Detailed NOESY/T-ROESY analysis as an effective method for eliminating spin diffusion from 2D NOE spectra of small flexible molecules

S.V. Efimov\textsuperscript{a}, I.A. Khodov\textsuperscript{a,b}, E.L. Ratkova\textsuperscript{b}, M.G. Kiselev\textsuperscript{b}, S. Berger\textsuperscript{c}, V.V. Klochkov\textsuperscript{a}

\textsuperscript{a}Institute of Physics, Kazan Federal University, Kremlevskaya 18, Kazan 420008, Russia
\textsuperscript{b}G.A. Krestov Institute of Solution Chemistry, Russian Academy of Sciences, Akademicheskaya St. 1, Ivanovo, 153045 Russia
\textsuperscript{c}Institute of Analytical Chemistry, Leipzig University, Linnéstraße 3, Leipzig D-04103, Germany

Abstract

An intriguing property of the multistep magnetization transfer, so-called spin diffusion, is that it can affect the results of NMR-based analysis of conformer distribution of small molecules in solution. Therefore, the contribution of spin diffusion should be subtracted in order to obtain accurate data on molecular conformations and their distributions. Several methods have been developed for this purpose, but many of them have a lack of versatility. These methods were critically analysed, and two approaches to eliminate spin diffusion were tested during the study of felodipine as the drug molecule of small size. QUIET-NOESY was found to be a powerful technique to solve this problem. The second method, combined analysis of two sets of spectra, NOESY and T-ROESY, was tested and proved to be the most correct way of obtaining exact internuclear distances in a flexible molecule in solution.

Keywords: 2D NOESY, spin diffusion, internal mobility, felodipine
1. Introduction

Nuclear Overhauser effect spectroscopy has become a powerful tool for determining conformations of both large [1, 2] and small [3, 4] molecules. NOE intensities, however, depend not only on distances between nuclei, but also on numerous factors, including fast and slow molecular motion, ability of free rotation of functional groups, etc. (various aspects of NOESY are described, for instance, in [5]). Obtaining internuclear distances as accurately as possible turns thus into a complicated problem requiring computer simulations of molecular flexibility and thorough analysis of experimental values of NOEs. One of the main sources of errors, spin diffusion, is considered; different approaches to eliminate its effect are tested in a study of a small drug molecule, felodipine.

Generally, observable properties of molecules undergoing fast conformational exchange can be represented as averaged values of corresponding parameters of individual conformers. In the case of NMR, this applies for chemical shifts and cross-relaxation rates. It is possible to reveal the amount of different conformers in solution, if sufficient experimental data on chemical shifts and/or NOE intensities have been obtained and the properties of individual conformers are known (e.g., from quantum chemistry calculations). The accuracy of estimates of the fractions depends on the dispersion of properties of the conformers (the more the dispersion, the easier to distinguish between them) and on the accuracy of the measurements. While the difference between individual structures is determined by the investigated object itself, a researcher can make effort to improve the measurement accuracy.

NOESY allows determination of atom–atom distances up to 5 Å, and thus gives direct information about spatial structure of a molecule. However, the distance range accessible to this method (from 2 to 5 Å) is relatively small, and typical distance error of 0.1–0.2 Å affords obtaining reliable results only in the case of macromolecules, when a large set of internuclear distances is measured and analysed simultaneously.

Low-molecular-weight compounds require a thorough measurement and anal-
ysis of NMR data. Spin diffusion is a phenomenon which can impede distance measurements. This effect occurs when the spin magnetization is transferred through intermediate nuclei, and becomes prominent at long mixing times. The indirect magnetization transfer $i \rightarrow k \rightarrow j$, which occurs simultaneously with the direct process $i \rightarrow j$, makes it impossible to calculate the internuclear distance between $i$ and $j$ from the observed cross-relaxation rate. It was revealed initially in complex macromolecular systems (proteins and nucleic acids). In practice it leads to an increase in integral intensities of cross-peaks [6], which can be misinterpreted in terms of spatial proximity of nuclei. The spin diffusion was considered to be negligible in small molecules. Butts and co-workers, however, noted that accurate determination of atom–atom distances of small, flexible molecules may also be hampered by the spin diffusion effect [7]. Here we report a similar observation for felodipine ($\text{C}_{18}\text{H}_{19}\text{Cl}_2\text{NO}_4$) in DMSO [8, 9].

In this paper we used two methods to overcome the influence of indirect magnetization transfer: analysis of combined T-ROESY/NOESY data [10–14] and suppression by the QUIET-NOESY pulse sequence [15–17]. The goal of this work is to test and compare several approaches invented to eliminate the effect of spin diffusion.

Various methods, both theoretical and experimental, were proposed to diminish this effect. One possibility is to allow for the spin diffusion on the stage of data analysis [18]. It may require knowledge of intensities of all diagonal and cross-peaks (full matrix analysis) or solving systems of nonlinear equations employing correction factors [19, 20]. Direct experimental approaches based on special manipulations with the spin magnetization include such methods as MINSY, BD-NOESY, QUIET-NOESY, etc. The third way is deuteration of the object, which is used in studies of large proteins. However, this methodology is inconvenient and expensive in the case of small organic molecules, including drug-like compounds.

Mentioned experimental methods all have their pros and cons. Minimization of the mixing time $\tau_m$ (initial rate approximation, proposed by Wüthrich in [21]) is not a good choice for small molecules since integral intensities of cross-peaks
are too weak in this case. The DEPT-NOESY method is not universal, as it is designed only for spin systems CH/CH$_2$ and CH/CH$_3$ [22]. Comparative analysis of the experiments MINSY [23], BD-NOESY [24], QUIET-NOESY [17, 25], and MYSINE [26] based on the first derivative of the matrix exponent showed that QUIET-NOESY (Quenching Undesirable Indirect External Trouble in Nuclear Overhauser Effect Spectroscopy) is the most sensitive and effective approach [27].

From these direct experimental methods, QUIET-NOESY pulse programme [15-17] was chosen for conducting experiments with suppression of spin diffusion. In addition, the effect of spin diffusion can be eliminated by joint analysis of simpler experiments such as NOESY and ROESY. The theoretical background which underlies this possibility is explained below.

2. Theory

2.1. Molecular Motion

Motions between nuclei can be described by a reorientation correlation function $G(t)$, which may take various forms depending on the types of the motion experienced by the molecule: overall rotation, fast or slow motions of certain segments of the molecule. Characteristic rate of the motion and the degree of its influence on atom positions (and observed NMR parameters which depend on the chemical bond orientations) are expressed through the correlation time $\tau$ and the order parameter $S$.

The conformational flexibility of felodipine molecules results in significant changes in the orientation of an involved pair of nuclei (i,j) (see section 4). Therefore, its fast motion order parameter $S_f$ will tend to zero. We can assume that $S_f \rightarrow 0$ and, additionally, that $\tau_f \ll \tau_s$ and $\tau_f \ll \tau_o$ ($\tau_o$ is the overall rotation correlation time of the whole molecular system; $\tau_f$ is the internal correlation time of fast motions of certain segments of the molecule (for example, fast-rotating methyl groups); $\tau_s$ is the internal correlation time of slow motions...
of molecular segments). Then the relation for the reorientation correlation function can be written in a form

\[ G(t) = S_f^2 \exp(-t/\tau_i) . \]  

(1)

The value \( \tau_i \) \( (\tau_i^{-1} = \tau_o^{-1} + \tau_s^{-1}) \) is the effective local rotation correlation time of proton pairs, and the spectral density function for this case looks like

\[ J^m(\omega) = \frac{\tau_i}{1 + m^2 \omega^2 \tau_i^2} . \]  

(2)

Note also that several other spectral density functions were proposed for different types of molecular motion [28–30].

The \( S_f \) value can be derived based on molecule coordinates obtained by quantum chemical calculation [20], using the following expression with spherical harmonics \( Y_{2k}(\theta, \phi) \) allowing for correct averaging of fast rotating groups

\[ S_f^2 = \frac{4\pi}{5} \left\langle \frac{1}{r_{ij}^6} \right\rangle^{-1} \sum_{k=-2}^{2} \left( \frac{Y_{2k}(\theta_{mol}, \phi_{mol})}{r_{ij}^6} \right)^2 . \]  

(3)

### 2.2. NOE Spectroscopy and Distance Determination

Intensities of diagonal and cross-peaks for all nuclear Overhauser effect spectra can be gathered in a matrix \( A \), which depends on the mixing time \( \tau_m \) as follows:

\[ A(\tau_m) = \exp(-R\tau_m)A_0 . \]  

(4)

Here \( A_0 \) corresponds to the zero mixing time; its elements \( a_{0i} \) are proportional to the equilibrium populations of individual spin states.

Elements of the cross-relaxation matrix \( \rho_{ij} \) and \( \sigma_{ij} \) are the longitudinal and cross-relaxation rates in a considered N-spin system. These relaxation rates depend on distances between spins and on the chaotic field experienced by the nuclei which, in turn, is defined by the character of molecular motion. The exact expression describing the cross-relaxation rate can also vary for different experimental schemes; for example, cross-relaxation rate in the case of the NOESY approach looks like

\[ \sigma_{ij} = \frac{1}{10} \hbar^2 \gamma^4 \left( \frac{\mu_0}{4\pi} \right)^2 \frac{n_i}{r_{ij}^{6}} (6J^2(\omega) - J^0(\omega)) . \]  

(5)
Determination of internuclear distances by comparing the cross-relaxation rates of a studied proton pair with a reference one within the initial rates approximation following the formula [31, 32]

\[ r_{ij} = r_{\text{ref}} \left( \frac{\sigma_{\text{ref}}}{\sigma_{ij}} \right)^{1/6} \]  

(6)
is not universal and can sometimes be inconvenient because there may be no independent sources to find the reference distance, or the internal mobility of the studied and reference atom pairs may be different (here \( r_{ij} \) is the interproton distance to be estimated and \( \sigma_{ij} \) is the corresponding 2D NOESY cross-relaxation rate; \( r_{\text{ref}} \) and \( \sigma_{\text{ref}} \) are the known interproton distance and its cross-relaxation rate, respectively). In the latter case, the distances can be found using local correlation times; this approach was proposed by Davis [33]. It was successfully applied by Poveda and co-authors in polysaccharide studies [10, 34–36], but several mistakes crept into the early papers (in the quartic equation, depending only on the correlation time and the spectrometer frequency, the numerator and the denominator were mixed up, and a quadratic term was typed instead of the term in the power of four in the denominator).

Further this approach was employed in the group of Widmalm [11–13]. To our knowledge, proper formulas for determination of local correlation times were published in 2009 [14].

Internal correlation time \( \tau_i \) can be calculated from the ratio of experimentally measured cross-relaxation rates \( \sigma_{ij}^{\text{NOE}} / \sigma_{ij}^{\text{ROE}} \) or \( \sigma_{ij}^{\text{NOE}} / \sigma_{ij}^{\text{T-ROE}} \), as expressions for them are known:

\[
\frac{\sigma_{ij}^{\text{NOE}}}{\sigma_{ij}^{\text{ROE}}} = \frac{5 + \omega^2 \tau_i^2 - 4 \omega^4 \tau_i^4}{5 + 22 \omega^2 \tau_i^2 + 8 \omega^4 \tau_i^4},
\]

(7)

\[
\frac{\sigma_{ij}^{\text{NOE}}}{\sigma_{ij}^{\text{T-ROE}}} = \frac{10 + 2 \omega^2 \tau_i^2 - 8 \omega^4 \tau_i^4}{10 + 23 \omega^2 \tau_i^2 + 4 \omega^4 \tau_i^4}.
\]

(8)

If the ratio of cross-relaxation rates (or, simply, of cross-peak intensities) are known from an experiment, internal correlation time may be calculated using Eq. (7) or (8). Order parameter is obtained from quantum chemical calculations following Eq. (3). Knowledge of correlation time and \( S^2 \) allows
calculating spectral density function (e.g., by the model-free approach [37, 38]), which, together with experimentally found cross-relaxation rates, yields finally internuclear distances by solving the Eq. (5). After that, conformer distribution may be estimated by comparing these effective (exchange-averaged) distances with corresponding values in different conformations (corresponding formulas can be found elsewhere [4]).

2.3. QUIET-NOESY

This pulse sequence contains a double-band selective pulse in the middle of the mixing time (Fig. 1). In other aspects it is a typical NOESY experiment in the laboratory frame, and its cross-peaks intensities are described by Eq. (5). It allows suppressing the spin diffusion for a certain pair of nuclei, which have separate signals in the NMR spectrum.
It was already mentioned that NOESY spectra can be misleading if two-step spin diffusion processes \(i - k - j\) are not properly taken into account. Several approaches have been proposed to overcome the adverse impact of the spin diffusion. The QUIET-NOESY sequence was found to be the most suitable solution to this problem.

Simultaneous inversion of the longitudinal magnetization is achieved by a cosine-modulated Gaussian cascade Q3. The Fourier image of a function of the type \(\cos(at) \exp(-bt^2)\) is the convolution of a Gaussian with the function \(\sqrt{\pi/2} (\delta(\omega - a) + \delta(\omega + a))\), which gives two Gaussians separated by \(2a\) in the frequency dimension. The so-called “quiet windows” are then formed in a 2D NMR spectrum on the intersections of the two frequency bands (ranges). These windows contain cross-peaks arising due to processes of direct magnetization transfer \(i - j\) only, while indirect pathways \(i - k - j\) are removed provided the chemical shifts of spins \(k, k_1, \ldots\) do not fall into these quiet windows. This is achieved by simultaneous inversion of the magnetizations of spins \(i\) and \(j\) within the two quiet windows, while spin magnetizations outside this regions (of spins \(k\)) are not disturbed. Obtained spectra are processed and analysed as ordinary NOESY spectra, for example, using Eq. (6).

3. Experimental Section

3.1. NMR Measurements

Felodipine and deuterated DMSO were purchased from Sigma Aldrich and used without further purification. Samples were prepared with concentration of 0.077 g/L. Solution volume was 0.6 mL. NMR experiments were performed on a Bruker Avance II 500 (Kazan) and on a Bruker Avance 700 NMR spectrometer (Leipzig) equipped with a 5 mm probe using standard Bruker TOPSPIN Software. Temperature control was performed using a Bruker variable temperature unit (BVT-2000) in combination with a Bruker cooling unit (BCU-05) to provide chilled air. Experiments were performed at 298 K without sample spinning.
Two-dimensional nuclear Overhauser effect spectroscopy (2D ge-NOESY) [39],
transverse rotating frame Overhauser enhancement spectroscopy (2D T-ROESY) [13],
and Overhauser effect spectroscopy with suppression of spin diffusion (2D QUIET-
NOESY) [17] experiments were performed with pulsed filtered gradient tech-
niques [40]. The spectra were recorded in a phase-sensitive mode with 2048
points in the F2 direction and 512 points in the F1 direction and acquired with
64 scans and relaxation delay of 2 s. Mixing time values for 2D ge-NOESY and
QUIET-NOESY were 0.30, 0.50, 0.70, and 0.90 s. Spin-lock delay values for
T-ROESY were 0.30, 0.50, 0.70, and 0.90 s. The spectra were recorded in a
phase-sensitive mode with 2048 points in the F2 direction and 512 points in the
F1 direction.

3.2. Structure Calculation

The six conformers of felodipine were taken from the work of Teberekidis
and co-authors [41], where they were predicted using quantum chemical cal-
culations. To explore an alternative way for the conformational analysis, we
performed exploration of felodipine conformational space using the stochastic
search algorithm implemented in the scan programme of TINKER molecular
modelling software (Version 6.2) [42]. The programme searches for minima of
the potential energy by the basin hopping method. Previously we found out that
for small drug-like molecules the scan programme with the MMFF force-field
provides the optimal combination of number/quality of predicted conformers in
comparison with other freely available programmes for the conformations gen-
eration (data not published). We performed the conformational search with the
following default parameters: the number of search directions is 5, the energy
window is 100 kcal/mol, and the convergence criterion is 0.0001 kcal/mol/Å. As
an initial guess of the felodipine geometry we used the first conformer obtained
from the QM calculations described above. The force-field parameters were as-
signed with the programme sdf2tinkerxyz. The comparison of results obtained
by both conformational search approaches is shown in Table 1. Aligning of the
structures and calculation of the root mean square displacement (RMSD) be-
Table 1: RMSD of felodipine conformers in the gas phase calculated by two approaches: (i) quantum mechanics (the data is taken from [41]), here the conformers are marked from A to F, and (ii) scan/TINKER, here the conformers are numbered from 1 to 6.

<table>
<thead>
<tr>
<th></th>
<th>scan / TINKER</th>
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<tr>
<td>QM</td>
<td>1  2  3  4  5  6</td>
</tr>
<tr>
<td>A</td>
<td>0.2 0.6 1.2 1.1 0.6 1.3</td>
</tr>
<tr>
<td>B</td>
<td>0.3 0.5 1.2 1.1 0.2 1.1</td>
</tr>
<tr>
<td>C</td>
<td>0.5 0.2 1.0 1.1 0.4 1.1</td>
</tr>
<tr>
<td>D</td>
<td>1.1 1.1 0.4 0.3 1.0 0.4</td>
</tr>
<tr>
<td>E</td>
<td>1.2 1.0 0.1 0.5 1.1 0.3</td>
</tr>
<tr>
<td>F</td>
<td>1.0 1.1 0.6 0.2 1.1 0.6</td>
</tr>
</tbody>
</table>

tween the generated conformers were performed using the VMD software. As one can see, conformers obtained by the scan programme are in a good agreement with the QM-determined structures (RMSD is about 0.2) but the ranking of the corresponding conformers differs between these approaches.

4. Results

Three approaches to determination of atom–atom distances and consequent calculation of conformation distribution are compared in the present work: NOESY, QUIET-NOESY, and T-ROESY. The mentioned methods were applied to a small flexible molecule of felodipine dissolved in dimethyl sulphoxide. Felodipine has six lowest-energy conformers differing by rotation of their aryl fragment. The six forms are shown in Fig. 2, and atom numbering which is used below is given in Fig. 3.

Data presented in Table 2 indicate that the results based on the QUIET-NOESY agree well with those obtained from analysis of NOESY/T-ROESY spectra, which proves that the latter approach can be successfully used to eliminate the effect of spin diffusion. Traditional NOESY analysis underestimates the distance by about 0.1 Å in the case of felodipine. It turns out that more
accurate consideration of spin diffusion in distance calculations has a significant
effect on resulting distribution of conformers.

It is worth noting that the distance H3b–H3c, which served as the reference
distance in analysis of NOESY and QUIET-NOESY data, was found independ-ently based on the combined experimental data of NOESY and T-ROESY,
and this result agreed quite well with the quantum chemistry data.

Moreover, experimentally found correlation times $\tau_{\text{eff}}$ and calculated gener-alized order parameters $S^2$ agree with each other reasonably well. These pa-rameters, which characterize the intramolecular mobility, are given in Table 2.
Order parameter for the atom pair H3b–H3c is 0.28, which points to a faster local motion of this fragment compared with the H4–H6' pair, where $S^2 = 0.4$
(1.43 times larger). Comparison of local correlation times gives a similar value: for H3b–H3c pair $\tau_{\text{eff}} = 168 \pm 3$ ps, while for H4–H6' it is $237 \pm 14$ ps, which is 1.41 times larger. This difference is caused by different nature of the intramolec-ular motion: relative rotation of methyl and methylene groups in the H3b–H3c fragment is faster than the conformation exchange of the whole molecule which is responsible for the mobility in the H4–H6' pair.

Table 2: Order parameters, correlation times, and distances for felodipine. QM and FF stand for results of quantum mechanics and force-field calculations; NMR-derived data are exchange-averaged values of individual conformers A–F

<table>
<thead>
<tr>
<th></th>
<th>$S^2$</th>
<th>$\tau_{\text{eff}}, \text{ps}$</th>
<th>QM</th>
<th>FF</th>
<th>Experimental distances, Å</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$r_{\text{A,B,E}}$</td>
<td>$r_{\text{C,D,F}}$</td>
<td>$r_{\text{1,3,5}}$</td>
</tr>
<tr>
<td>H3b–H3c</td>
<td>0.28</td>
<td>168 ± 3</td>
<td>2.69</td>
<td>2.69</td>
<td>2.72</td>
</tr>
<tr>
<td>H4–H6'</td>
<td>0.40</td>
<td>237 ± 14</td>
<td>2.12</td>
<td>3.73</td>
<td>2.23</td>
</tr>
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</table>

Revealed distribution of conformations shows that neither of the felodipine conformer groups, (A,B,E) or (C,D,F), dominates in the solution of moderate concentration in DMSO (Fig. 4). The (A,B,E) group has a slightly bigger fraction, but the achieved measurement error does not allow us to make a reliable conclusion. In the light of this finding, our earlier data [9] showing that one group strongly dominates in the saturated solution points to an interest-
Figure 2: Six possible conformers of felodipine. Two groups can be distinguished differing by the orientation of the upper dichlorophenyl ring relative to the other part of the molecule. In this paper we do not distinguish individual conformations within these two groups, (A,B,E) and (C,D,F). Conformations were calculated by Teberekidis and Sigalas [41].
Figure 3: Chemical formula and atom numbering of felodipine. Arrows show the flexible sites with different characteristic motion rates.

ing phenomenon: preferred conformation depends on interactions between drug molecules and/or solvent effect.

5. Discussion

Since cross-relaxation rates rise sharply when interacting nuclei become closer, minor conformers can contribute significantly to observed values of $\sigma_{ij}$ (and hence to calculated distances $r_{ij}$) even in small concentrations, if they have favourable positions of the considered nuclei. In the case of felodipine there is a possibility for spin diffusion in conformations C, D, and F, because the distances in the pair of interest H4–H6’ and in the pairs H4–H1 and H1–H6’ are all similar (3.7, 4.7, and 3.4 Å, see the scheme in Fig. 5). Thus, the key effective distance H4–H6’ used in calculation of the conformer fractions appears smaller than it is. If the reason for this deviation (namely, spin diffusion) is not considered, the calculation will overestimate the population of the conformers where the analyzed atom-atom distance is smaller; these are A, B, and E in the case of felodipine.

Knowledge of conformers’ structures is also of great importance if one wishes to accurately calculate the distribution, since both experimentally found and theoretically predicted geometrical restraints determine the fractions of all conformers. Quantum chemical calculations are hence indispensable in this kind of
Figure 4: Conformer distributions of felodipine obtained using different analysis methods based on effective distances given in Table 2.
Figure 5: Disposition of atoms facilitating spin diffusion in conformers C, D, F of felodipine. The direct magnetization transfer pathway corresponds to the distance of interest, H4–H6', which is used in calculating the conformer fractions.
studies. The simulation employing a force field gives results which are close to that predicted by QM and agree with experimental observations. Certainly, it cannot replace QM simulations due to its limited accuracy, but it gives a good approximation to structures and even solvation thermodynamics [43] just within several seconds, which is the main advantage of this method.

Our results show that the combined analysis of NOESY and T-ROESY data yields atom–atom distances which are not biased by the spin diffusion effect, and hence gives an opportunity to find conformer distribution in solution more accurately. Furthermore, it gives information on parameters of intramolecular mobility, including conformational exchange, in the case of small molecules.

The QUIET-NOESY approach, in the end, gives results very similar to those obtained from NOESY/T-ROESY spectra. Any of them may be chosen if accurate determination of atom–atom distances in a small molecule should be done. However, implementation of the former experiment requires creating selective or band-selective pulses, which should be tuned to the resonances of interest in any given compound. The latter approach is simpler, and though it requires recording a double set of spectra, the total duration of the measurements will be shorter than in the QUIET-NOESY method if spin diffusion needs to be eliminated in several proton pairs independently. Additional merit of the NOESY/T-ROESY approach is that it allows estimating the internal molecular mobility (correlation time).

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