**Application of Unsymmetrical Indirect Covariance NMR
Methods to the Computation of the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH and
 $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HMBC-IMPEACH Correlation Spectra**

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Abstract

Utilization of long-range ^1H - ^{15}N heteronuclear chemical shift correlation has continually grown in importance since the first applications were reported in 1995. More recently, indirect covariance NMR methods have been introduced followed by the development of unsymmetrical indirect covariance processing methods. The latter technique has been shown to allow the calculation of hyphenated 2D NMR data matrices from more readily acquired non-hyphenated 2D NMR spectra. We recently reported the use of unsymmetrical indirect covariance processing to combine ^1H - ^{13}C GHSQC and ^1H - ^{15}N GHMBC long-range spectra to yield a ^{13}C - ^{15}N HSQC-HMBC chemical shift correlation spectrum that could not be acquired in a reasonable period of time without resorting to ^{15}N -labeled molecules. We now report the unsymmetrical indirect covariance processing of ^1H - ^{13}C GHMBC and ^1H - ^{15}N IMPEACH spectra to afford a ^{13}C - ^{15}N HMBC-IMPEACH spectrum that has the potential to span as many as 6 to 8 bonds. Correlations for carbon resonances long-range coupled to a protonated carbon in the ^1H - ^{13}C HMBC spectrum are transferred *via* the long-range ^1H - ^{15}N coupling pathway in the ^1H - ^{15}N IMPEACH spectrum to afford a much broader range of correlation possibilities in the ^{13}C - ^{15}N HMBC-IMPEACH correlation spectrum. The indole alkaloid vincamine is used as a model compound to illustrate the application of the method.

Introduction

Long-range ^1H - ^{15}N 2D NMR methods have become important tools in structure elucidation since the first experiments were reported at natural abundance in 1995.^{1,2} Long-range ^1H - ^{15}N methods have been reviewed several times.³⁻⁷ The acquisition of long-range ^1H - ^{15}N data has become sufficiently prevalent that several pulse sequences have recently been reported that allow the simultaneous acquisition of ^1H - ^{13}C and ^1H - ^{15}N GHMBC spectra.^{8,9}

Recently, another new area of investigation, covariance NMR spectroscopy, has been receiving considerable attention.^{10,11} The work of greatest applicability to small molecule spectroscopy is probably the 2004 communication of Zhang and Brüschweiler that described the calculation of a ^{13}C - ^{13}C homonuclear correlation spectrum derived from an HSQC-TOCSY spectrum.¹¹ That communication stimulated our analysis of artifacts that occur in the indirect covariance processed spectra due to proton resonance overlaps in the F_2 frequency domain.¹² In an effort to eliminate artifacts, we also reported the development of unsymmetrical indirect covariance processing, a method that allows a pair of 2D NMR data matrices to be coprocessed. In the case of inverted direct response HSQC-TOCSY spectra, the negative direct response component of the data can be coprocessed with the positive relayed response component affording a covariance spectrum in which one type of overlap artifact is eliminated and the second is diagonally asymmetrical, allowing those responses to be eliminated by conventional symmetrization. We have subsequently shown that unsymmetrical indirect covariance processing can also be used to coprocess discretely acquired 2D NMR spectra to afford spectra corresponding to various 2D-NMR experiments such as *m,n*-ADEQUATE,¹³ HSQC-COSY,^{14,15} and

most recently, HSQC-NOESY.¹⁶ In a further extension of the unsymmetrical indirect covariance processing method, we recently reported the application of the technique in the computation of ^{13}C - ^{15}N correlation spectra through the mathematical combination of multiplicity-edited ^1H - ^{13}C GHSQC and ^1H - ^{15}N GHMBC spectra.^{17,18} We now wish to communicate the results we have obtained for the alkaloid vincamine (**1**), which was previously studied by long-range ^1H - ^{15}N GHMBC methods.¹⁹ Specifically, we wish to contrast the results obtained by unsymmetrical coprocessing of ^1H - ^{13}C GHSQC and ^1H - ^{15}N IMPEACH spectra with those obtained by coprocessing ^1H - ^{13}C GHMBC and ^1H - ^{15}N IMPEACH-MBC (^1H - ^{15}N IMPEACH hereafter) to the latter coprocessed spectra providing a spectrum that can be described as a ^{13}C - ^{15}N HMBC-IMPEACH correlation matrix.

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Experimental

All NMR data were recorded using a sample prepared by dissolving approximately 10 mg of vincamine dissolved in ~180 μL d_6 -DMSO, after which the solution was transferred *via* a TeflonTM (Hamilton) needle to a 3 mm NMR tube

(Wilmad). All of the data were acquired using a Varian three channel 500 MHz NMR spectrometer equipped with a gradient inverse triple resonance NMR probe. Spectra were recorded with identical F₂ (proton) spectral widths. The ¹H-¹³C GHSQC spectrum was acquired as 1024 x 96 data points; the 10 Hz ¹H-¹³C GHMBC data were recorded as 2048 x 160 data points; and the ¹H-¹⁵N IMPEACH-MBC data were recorded as 1024 x 96 data points. The multiplicity-edited GHSQC and GHMBC pulse sequences used were directly from the Varian pulse sequence library. The IMPEACH-MBC pulse sequence used was that described by Hadden, Martin, and Krishnamurthy²⁰ without any further modification. All three of the 2D NMR data sets were processed to afford final spectra consisting of 2048 x 512 points. The data were linear predicted in the 2nd dimension to twice the number of acquired points followed by zero-filling to 512 points prior to Fourier transformation. The unsymmetrical indirect covariance processing was performed using ACD/Labs SpecManager v10.02. The approximate computation time was ~5 s on a Dell Latitude D610 computer with 1 Gb of RAM and a 1.7 GHz processor.

The unsymmetrical indirect covariance matrix can be calculated by

$$\mathbf{C} = \mathbf{R}_N * \mathbf{R}_C^T \quad [1]$$

where $\mathbf{R}_N$ and $\mathbf{R}_C$ correspond to the real data matrices from the long-range ¹H-¹⁵N GHMBC and ¹H-¹³C multiplicity-edited GHSQCAD spectra, respectively. In the present report, the GHSQCAD data are plotted with CH and CH₃ resonances with positive phase and CH₂ resonances with negative phase. The ¹H-¹³C data matrix is transposed to $\mathbf{R}_C^T$ during processing. The data were acquired and processed so that there were equal

numbers of columns in the data sets, *i.e.* $\mathbf{R}_N$ is $N * M_1$ and $\mathbf{R}_C$ is $N * M_2$ to allow the multiplication of the data matrices. In the present example, F_2 spectral widths were identical although that is not an absolute requirement. By definition, the following formula is used to calculate each element C_{ij} (i and j are row indices in the initial matrices, correspondingly, $\mathbf{R}_N$ and $\mathbf{R}_C$) of data matrix $\mathbf{C}$:

$$C_{ij} = (\mathbf{R}_N)_{ij} * [(\mathbf{R}_C)_{ij}]^T = (\mathbf{R}_N)_{i1} * (\mathbf{R}_C)_{j1} + (\mathbf{R}_N)_{i2} * (\mathbf{R}_C)_{j2} + \dots + (\mathbf{R}_N)_{iN} * (\mathbf{R}_C)_{jN} \quad [2]$$

Bruce – did I get this the way you intended????

Each element of matrix $\mathbf{C}$ is the sum of products of values $(\mathbf{R}_N)_{ik}$ and $(\mathbf{R}_C)_{jk}$. A necessary condition is to have non-zero elements in equivalent positions in the rows of $(\mathbf{R}_N)_i$ and $(\mathbf{R}_C)_j$. For two “ideal” 2D NMR spectra, assuming zero noise in the data matrices, the sum of a matrix element will be non-zero when rows $(\mathbf{R}_N)_i$ and $(\mathbf{R}_C)_j$ have crosspeaks in the same position.

Results and Discussion

The application of unsymmetrical indirect covariance processing to combine discretely acquired 2D NMR spectra arose from an investigation of artifacts in ^{13}C - ^{13}C correlation plots that arise from indirect covariance processed inverted direct response (IDR) GHSQC-TOCSY spectra.¹³ Significant time savings have been demonstrated in the calculation of GHSQC-COSY^{14,15} and GHSQC-NOESY¹⁶ as compared to the direct acquisition of these data *via* the hyphenated 2D NMR experiments. For experiments such as ^{13}C - ^{13}C INADEQUATE²¹ or m,n -ADEQUATE,²² the equivalent data matrix calculated by combining ^1H - ^{13}C GHSQC and GHMBC spectra¹³ allows even greater spectrometer

time savings to be realized because of the low statistical probability (1:10,000) of two ^{13}C nuclides being in the structure of a single molecule. At natural abundance, ^{13}C - ^{15}N experiments are hampered by even lower statistical probability because of the 0.37% natural abundance of ^{15}N vs. ^{13}C at 1.1 %. Based on relative natural abundance, the probability of a ^{13}C and ^{15}N being in the same molecule is slight, ~1:27,000. The likelihood of ^{13}C and ^{15}N being in positions in a given structure and amenable to correlation *via* $^1\text{J}_{\text{CN}}$ or $^n\text{J}_{\text{CN}}$ where $n = 2-4$ is, of course, correspondingly lower. Consequently, direct and long-range ^{13}C - ^{15}N experiments have not been reported to date, although experiments of this type are quite important in the study of $^{13}\text{C}/^{15}\text{N}$ doubly labeled proteins.²³ We were thus very interested in exploring the combination of ^1H - ^{13}C and ^1H - ^{15}N 2D NMR experiments *via* unsymmetrical indirect covariance methods. Our first investigation along these lines yielded a ^{13}C - ^{15}N long-range correlation plot for strychnine calculated from a multiplicity-edited ^1H - ^{13}C GHSQC spectrum and a ^1H - ^{15}N GHMBC spectrum.¹⁶ It has been shown previously that ^1H - ^{15}N IMPEACH-MBC²⁴ and CIGAR-HMBC²⁵ experiments provide better experimental access to long-range ^1H - ^{15}N correlation information because of the accordion-optimization of the long-range magnetization transfer delay.

Using an approximately 10 mg sample of vincamine (**1**) dissolved in 180 μL d_6 -DMSO, ^1H - ^{13}C GHSQC and ^1H - ^{15}N IMPEACH-MBC (3-8 Hz optimized) spectra were acquired and processed to yield identically digitized 2D NMR data matrices in the F_2 frequency domain. The data sets were also equivalently digitized in the F_1 frequency domain although this is not a requirement for the unsymmetrical indirect covariance processing algorithm (ACD/Labs SpecManager v10.02).

$^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH

Discretely acquired coherence transfer experiments of the type $A \rightarrow B$ and $A \rightarrow C$ can be manipulated to indirectly afford a $B \leftrightarrow C$ correlation spectrum using unsymmetrical indirect covariance processing techniques as in our previous work¹³⁻¹⁸ or using projection reconstruction methods described by Kupče and Freeman.^{9,26,27} Figure 1 shows the multiplicity-edited ^1H - ^{13}C HSQC and the 3-8 Hz optimized ^1H - ^{15}N IMPEACH spectra flanking the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH correlation spectrum indirectly calculated by unsymmetrical indirect covariance processing. Responses arising *via* $^2J_{\text{NH}}$ couplings correspond to direct $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ correlations; responses arising *via* $^3J_{\text{NH}}$ and $^4J_{\text{NH}}$ heteronuclear coupling pathways correspond to $^2J_{\text{CN}}$ and $^3J_{\text{CN}}$ correlation responses, respectively. All of the expected $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ correlations based on the correlations observed in the ^1H - ^{15}N IMPEACH spectrum are observed in the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH correlation spectrum with the exception of a correlation for the 14-hydroxyl proton. The 14-hydroxyl proton is not directly bound to a ^{13}C resonance and hence cannot yield a correlation response in the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH correlation spectrum. The phase of the responses in the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH correlation spectrum is defined by the multiplicity-editing of the ^1H - ^{13}C HSQC spectrum. Responses correlating methylene carbons to nitrogen are inverted and displayed in red; responses correlating methine and methyl (none of the latter occur in the structure of vincamine) carbons to nitrogen are positive and plotted in black. It should also be noted that the 3-8 Hz optimized ^1H - ^{15}N IMPEACH spectrum of vincamine (**1**) contains several responses not observed in the 10 Hz optimized GHMBC spectrum previously reported.¹⁹

Figure 1.

Figure 1. The $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH correlation spectrum of vincamine (**1**) obtained *via* the unsymmetrical indirect covariance coprocessing is shown in the top right panel. The spectrum was derived from the multiplicity-edited ^1H - ^{13}C GHSQC (bottom right panel) and 3-8 Hz optimized ^1H - ^{15}N IMPEACH spectra (top left panel). The main body of the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH spectrum was plotted with a 3% threshold value. The boxed regions were plotted with a 0.7 % threshold to minimize t_1 noise in the F_1 frequency domain from the more intense correlation responses. The correlation from the 14-hydroxyl proton to the N1 indole nitrogen is not observed in the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH spectrum since this proton is not directly bound to a carbon resonance. The phase of responses in the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH is governed by the multiplicity-editing of the ^1H - ^{13}C GHSQC spectrum used in the unsymmetrical indirect covariance processing. Methylene resonances are plotted in red and have negative phase; methine and methyl (none of the latter afford responses in the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ spectrum of vincamine) have positive phase and are plotted in black.

Figure 2. Correlations observed in the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH spectrum of vincamine (**1**). The correlation from the 14-hydroxyl resonance (red arrow) is not observed in the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ correlation spectrum since this proton is not directly bound to a ^{13}C resonance.

Correlations observed in the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH correlation spectrum are summarized on the structure shown in Figure 2. In the context of the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HMBC-IMPEACH discussed below, it is worth noting that there are no correlations observed in the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH spectrum that link the two nitrogen resonances, which would be desirable if this were an unknown structure in the process of being elucidated.

$^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HMBC-IMPEACH

The absence of an intense correlation such as the 14-hydroxyl proton to the N1 resonance, in conjunction with a desire to experimentally access a larger segment of the

molecular structure prompted the exploration of the combination of ^1H - ^{13}C HMBC and ^1H - ^{15}N IMPEACH 2D NMR experiments *via* unsymmetrical indirect covariance processing methods. While we have employed ^1H - ^{15}N IMPEACH data set in this study, any long-range ^1H - ^{15}N correlation experiment can be employed.

A fundamental premise of calculating a $^{13}\text{C}\leftrightarrow^{15}\text{N}$ HMBC-IMPEACH correlation data matrix was to explore the transfer of long-range ^1H - ^{13}C connectivity information from a given proton resonance in the ^1H - ^{13}C HMBC to ^{15}N in the final $^{13}\text{C}\leftrightarrow^{15}\text{N}$ HMBC-IMPEACH spectrum. As an example, consider the extensive long-range ^1H - ^{13}C correlations observed for the 15-methylene AB spin system in the GHMBC spectrum of vincamine (**1**) summarized in Figure 3.

Examining the N1 chemical shift in the $^{13}\text{C}\leftrightarrow^{15}\text{N}$ HMBC-IMPEACH spectrum shown in Figure 4 (top right panel) we note that all of the long-range correlations anticipated (Figure 3) are indeed observed in the $^{13}\text{C}\leftrightarrow^{15}\text{N}$ correlation spectrum, including a correlation to the C3 resonance, which is pivotally located between the N1 and N4 resonances of vincamine, and thus capable of potentially providing the means of linking the two nitrogens in the carbon skeleton. A weak correlation is also observed for the C15 methylene resonance, which must be transferred to N1 *via* some long-range ^1H - ^{15}N coupling pathway, most probably a $^3J_{\text{NH}}$ coupling from H3 to N1. The weak correlations from C11 and C12 to N1 observed in Figure 1 are not observed in the $^{13}\text{C}\leftrightarrow^{15}\text{N}$ HMBC-IMPEACH spectrum shown in Figure 4.

By combining the long-range couplings of a ^1H - ^{13}C GHMBC experiment, which can routinely span two to four bonds, with those of a ^1H - ^{15}N IMPEACH experiment, which typically spans two or three bonds, an investigator has the means of visualizing

correlations across five or more bonds directly in the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HMBC-IMPEACH spectrum. In comparison, the same connectivity information can be indirectly extracted from the contributing 2D NMR spectra.

Figure 3. Long-range ^1H - ^{13}C correlations observed from the 15-methylene AB spin system in the ^1H - ^{13}C GHMBC spectrum of vincamine (**1**). ^1H - ^{13}C long-range correlations are denoted by black arrows; the correlation from $\text{C}15 \leftrightarrow \text{N}1$ is denoted by the red arrow.

Figure 4.

Figure 4. The $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HMBC-IMPEACH correlation spectrum of vincamine (**1**) obtained *via* the unsymmetrical indirect covariance coprocessing is shown in the top right panel. The spectrum was derived from the ^1H - ^{13}C GHMBC (bottom right panel – the C15 methylene correlations are within the red boxed region) and 3-8 Hz optimized ^1H - ^{15}N IMPEACH spectra (top left panel). The $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HMBC-IMPEACH correlation spectrum contains correlations to the N1 nitrogen resonance for all of the ^{13}C resonances to which the 15-methylene protons are long-range coupled. In addition, there are also correlations from C3, C14, and C16 to both of the nitrogen resonances of the vincamine (**1**) skeleton, which could be beneficial in the structural characterization of an unknown. In comparison with the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH spectrum shown in Figure 1, which affords $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ correlations across up to three bonds, the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HMBC-IMPEACH spectrum can span up to four bonds *via* the long-range ^1H - ^{13}C correlations in the GHMBC spectrum plus three and in some cases four additional bonds *via* the long-range ^1H - ^{15}N coupling pathways in the ^1H - ^{15}N IMPEACH spectrum providing experimental access across 6 or more bonds.

Figure 5. Long-range ^1H - ^{13}C (black arrows) and ^1H - ^{15}N (red arrows) correlation pathways observed in the GHMBC and IMPEACH-MBC spectra, respectively, of vincamine, **1**. Responses in the ^{13}C - ^{15}N HMBC-IMPEACH arise *via* the coherence transfer between proton and carbons in the GHMBC spectrum that are observed at the ^{15}N shift (IMPEACH) to which the proton in question is long-range coupled. In the case of $^{13}\text{C}15 \leftrightarrow ^{15}\text{N}1$ the correlation pathways are readily analyzed (Figure 3) since there is a single major ^1H - ^{15}N coupling. In the case of the correlations to the N4 resonance, however, there are multiple potential pathways through which the long-range connectivity information from the HMBC experiment can be transferred to the nitrogen in the ^{13}C - ^{15}N correlation spectrum.

Conclusions

The ^{13}C - ^{15}N HSQC-IMPEACH heteronuclear shift correlation data presented in Figure 1 should be readily and directly applicable in structure elucidation studies of alkaloids and other unknown, nitrogen-containing molecules and heterocycles. The interpretation of the heteronuclear chemical shift correlation responses observed in a $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-HMBC or $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HSQC-IMPEACH spectrum is straightforward. In contrast, it is more difficult to assess the potential utility of the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HMBC-IMPEACH correlation spectrum in structure elucidation problems because of the multiple potential correlation pathways that can lead to responses in the spectrum, for example the correlation responses to the N4 resonance shown in Figure 5. In the long term, the $^{13}\text{C} \leftrightarrow ^{15}\text{N}$ HMBC-IMPEACH heteronuclear shift correlation spectrum may be more readily applicable in the confirmation of a partially established. We are exploring potential applications of both types of experiments, which will serve as the basis of future reports. We are also continuing to explore other potential means of employing unsymmetrical indirect covariance processing methods to indirectly determine $\text{B} \leftrightarrow \text{C}$ coherence pathways from more readily measured $\text{A} \rightarrow \text{B}$ and $\text{A} \rightarrow \text{C}$ coherence transfer experiments.

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