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Journal of Magnetic Resonance 160 (2003) 120–125
JMR Journal of Magnetic Resonance
www.elsevier.com/locate/jmr

The new HMQC-based technique for the quantitative determination of heteronuclear coupling constants. Application for the measurement of $^3J(H'_i, P_{i+1})$ in DNA oligomers

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Received 5 June 2002; revised 24 October 2002

Abstract

A new general *J*-HMQC-based technique is presented, which allows an accurate determination of heteronuclear coupling constants. The most important feature of this new approach includes acquisition of the two data sets with and without the additional π(S)-pulse at the end of coupling evolution period. This enables preservation and separation of the two orthogonal terms of coupling evolution, which are manifested by in- and antiphase cross-peaks, respectively. The coupling magnitudes are evaluated by the non-linear least-squares fitting of the ratios of integrated signal volumes for both kinds of signals. The effectiveness of the new sequence is demonstrated by determination of the $^3J(H3'_i, P_{i+1})$ couplings in DNA octamer duplex $d(GCGTACGC)_2$ sample. Additionally, the ability of the new method for the measurement at the natural abundance level of ^{13}C nuclei is presented for the β-cyclodextrin.

Keywords: HMQC; Coupling constants; DNA; $^3J(H3'_i, P_{i+1})$; β-Cyclodextrin

1. Introduction

The homo- and heteronuclear spin–spin coupling constants provide valuable information for characterization of intramolecular bonding conformation and dynamics. The most important applications utilizing the coupling values include determination of scalar vicinal three-bond couplings [1] and the measurement of residual dipolar couplings in partially oriented media [2]. However, especially in cases where couplings and transverse relaxation rates are of comparable magnitudes, dedicated methods are required to obtain precise and accurate values of the coupling constants. There are two general approaches for the determination of coupling constants. The first involves analysis of the frequency domain spectra, e.g., E.COSY-type techniques or reference deconvolution [1,3], and second, referred to as *J*-correlation spectroscopy [4], utilizes for coupling evaluation the dependence of signal intensity as a function of evolution time. The *J*-correlation methods require pulse sequences with single or multiple coupling evolution periods and well-defined transfer amplitude of either in- or antiphase signals.

Although the *J*-correlation techniques usually require a long experimental time for the collection of number of spectra with appropriately good signal-to-noise ratio, they offer superior precision of the measured coupling magnitudes. The simplest application of this type, used for the measurement of heteronuclear coupling constants, is based on comparison of the signal intensity acquired with and without refocusing S-spin pulses for the single evolution time τ. The methods of this type usually exploit constant-time evolution periods. The experiments based on CT-COSY [5], CT-NOESY [6], and CT-HMQC [7,8] were successfully applied to the measurements of ^{31}P–^1H couplings in oligonucleotides. Another approach requires the determination of cross-

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doi:10.1016/S1090-7807(02)00142-8

peak intensities for a set of different τ delays. For example, this homo- [9] or heteronuclear [10,11] coupling evolution period τ can be inserted into the HSQC or HMQC scheme. The coupling magnitudes should be fitted to the function of $A\cos(\pi J\tau)\exp(-\tau R_2)$ or, depending on implementation, $A\sin(\pi J\tau)\exp(-\tau R_2)$, where A is the maximum signal intensity, R_2 is the effective transverse relaxation rate, and J is the actual coupling magnitude. The exponential factor of $\exp(-\tau R_2)$, describing transverse relaxation, could be omitted by application of J-HMQC sequence with constant time coupling evolution [12–14], however, at the expense of substantially reduced signal-to-noise ratio for long τ periods.

In the present work, we propose the simple and general technique, based on two-parameter fitting of the function $A|\tan(\pi J\tau)|$ in multiple points. The new experiment could be applied for rare spins at the natural isotopic abundance level, is free of disadvantages originating from constant time evolution, and consists only of a few pulses. Similar methodology of fitting of tangent dependence was recently proposed for the determination of homonuclear couplings using ratios of cross- and autocorrelation peaks in CT-COSY experiment [15].

The factor most limiting the applicability of J-correlation methods is the transverse relaxation rate of I-spin (usually ^{1}H) nuclei. Thus, we decided to verify the technique presented in this work by determination of important $^3J(H3'_i, P_{i+1})$ coupling constants in DNA oligomer. The parameterization of Karplus equation, relating this coupling to the backbone angle ε, is well known [16,17], and the same measurement could provide additional information on residual dipolar coupling in the partially oriented phase [6].

There are several approaches for the determination of this significant coupling which include: (i) direct evaluation of couplings from splittings in frequency domain spectra, using selective pulses [18] or J-scaled CT-HMBC [19], (ii) P-FIDS-CT-HSQC method [20] designed for ^{13}C-labeled samples, and (iii) already mentioned techniques, which rely on comparison of signal intensities with and without evolution of couplings involving ^{31}P for the samples at natural ^{13}C abundance [5,6] and ^{13}C-enriched [7,8].

2. Results and discussion

The pulse-sequence schemes for the new experiments are depicted in Fig. 1. The presented approach is derived from the HMQC technique with variable refocusing delay τ. The two sequences presented differ in application of States–TPPI quadrature in (a) and echo–antiecho PFG coherence selection, in the way proposed for MBOB method [21], in (b). These variants of HMQC

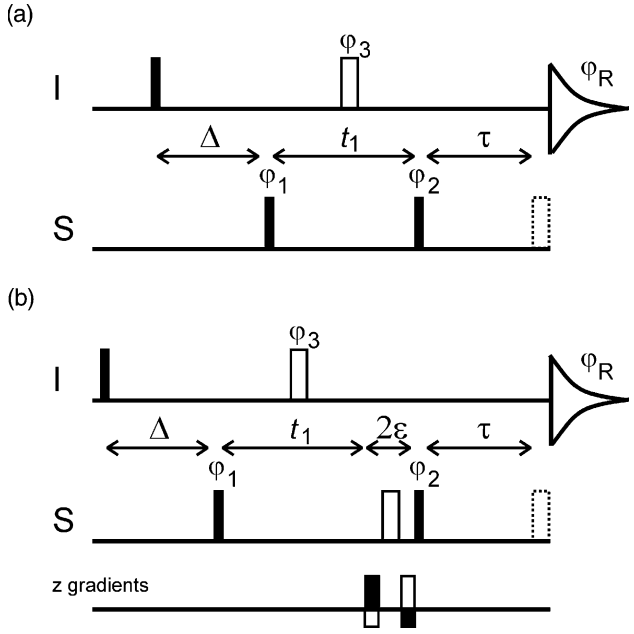

Fig. 1. Pulse sequences of the new experiment. (a) Sequence with States–TPPI quadrature in F_1, applied to φ_1 (b) with gradient echo–antiecho coherence selection. In both cases, four data sets should be collected for each t_1 increment in the interleaved mode. The selection of in- and antiphase spectrum is accomplished by addition and subtraction of the data sets, acquired with and without additional π(S)-pulse prior to FID acquisition, respectively. This π(S)-pulse is represented by dotted box. Dark-filled and open bars represent π/2 and π pulses, respectively. All pulses are applied along the rotating-frame x-axis unless indicated differently. The delay Δ should be optimized for maximum amplitude of coherence transfer. τ is a variable delay for heteronuclear coupling evolution. The basic phase cycle was $\phi_1 = x, -x,\ \phi_2 = 2x,\ 2(-x),\ \phi_3 = 4x,\ 4y,\ 4(-x),\ 4(-y)$, and receiver $\phi_R = x, -x, -x, x, -x, x, x, -x$. In the sequence (b) ε includes the rectangular-shaped gradient pulse and a 100 μs recovery time. The gradient ratio should be set to: $+(\gamma I + \gamma S) : -(\gamma I - \gamma S)$ and $-(\gamma I - \gamma S) : +(\gamma I + \gamma S)$, for heteronuclear echo and antiecho, respectively.

technique allow one to obtain the nearly pure absorption lineshapes along F_1, combined with absolute value mode in F_2 dimension. In both cases, for each t_1 increment, the two States–TPPI or echo–antiecho data sets are acquired twice—in the presence and in the absence of π(S)-pulse at the and of τ period, respectively. The separation of in- and antiphase coherences is accomplished by the addition or substraction of the data sets different by application of additional π(S)-pulse. This approach, of appropriate combination of spectra collected with and without sign inversion of antiphase coherences, was originally implemented in HECADE method [22,23] in order to simplify cross-peak patterns due to active coupling.

In the case of sequence 1(a), the signals are obviously attenuated by a factor of $\exp[-R_2(\Delta + t_1 + \tau)]$ due to the transverse relaxation. Additionally, the cross-peaks are affected by phase distortions as a result of homonuclear I-spin coupling modulation, and not refocused I-spin chemical shift evolution, during the periods of

$(\Delta + t_1 + \tau)$ and $(\Delta - \tau)$, respectively. (For the sequence 1(b) the period of 2ε should also be considered.) However, for both in- and antiphase signals, these effects could be assumed in the first estimation to be equal, and thus, not relevant in their ratio.

The resulting integral intensities of in- (V_{IP}) and antiphase cross-peaks (V_{AP}) are proportional to $\sin(\pi J\tau)$ and $\cos(\pi J\tau)$, respectively. In the case of equal magnitudes of the respective proportionality coefficients, the coupling constants could be evaluated from single measurement as: $J = \arctan(V_{IP}/V_{AP})/\pi\tau$. However, owing to possibly different shapes of the in- and antiphase cross-peaks, it is more practical to obtain the couplings by two-parameter fitting of Eq. (1), where A is the normalization factor

$$V_{IP}/V_{AP} = A|\tan(\pi J\tau)|. \quad (1)$$

The plots of (V_{IP}/V_{AP}) ratios against τ, and the curves resulting the fitting procedure, for data acquired for DNA sample $d(GCGTACGC)_2$ using the sequence 1(a) are shown in Fig. 2. Additionally, for comparison, the same coupling constants were measured by CT-COSY [5] and CT-NOESY [6] methods. All obtained coupling magnitudes are collected in Table 1. The $^3J(H3'_i, P_{i+1})$ coupling constants determined by different methods agree within 0.3 Hz and lie in the range of 3–6 Hz, which is a typical value for this type of molecules.

In addition, we have applied sequence 1(b) with PFG coherence selection for the measurement of important $^3J(H1, C4')$ coupling constant between the anomeric proton and carbon from the adjacent glycosidic unit in the β-cyclodextrin molecule at natural ^{13}C isotopic abundance. The magnitude obtained for $^3J(H1, C4')$ coupling is equal to 5.65 ± 0.1 Hz.

For the single τ period, the simultaneous acquisition of in- and antiphase coherences followed by its separation relying on data set combination improves the achieved signal-to-noise ratio by a factor of $\sqrt{2}$, in comparison to techniques which require the two independent measurements. The presented *J*-HMQC sequence is, however, longer by a period of $(\Delta + t_1)$, when compared with constant-time homonuclear techniques [5,6], where the coupling evolution period τ and t_1 are combined together. This extensive length of the pulse sequence is insignificant in the case of determination of relatively big one-bond couplings, but it may cause considerable sensitivity losses due to effective transverse relaxation when the experiment is applied for the determination of small coupling constants in larger biomolecules.

The evaluation of coupling magnitudes from a number of experiments acquired for the set of different τ periods is obviously more time consuming than the application of, e.g., CT-COSY [5] and CT-NOESY [6]

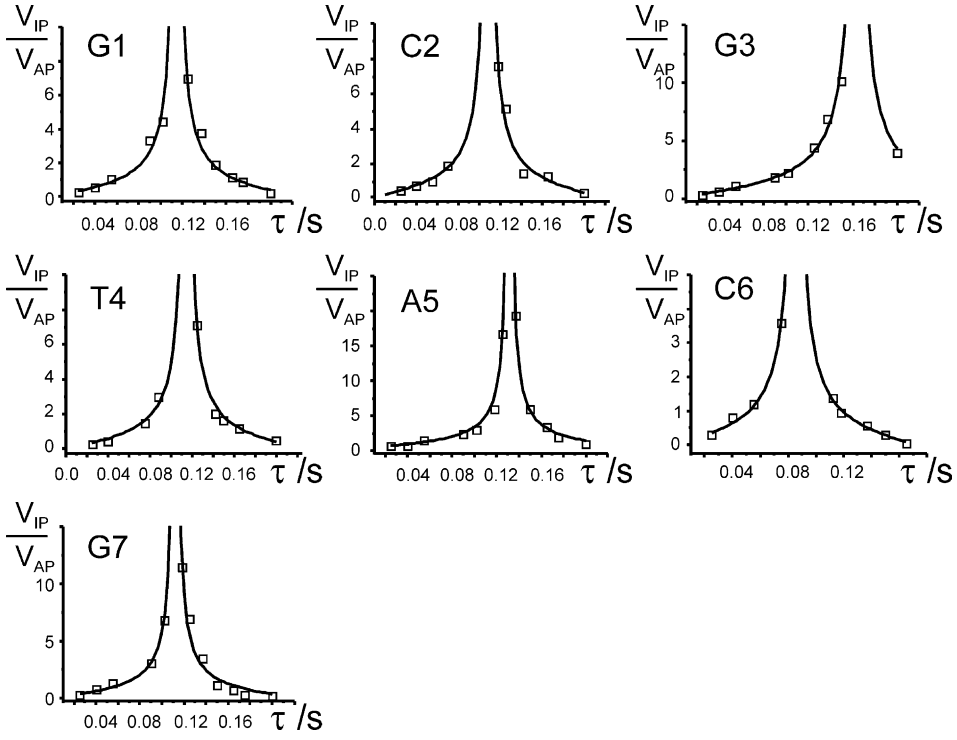

Fig. 2. Comparison of plots of the ratio of in- and antiphase signal volumes (V_{IP} and V_{AP}, respectively) against refocusing delay τ, measured for the sample of $d(GCGTACGC)_2$ using experiment from Fig. 1a. Experimental points are represented by empty squares. The curves are obtained by the two-parameter nonlinear least-squares fitting of Eq. (1), the corresponding coupling magnitudes are collected in Table 1. In all cases, the normalization parameter A was obtained in the range of 0.8–0.95, owing to a slightly reduced integral intensity of antiphase cross-peaks.

Table 1
Comparison of the $^3J(H3'_i, P_{i+1})$ coupling constants obtained for the DNA octamer $d(GCGTACGC)_2$ using the proposed *J*-HMQC experiment and other techniques

H3′ nucleoside	$^3J(H3'_i, P_{i+1})$[a] *J*-HMQC	$^3J(H3'_i, P_{i+1})$[b] CT-NOESY [6]	$^3J(H3'_i, P_{i+1})$[c] CT-COSY [5]
G1	4.36	4.2	4.3
C2	4.61	4.9	[d]
G3	3.05	3.1	2.8
T4	4.36	4.3	[d]
A5	3.80	4.0	3.5
C6	5.85	5.5	5.8
G7	4.46	4.5	4.3

[a] Estimated, on the basis of integral precision, error is ca. ±0.1 Hz (the standard deviations obtained in least-squares fitting lie in the range of 0.02–0.06 Hz).
[b] ±0.2 Hz.
[c] ±0.3 Hz.
[d] Not detected owing to signal overlap.

constant-time techniques for single evolution delay. Thus, procedure involving only two-parameter least-squares fitting described in this work, increases the precision of the measurement at the expense of a substantially longer experimental time. Moreover, the discontinuity of tangent function improves the accuracy of the proposed technique.

The obtained coupling magnitudes could be affected by different, in general, relaxation rates of in- and antiphase signal components [24]. However, these effects are expected to be of minor importance for the molecules studied in this work, whereas for larger molecules corrections might be necessary. In the case of proposed *J*-HMQC technique, the S-spin flip rate should be considered and involved as the additional parameter into fitting function Eq. (1), as described in [25–27].

In the case of imperfect S-spin inversion caused by the π-pulse in the second experiment, the effective inversion ratio k, $0 \leqslant k \leqslant 1$ should be taken into account. This phenomenon does not affect the experiment without S-spin π-pulse, but in the second data set the amplitude of antiphase signals would be attenuated by a factor of k. Thus, the intensity of antiphase signals, calculated from difference of the two experiments, would be reduced by $(1-k)$. Consequently, the intensity of in-phase cross-peaks, obtained from the sum of both experiments, consisting in the ideal case of pure in-phase signals, would increase by an additional amount of $(1-k)$ of the antiphase part. The ratio of signal volumes in the sum (V_S) and difference (V_D) of both experiments is therefore for $k < 1$ defined by Eq. (2)

$$V_S/V_D = A[|2\sin(\pi J\tau) + (1-k)\cos(\pi J\tau)|]/[(1+k)|\cos(\pi J\tau)|]. \quad (2)$$

We have tested the sensitivity of the proposed method to the nonideal inversion pulse. The application of Eq. (2) for fitting data obtained for $d(GCGTACGC)_2$ sample with k of 0.85 did not change the fit quality, and *J*-magnitudes obtained in this way were different only by approximately 0.01–0.04 Hz from those obtained using Eq. (1).

In summary, we have shown the ability of proposed approach for the accurate determination of $^3J(^{31}P, ^1H)$ coupling constants in DNA oligomers and of $^3J(^{13}C, ^1H)$ in medium-sized organic molecules at natural isotopic abundance. The high precision and accuracy of the new technique is combined with relatively good sensitivity and obtained by the two-parameter only least-squares fitting of the ratios of in- and antiphase signal intensities. The proposed experiment is simple and in general could be applied to a wide range of molecules for determination of any one- and multiple bond heteronuclear coupling magnitudes when it is possible to obtain only a resolved cross-peak in HMQC spectra.

3. Experimental

All spectra presented, except CT-NOESY measurement, were recorded at 303 K on a Varian Unity Plus 500 spectrometer equipped with a Performa I z-PFG unit and a standard 5 mm ID_PFG probehead. 8, 12, and 39 μs high power ^1H, ^{13}C, and ^{31}P $\pi/2$ pulses, respectively, were employed. The CT-NOESY [6] experiment was performed on a Varian Unity Inova 500 spectrometer equipped with a Performa II z-PFG unit and a 5 mm Nalorac Z-SPEC probehead, using 10.4 and 18.5 μs high power ^1H and ^{31}P $\pi/2$ pulses, respectively. The samples of 4.3 mM selfcomplementary DNA octamer duplex $d(GCGTACGC)_2$, at pH 7.8, and 20 mM β-cyclodextrin in D_2O were used.

For the DNA sample the 14 experiments for different τ delays chosen from the range of 25–200 ms were recorded according to scheme from Fig. 1a. Sixteen to sixty-four scans were coherently added for each data set for 32 t_1 increments. The maximum t_1 and t_2 times were set to 32 and 256 ms, respectively. A relaxation delay of

1.5 s was used and Δ was set to 70 ms. The data matrix containing 32×512 complex points in t_1 and t_2 was zero-filled to 256×1024 complex points. Cosine and $\pi/4$ shifted sine weighting functions were applied, prior to Fourier transformation in t_1 and t_2, respectively. In the case of β-CD sample, the PFG experiment from Fig. 1b was used. The spectra were recorded for eight different τ periods lying in the range of 40–140 ms. Sixteen scans were acquired for each data set for 32 t_1 increments. The t_1 and t_2 times were sampled to 4 and 254 ms, respectively. A relaxation delay of 2 s was used and Δ was set to 70 ms. The data matrix containing 32×320 complex points in t_1 and t_2 was zero-filled to 256×1024 complex points. Cosine and $\pi/4$ shifted sine weighting functions were applied prior to Fourier transformation in t_1 and t_2, respectively.

In all cases, data were processed to obtain near absorption lineshapes along F_1 and then magnitude calculation was employed. To remove small positive offset observed due to magnitude calculation, the baseline correction of 2D spectra was applied, using the abc program [28], distributed with VNMR software (userlib section). Signal volume integration was performed using the standard VNMR 6.1B software. After the baseline correction, the average integrals from empty region of spectra were near zero. The highest relative errors were obtained for data points recorded for τ close to $0.5/J$ and, consequently, with near zero $\cos(\pi J\tau)$ modulated part. Thus, these points were rejected during the fitting procedure. The CT-COSY and CT-NOESY experiments were set according to published procedures [5,6].

Acknowledgments

W.K. thanks to the Warsaw University for the support by Grant: 120-501/68/BW-1522/14/2001.

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