Two-dimensional concurrent HMQC-COSY as an approach for small molecule chemical shift assignment and compound identification

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Abstract  Chemical shift assignment is the first step toward the structure elucidation of natural products and other chemical compounds. We propose here the use of 2D concurrent HMQC-COSY as an experiment for rapid chemical shift assignment of small molecules. This experiment provides well-dispersed $^1$H-$^{13}$C peak patterns that are distinctive for different functional groups plus $^1$H-$^1$H COSY connectivities that serve to identify adjacent groups. The COSY diagonal peaks, which are phased to be absorptive, resemble $^1$H-$^{13}$C HMQC cross peaks. We demonstrate the applicability of this experiment for rapidly and unambiguously establishing correlations between different functional groups through the analysis of the spectrum of a metabolite (jasmonic acid) dissolved in CDCl$_3$. In addition, we show that the experiment can be used to assign spectra of compounds in a mixture of metabolites in D$_2$O.

Keywords  2D HMQC-COSY · Chemical shift assignment · Compound identification · Metabolite mixture

Introduction

2D COSY spectra (Aue et al. 1976) provide important information on the connectivity between different functional groups; however, 2D $^1$H-$^1$H COSY spectra suffer from phasing issues, arising from intrinsic differences in the relative phase of diagonal and cross peaks, and peak overlap issues, resulting from the low proton chemical shift dispersion. Heteronuclear 2D HSQC or HMQC experiments yield better-resolved spectra of metabolites and metabolite mixtures because of the higher chemical shift dispersion of carbon; however, these experiments do not offer information on the connectivity between different functional groups. Heteronuclear multiple-bond correlation spectra, HMBC type experiments (Bax and Summers 1986) can show heteronuclear long-range “indirect” connectivities, but these are not always unambiguous. The INADEQUATE experiment (Bax et al. 1980), which makes use of direct $^{13}$C-$^{13}$C coupling, can provide very important information on $^{13}$C-$^{13}$C connectivities within the molecular skeleton, but its application is limited by the low sensitivity of the experiment at natural abundance $^{13}$C.

To overcome the peak overlap issue in 2D COSY, the 3D $^{13}$C-edited HMQC-COSY experiment was proposed and demonstrated on kanamycin A with natural abundance $^{13}$C (Fesik et al. 1989). A $^{15}$C-resolved COSY experiment involving selective excitation of a particular carbon signal by the SELINCOR technique was proposed as a means for overcoming spectral overlaps (Facke and Berger 1995). The 3D HMQC-COSY experiment was later modified with gradient-enhanced coherence order selection and further demonstrated on sucrose and menthol to facilitate assignments (Hurd and John 1991).

In 2D HMQC-COSY, the initial $^1$H magnetization is transferred concurrently to $^{13}$C through $^{1}$J$_{CH}$ (HMQC) and to $^1$H through $^{3}$J$_{HH}$ (or $^3$J$_{HH}$ COSY). The $^1$H-$^{13}$C multiple-quantum period is exploited for both $^{13}$C chemical shift coding and for achieving a maximal COSY effect. Similar information on the connectivity can be obtained from HSQC-COSY to HSQC-TOCSY (Bax and Davis 1985) experiments. For HSQC-COSY and HSQC-TOCSY, the initial $^1$H magnetization is transferred sequentially to...
bonded $^{13}$C’s (by HSQC) and to homonuclear $J$ coupled $^1$H’s (by COSY or TOCSY). Considering the relatively small 3-bond $^{1}H-^{1}H$ homonuclear $J$-couplings, usually the coupling Hamiltonian has to be allowed to evolve for a considerably long time to achieve reasonable COSY or TOCSY effects. In addition, to achieve reasonable resolution along the indirect $^{13}$C-dimension, magnetization on $^{13}$C (single-quantum coherence) has to evolve for a long $t_1$ time.

Here we present 2D HMOC-COSY experiments that can be run as constant-time (Fig. 1a) or non-constant-time (Fig. 1b) versions and used for rapid spectral assignment of small molecules. In the constant-time version of 2D HMOC-COSY to achieve maximum COSY effect, given the relatively small 3-bond $^1H-^{13}$C heteronuclear multiple quantum generation period (a–b) and the $^{1}H-^{13}$C multiple quantum constant-time period. In the non-constant-time version of 2D HMOC-COSY, the $^1H-^{13}$C homonuclear $J$-coupling runs in accordian mode (Mandel and Palmer 1994). The constant-time version is superior for the analysis of small molecule spectra because of its higher COSY transfer efficiency with small $J$-couplings and its higher resolution along the indirect $^{13}$C-dimension compared to the non-constant-time version.

We illustrate the utility of this approach for the spectral assignment of a small molecule and show how the experiment can be used for identifying compounds in mixtures of metabolites.

**Materials and methods**

Jasmonic acid (Sigma–Aldrich) was dissolved in CDCl$_3$ at a concentration about 60 mM. The constant-time 2D HMOC-COSY spectrum of jasmonic acid was recorded at 25°C on a Bruker Avance 500 MHz spectrometer equipped with a $z$-gradient triple resonance CPTXO probe optimized for $^{13}$C detection. The radio-frequency pulses on $^1H$ and $^{13}$C were applied at 4.7 and 73 ppm, respectively. $^{13}$C decoupling was carried out using GARP with a field strength of $\gamma B_2 = 3.38$ kHz. 1024 $\times$ 150 complex data points were collected along $^1H$ and $^{13}$C dimensions (spectral widths of 14 and 80 ppm, respectively) with 4 scans per FID and an inter-scan delay of 4 s, resulting in total data acquisition time of about 1 h. The NMRPipe package (Delaglio et al. 1995) was used to process spectra with linear prediction and zero-filling along the $^{13}$C dimension.

$^{13}$C GARP decoupling was carried out with a field strength of $\gamma B_2 = 3.38$ kHz. 1024 $\times$ 120 complex data points were recorded along the $^1H$ and $^{13}$C dimensions (spectral widths of 14 and 80 ppm, respectively) with 4 scans per FID and an inter-scan delay of 4 s, resulting in total data acquisition time of about 1 h. The NMRPipe package (Delaglio et al. 1995) was used to process spectra with linear prediction and zero-filling along the $^{13}$C dimension.

**Results and discussion**

NMR pulse sequences

In the constant-time 2D concurrent HMOC-COSY pulse scheme (Fig. 1a), the initial $^1H^a$ polarization is excited by a 90° pulse on $^1H$ followed by a multiple quantum generation period, where magnetization is transferred to $^1H^a-^{13}$C$^a$ multiple quantum coherence at point $b$. By synchronously
shifting the 180° 13C pulse (ϕ2) and 180° 1H pulses (as indicated by the arrows) during the constant time period T (points b–c shown in the dashed rectangle), the chemical shift of 13C is encoded in time and concurrently the 1H–1H homonuclear J-coupling Hamiltonian evolves. Note, that besides refocussing the chemical shift evolution of 1H, insertion of the two 180° 1H pulses during the constant time also refocusses the evolution of other passive heteronuclear J-couplings between 13C and other multiple bond coupled protons. Thus, a narrower line width along the 13C dimension can be obtained. Application of a second 90° pulse on 13C (ϕ2) transfers 1H–13C MQ coherence back to 1H. Following the 2τ reverse HMQC period, magnetization on 1H evolves in-phase relative to 13C. For the entire 2τ + T + 2τ time period (point a–d), 1H–1H homonuclear J-coupling Hamiltonian evolves and part of the magnetization arising from 1H evolves to be antiphase relative to the homonuclear coupled third party proton 1H. COSY transfer from 1H to 1H is then achieved by the 90° pulse on 1H (ϕ5). The period 2δ inserted before detection serves to accommodate the refocusing gradient g2. The relevant coherence transfer pathway can be described as:

\[ H_{c2}^{090} \xrightarrow{180°,180°,2\tau} 2H_{c3}^{2\delta}C_{c}^{0}\cos[πJ_{1H-1H}\cdot2\tau] \]
\[ + 4H_{c1}^{090}H_{c2}^{090}C_{c}^{0}\sin[πJ_{1H-1H}\cdot2\tau] \]
\[ + 4H_{c3}^{090}H_{c2}^{090}C_{c}^{0}\cos[πJ_{1H-1H}\cdot2\tau] \]
\[ + 4H_{c3}^{090}H_{c2}^{090}C_{c}^{0}\sin[πJ_{1H-1H}\cdot2\tau] \]
\[ \xrightarrow{180°,180°,2\tau} H_{s1}^{090}(ω_{C},γ_{C}g_{1})\cos[πJ_{1H-1H}\cdot(T+2\tau)] \]
\[ + 4H_{c1}^{090}H_{c2}^{090}(ω_{C},γ_{C}g_{1})\sin[πJ_{1H-1H}\cdot(T+2\tau)] \]
\[ + 4H_{c3}^{090}H_{c2}^{090}(ω_{C},γ_{C}g_{1})\sin[πJ_{1H-1H}\cdot(T+2\tau)] \]
\[ + 4H_{c3}^{090}H_{c2}^{090}(ω_{C},γ_{C}g_{1})\sin[πJ_{1H-1H}\cdot(T+2\tau)] \]
\[ \xrightarrow{180°,180°,2\tau} H_{s1}^{090}(ω_{C},γ_{C}g_{1})\cos[πJ_{1H-1H}\cdot(T+4\tau)] \]
\[ + 2H_{c1}^{090}H_{c2}^{090}(ω_{C},γ_{C}g_{1})\sin[πJ_{1H-1H}\cdot(T+4\tau)] \]
\[ + 4H_{c3}^{090}H_{c2}^{090}(ω_{C},γ_{C}g_{1})\sin[πJ_{1H-1H}\cdot(T+4\tau)] \]
\[ + 4H_{c3}^{090}H_{c2}^{090}(ω_{C},γ_{C}g_{1})\sin[πJ_{1H-1H}\cdot(T+4\tau)] \]
\[ + 4H_{c3}^{090}H_{c2}^{090}(ω_{C},γ_{C}g_{1})\sin[πJ_{1H-1H}\cdot(T+4\tau)] \]

The term (ω_{C},γ_{C}g_{1}) arises from the coherence encoded by the chemical shift of 13C(ω_{C}) and dephased by gradient g_{1}. Gradient selection is then achieved by application of the refocusing gradient g_{2} [last line in Eq. (1)]. Note that the homonuclear J-coupling evolution is ignored during the short gradient refocusing period 2δ.

The COSY diagonal peak, represented by the first term at point e, \[ H_{s1}^{090}(ω_{C})\cos[πJ_{1H-1H}\cdot(T+4\tau)] \], is 90° out of phase relative to the COSY cross peak, represented by the second term at point e, \[ 2H_{c1}^{090}H_{c2}^{090}(ω_{C})\sin[πJ_{1H-1H}\cdot(T+4\tau)] \]. Therefore, whereas the COSY diagonal peaks are phased to be absorptive, the COSY cross peaks show a typical pattern of antiphase dispersion (see the line shape of H12 in the dashed rectangle in Fig. 2b). The COSY diagonal peak term \[ H_{s1}^{090}(ω_{C})\cos[πJ_{1H-1H}\cdot(T+4\tau)] \] evolves under the chemical shift of 1H during detection, which will appear as normal 1H–13C HMBC cross-peaks. The COSY cross peak term \[ 2H_{c1}^{090}H_{c2}^{090}(ω_{C})\sin[πJ_{1H-1H}\cdot(T+4\tau)] \] will evolve under the chemical shift of 1H during detection. It will thus show cross peaks at the chemical shift of 13C and chemical shift of the coupled proton 1H. This indirect correlation provides information similar to that of an HMBC experiment. Similarly, magnetization initially from 1H, will yield a COSY diagonal peak at 1H–13C and a COSY cross peak at 1H–13C.

Therefore, in the 2D HMQC-COSY spectrum, neighboring (1H–1H homonuclear coupled) functional groups 1H–13C and 1H–13C will yield two COSY diagonal peaks at the chemical shifts of 13C/1H and 13C/1H, respectively, which can be correlated by the two COSY cross peaks at the chemical shifts of 13C/1H and 13C/1H. The COSY diagonal and cross peaks can be recognized by their line shape: the COSY diagonal peaks are phased to be absorptive, and the COSY cross peaks show an antiphase dispersive line shape. They also can be differentiated simply by comparison to conventional HSQC/HCOSY spectra: the extra peaks shown in HMQC-COSY are the COSY cross peaks, giving HMBC type information. The feature of high chemical shift dispersion along the 13C dimension and HMBC type information contained in the HMQC-COSY spectrum renders the HMQC-COSY experiment a robust approach for chemical shift assignments and also for identifying compounds in metabolite mixtures as demonstrated below.

In the non-constant-time version of HMQC-COSY, the constant-time period (dashed block in Fig. 1a) is replaced by non-constant-time block (Fig. 1b). The short delay \[ δ_1 = 1.21 ms \] is just to accommodate the coherence selective pulsed field gradients g_{1}. As the chemical shift of 13C is encoded in time, the effective evolution time for the 1H–1H homonuclear J-coupling Hamiltonian also varies with \[ t_{1} \] from \((2π + 4δ_{1} + 2τ)\) to \((2π + t_{1} + 2δ_{1} + 2τ)\) (point a–d). 1H–1H homonuclear J-coupling evolution in the accordion mode allows the COSY transfer in the non-constant-time 2D HMQC-COSY to be more efficient for very diverse values of \[ J_{HH} \].

Chemical shift assignment of jasmonic acid

We illustrate the application of this approach with data from jasmonic acid (see Fig. 2a for the structure and atom
nomenclature of this compound as well as its 2D HMQC-COSY). The signal from the methyl group (C12–H12, labelled peak 12) of jasmonic acid is easily identified on the basis of its characteristic $^1$H and $^{13}$C chemical shifts. The assignment of the methyl group can be confirmed by reference to 1D $^1$H (Fig. 2a) and $^{13}$C DEPT-135 (Fig. 2a').
Walking along the $^1$H dimension to the left of peak 12 at the $^{13}$C chemical shift of the methyl group (14.21 ppm) leads to a cross peak at $^1$H = 2.058 ppm, which corresponds to the indirect interaction of C12 and H11 (C12–H11). Then walking down from peak C12–H11 along the $^{13}$C dimension leads to a cross peak at $^{13}$C = 20.80 ppm (peak 11, the direct H11–C11 correlation). Next, walking leftward from peak 11 along the $^1$H dimension leads to a cross peak at $^1$H = 5.463 ppm, which corresponds to C11–H10. Again, the downward arrow from peak C11–H10 along the $^{13}$C dimension leads to a cross peak at $^{13}$C = 5.463 ppm, which corresponds to C11–H10. Again, the downward arrow from peak C11–H10 along the $^{13}$C dimension leads to a cross peak at $^{13}$C = 20.80 ppm, which corresponds to C11–H10. Again, the downward arrow from peak C11–H10 along the $^{13}$C dimension leads to a cross peak at $^{13}$C = 5.463 ppm, which corresponds to C11–H10. 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negative. Only the quaternary carbons of jasmonic acid (C3 and C7) remain undetected and unassigned by the 2D HMQC-COSY approach. They can be found by 1D 13C NMR detection.

Compound identification from metabolite mixture

The 2D HMQC-COSY experiment can also be used to identify individual compounds in a mixture of metabolites. We illustrate this by reference to results with a mixture of 63.75 mM alanine (A), 88.47 mM methionine (M), and 124.36 mM sodium 3-hydroxybutyrate (HB) in D2O. Figure 3a shows the 2D HMQC-COSY spectrum of the mixture. As before, the directly correlated peaks are absorptive while the indirectly correlated peaks have an antiphase dispersive lineshape. Following the procedure outlined above for jasmonic acid, the spin networks of A, M, and HB were identified, as illustrated by the dotted, solid, and broken arrows, respectively, in Fig. 3a and b. Slices from the 2D HMQC-COSY spectrum of the mixture taken along the 1H dimension at the chemical shifts of M C, C, C, and C (Fig. 3c) show how this spin system can be identified selectively; the excellent alignment of the chemical shifts of H, H, and H from these slices indicates that these groups belong to the same spin network. Because C shows indirect correlation with both H and H, whereas C and C, each show indirect correlations only with H, the spin network is connected as C–C–H. The slice at M C does not show any indirectly correlated peak because its attached proton is not coupled to any other group by efficient COSY transfer; thus, C is isolated from the rest of the spin network. Having identified the spin network and associated chemical shifts, the identity of the compound can be determined from a metabolite standards database.

Conclusion

The 2D HMQC-COSY experiment presented here offers a robust approach for assigning 1H and 13C NMR signals from individual compounds or mixtures of compounds. Because of the high chemical shift dispersion along the carbon dimension, this experiment produces distinct peak patterns for different functional groups through 1H–13C multiple quantum coherence while retaining the important information on peak connectivity through 1H–1H COSY. We have implemented this method as a routine experiment for chemical shift assignment of standard compounds being added to the BMRB metabolomics database (http://www.bmrb.wisc.edu/metabolomics) in conjunction with 2D 1H–13C HMBC for assignment of quaternary carbons. Although the spectra shown here were of compounds at high concentration (60 mM), we have used the approach with samples at 2 mM concentration with only 4 transients. With more extensive time averaging, we expect that 2D HMQC-COSY can be used for the assignment of compounds at concentrations as low as 500 μM. We anticipate that the experiment may find wider application in NMR studies of metabolites and natural products.

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