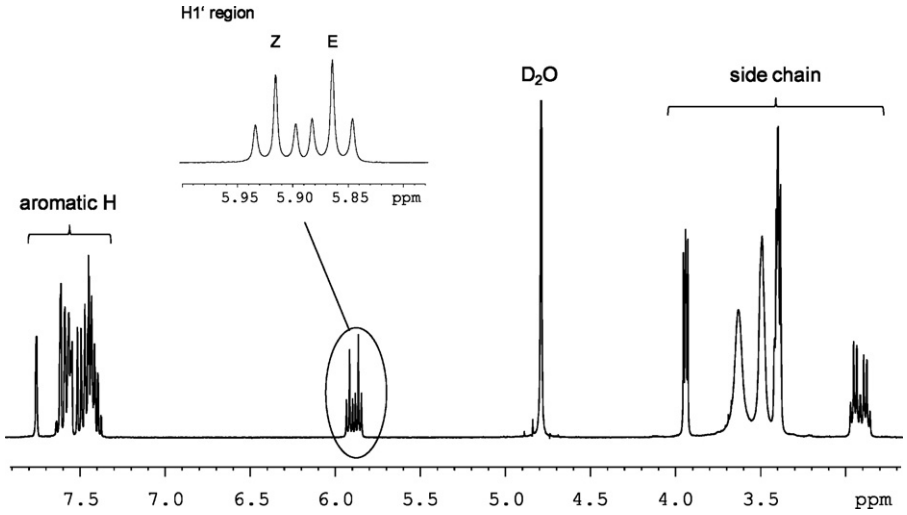


Fig. 5. ^{1}H NMR spectra of flupentixol dihydrochloride in D_2O (400 MHz). The inset shows an expansion of the H1′ region which contains the ^{1}H signals of E- and Z-flupentixol.

3.2. Applications

For separation of the resonances of the multi-component drug codergocrine mesilate and the flupentixol isomers we applied NMR experiments in solvents with significant differences in their polarity and aromaticity to achieve distinctive solvent effects concerning the chemical shift of certain protons; to demonstrate the solvent effect initial experiments should be carried out using a polar (D_2O, DMSO-d_6), a non-polar ($CDCl_3$) and an anisotropic solvent (C_6D_6).

3.3. Codergocrine mesilate

Codergocrine mesilate is a mixture of the methanesulfonate salts of three dihydroergopeptide alkaloids, namely dihydroergocornine (Cor), dihydroergocristine (Cr) and dihydroergocryptine, in a ratio of about 1:1:1. Dihydroergocryptine is composed of two regio isomers, called α-dihydroergocryptine (α) and β-dihydroergocryptine (β). The structures of all these components are of high similarity; they only differ in the side chain in position 5′ (Fig. 1).

In the following we will present the steps to be taken in order to achieve the optimal signal separation of the multi-component drug codergocrine mesilate. For optimal signal separation of all four components of codergocrine mesilate, initial measurements were carried out using a variety of deuterated solvents (e.g. polar, non-polar, aromatic solvents) to show their particular impact on the chemical shift. Due to the poor solubility of codergocrine mesilate in D_2O, ACN-d_3, $CDCl_3$ and C_6D_6, small amounts of DMSO-d_6 or CD_3OD were added as solubilizer to achieve complete solubility of the analyte. To reliably assign the ^{1}H NMR resonances in the spectrum of codergocrine mesilate, supplemental 1D and 2D experiments such as DEPT, COSY, HMQC, and HMBC were carried out, which confirm the signal assignment results described in the literature [33,34].

The entire spectrum measured in aprotic DMSO-d_6 is displayed in Fig. 2. It revealed that a quantitative determination of the four components of codergocrine mesilate on basis of their different side chains attached to position 5′ was impossible. Strong signal overlap of the aliphatic protons (CH_X) and even the aromatic protons of dihydroergocristine showed that these protons were not suitable for quantification (Fig. 2).

However, looking at the ^{1}H NMR spectrum of codergocrine mesilate in DMSO-d_6 (Fig. 2: inset) the signals of the tertiary proton in position 5′ could be suitable for quantitative analysis by means of NMR spectroscopy. The resonances of the proton in position 5′ of dihydroergocristine and dihydroergocornine, recognized as doublet and triplet, respectively, were clearly separated, only the signals of the protons of α- and β-dihydroergocryptine overlap.

Using protic D_2O or CD_3OD the ^{1}H spectra were similar to the ^{1}H spectrum recorded in DMSO-d_6 (Fig. 3a). The H5′ resonances of the two regioisomers overlap, only the signals of dihydroergocristine and dihydroergocornine were clearly separated. Nevertheless DMSO-d_6 was preferred because there are no solvent signals in the region of interest which could possibly interfere with the signals of H5′. Applying CD_3OD, complete

Fig. 3. ^{1}H NMR spectra oft he H5′ region of codergocrine mesilate ordered by improved signal separation, for the following solvents: (a) DMSO-d_6 (left), CD_3OD (right); (b) $CDCl_3$:DMSO-d_6; (c) C_6D_6:DMSO-d_6. In cases of solvent mixtures, the corresponding ratio is given in boxes. Optimal signal separation is achieved using a mixture of C_6D_6 and DMSO-d_6 in a ratio of 10:1 (v/v).

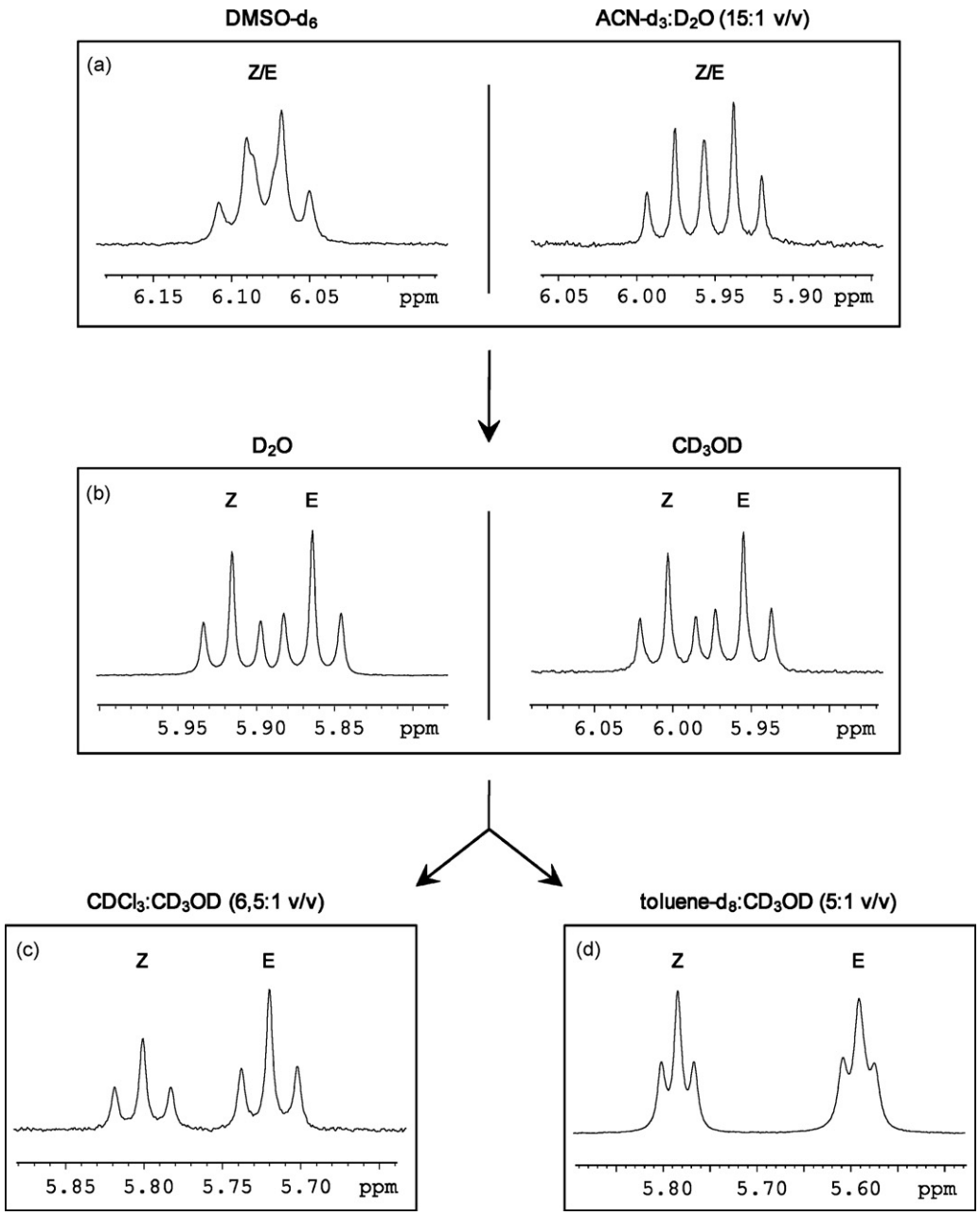


Fig. 6. ^{1}H NMR spectra of the H1′ region of flupentixol dihydrochloride, ordered by improved signal separation for the following solvents: (a) DMSO-d_6 (left), ACN-d_3:D_2O (right); (b) D_2O (left), CD_3OD (right); (c) $CDCl_3$:CD_3OD; (d) toluene-d_8:CD_3OD. In the case of solvent mixtures, the corresponding ratio is given. Optimal signal separation is achieved using a mixture of toluene-d_8 and CD_3OD in a ratio of 5:1 (v/v).

baseline separation between the HOD signal of CD_3OD and dihydroergocristine could not be achieved (Fig. 3a: right spectrum). Using D_2O, the solvent signal overlapped with the H5′ signal of dihydroergocristine (data not shown).

Using $CDCl_3$, separation of the H5′ protons of α- and β-dihydroergocryptine was noticeable and the corresponding signals of dihydroergocristine and dihydroergocornine were clearly separated, too. Varying the amount of the polar solubilizer, signal separation was optimal for low amounts of DMSO-d_6 (Fig. 3b: right expansion). Nevertheless, a complete baseline separation of α- and β-dihydroergocryptine was not achieved.

Applying C_6D_6 in combination with small amounts of DMSO-d_6, complete baseline separation between the H5′ resonances of α- and β-dihydroergocryptine is possible. Due to C_6D_6, the H5′ signal of α-dihydroergocryptine was downfield shifted relative to the other H5′ resonances. When using a solvent mixture with only small amounts of DMSO-d_6 (C_6D_6-DMSO-d_6 (30:1, v/v)), the two H5′ signals of α-dihydroergocryptine and dihydroergocristine overlap (Fig. 3c). Increasing the amount of DMSO-d_6, the signal of α-dihydroergocryptine was upfield shifted relative to the other resonances (Fig. 3c). Optimal signal separation was achieved using a mixture of C_6D_6 and DMSO-d_6 in a ratio of 10:1 (v/v) (Fig. 3c).

To determine the accuracy of the quantitative analysis of codergocrine mesilate by means of the integration of the H5′ signals, a validated HPLC method which is prescribed in the United States Pharmacopoeia [35] was used as an orthogonal method. For both methods, the content of each alkaloid was calculated using the normalization method (Table 1). Comparison reveals that the results of NMR spectroscopy and HPLC are in perfect agreement.

Table 2
Results obtained by qNMR and HPLC—the content of both isomers as well as of flupentixol dihydrochloride were calculated by the absolute method using certified reference compounds.

	Z-flupentixol [%]	E-flupentixol [%]	Content	
			MW [%]	RSD [%]
^{1}H NMR	41.7	49.8	91.5	0.2
HPLC	41.2	50.7	91.9	0.2

3.4. Flupentixol dihydrochloride

Flupentixol dihydrochloride belongs to the group of E/Z-isomers of thioxanthene derivatives occurring in approximately equal amounts as E- and Z-flupentixol dihydrochloride. Using the example of flupentixol it shall be demonstrated that NMR spectroscopy is also suited for the quantification of components with the same chemical structure (Fig. 4).

The ^{1}H NMR signals of the aliphatic and aromatic protons of flupentixol were assigned by supplemental 1D and 2D experiments; spiking experiments using pure Z-flupentixol facilitates the signal assignment of both isomers.

First experiments in D_2O revealed that a quantitative analysis of both isomers is feasible in principle using the ^{1}H triplet resonance of the proton in position 1′; however, applying D_2O a complete separation suitable for quantification was not achieved (Fig. 5b: inset). Due to the poor solubility of flupentixol dihydrochloride in ACN-d_3, $CDCl_3$, C_6D_6, and toluene-d_8, small amounts of CD_3OD and D_2O, respectively, were added as solubilizer to achieve complete solubility of the analyte.

Using polar aprotic solvents such as pure DMSO-d_6 and ACN-d_3-mixtures with D_2O, respectively, the H1′ region was characterized by a strong signal overlap, which makes the assignment of the triplet shape impossible (Fig. 6a).

Applying pure CD_3OD, the signal separation of the two H1′ resonances is similar to the ^{1}H spectra recorded in D_2O; the triplet shape of the H1′ signals is noticeable; however, a complete baseline separation could not be achieved (Fig. 6b).

When applying a non-polar solvent like $CDCl_3$ in combination with small amounts of CD_3OD the signal separation of the H1′ resonances of E- and Z-flupentixol could be improved considerably due to large differences in chemical shifts (Fig. 6c). Non-polar solvents having an aromatic structure, such as C_6D_6 or toluene-d_8, are preferred because of substantial enhancements in the signal separation of the H1′ protons. Optimal signal separation of the H1′ resonances of E- and Z-flupentixol could be achieved using a mixture of toluene-d_8 and CD_3OD in a ratio of 5:1 (v/v) (Fig. 6d), therefore leading to increased accuracy in the integration process in contrast to measurements in $CDCl_3$. Replacing toluene-d_8 by C_6D_6, the obtained ^{1}H spectra were similar to the spectra recorded in toluene-d_8, however applying C_6D_6 the line shape of the corresponding triplets is worse (data not shown).

The signal separation is robust to a variation of the amount of the polar solubilizer CD_3OD; the respective signal intensities of the H1′ resonances when applying mixtures of toluene-d_8 and CD_3OD in varying compositions are in perfect agreement. In the case of flupentixol as an example predominantly CD_3OD is used as a solubilizer.

The quantitative analysis of flupentixol dihydrochloride was carried out using the official HPLC method prescribed in the European Pharmacopoeia [36]; absolute results of each isomer as well as the content of flupentixol were achieved applying a certified flupentixol reference standard with defined purity. The HPLC results were in agreement with those obtained by the aforementioned qNMR method, using ethyl-4-(dimethylamino) benzoate as internal standard (Table 2).

4. Conclusion

In this study we have demonstrated for the codergocrine mixture and the flupentixol isomers that significant variations in the chemical shift may occur by an exchange of solvents or by using a mixture of solvents with different chemical characteristics concerning polarity and aromaticity due to specific solute–solvent effects. However, because of the requirement of complete solubility of the analyzed component, the choice of suitable solvents may be limited; therefore the addition of small amounts of a solubilizer is often necessary. Additionally, this solubilizer may also improve the signal separation.

Even though mixtures of deuterated solvents may result in “wiggly” lock signals and, thus, in difficulties with locking and shimming. This was not observed in the examples described here. Additionally, one has to keep in mind that the exchange of the solvent may require a new calibration and confirmation in signal purity by 2D experiments. Nevertheless, the optimization of the solvent often enables quantification of drug components more easily than highly sophisticated pulse sequences in NMR spectroscopy of a separation in HPLC, beside the fact of providing structural information.

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