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Rapid Analysis of Organic Compounds by Proton-Detected Heteronuclear Correlation NMR Spectroscopy at 40 kHz Magic-Angle Spinning**

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Keywords

analytical methods; drugs; solid-state NMR spectroscopy; pharmaceuticals; natural abundance

High-resolution magic-angle spinning (MAS) solid-state NMR (SSNMR) spectroscopy is a powerful tool for analyzing the structural and dynamical properties of organic compounds; for example, unique insights into solid phase polymorphism/pseudo-polymorphism, hydrogen bonding, and stereoisomerism have been achieved,[1,2] and these results apply to important classes of pharmaceutical solids in both pure and dosage forms.[3] Most studies have employed simple one-dimensional ^{13}C spectra, assigning resonances in comparison to solution state spectra.[3,4] Unambiguous assignments are usually not possible when the solid and solution spectra differ substantially. Spectral editing experiments, which distinguish carbon types (C, CH, CH_2 , CH_3), aid in confirming some assignments but are not always reliable, for example when significant molecular dynamics are present.[3]

Two-dimensional techniques—as commonly employed for isotopically enriched materials in the solid state or at natural abundance in the solution state—greatly accelerate data interpretation on the basis of improved spectral resolution and rich spin connectivity information. For example, a ^{13}C - ^{13}C refocused INADEQUATE spectrum of oxybuprocaine hydrochloride was utilized to assign resonances to two molecules in the asymmetric unit.[5] However, that experiment required 5 days of data collection and 80 mg of material, fairly representing the sensitivity challenge presented by the low natural ^{13}C abundance. Heteronuclear correlation (HETCOR) experiments involving ^1H and rare nuclei ^{13}C or ^{15}N enhance sensitivity for resonance assignments (e.g. one day for ~20 mg of sample).[6,7] Inverse proton detection at 30 kHz MAS rate has been demonstrated on a polymer and a ^{13}C -labeled heptapeptide, with sensitivity enhanced by approximately 2-3 times.[8] At even faster MAS rates (~40 kHz) with probes optimized for ^1H sensitivity, we have shown further improvements in sensitivity and proton resolution,[9,10] as recently applied to natural abundance peptide and protein pharmaceuticals.[11] Using the same method, Pruski and coworkers have also dramatically accelerated data collection for surface-bound molecules.[12]

Here we apply proton-detected 40 kHz MAS HETCOR experiments on organic compounds for rapid data acquisition (30 m) with small sample quantities (~6 mg, consisting of <4 mg of active pharmaceutical ingredient (API)). The reduced sample volume (8 μl) of 1.6-mm rotors is compensated by a high filling factor of a small sample coil, resulting in sensitivity

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comparable to that observed with much larger sample quantities.[9] In addition to facilitating proton detection, fast MAS results in spinning-side-band free spectra, which are easier to interpret; therefore analysis of resonance assignments, polymorphism, dynamics, and hydrogen bonding is readily achieved. Demonstrations here include tablet formulations of ibuprofen and acetaminophen; using our recent solvent suppression technique MISSISSIPPI, [13] sufficient attenuation of water and other unwanted proton signals is achieved so that the results will readily translate to hydrated formulations such as creams, ointments, and suspensions.[11]

Ibuprofen, a widely used nonsteroidal anti-inflammatory drug, has known three-dimensional crystal structure[14] and is frequently used as a model compound to study formulation impacts on physical and chemical properties of the drug.[15-17] Its ^1H and ^{13}C resonance assignments have been completed only recently with the utilization of ^1H - ^{13}C HETCOR experiments, which have also been proven very powerful in discerning acid and sodium forms of ibuprofen.[15] However, long data acquisition from three to six days was necessary for these ^{13}C -detected HETCOR experiments with over 20 mg of sample.[15]

With the sensitivity gain offered by ^1H -detection, we have acquired a high-quality ^{13}C - ^1H HETCOR spectrum (Figure 1) in 33 min on 6 mg ibuprofen formulation, which contains less than 4 mg of the active pharmaceutical ingredient (API). Excipients do not interfere with the API peaks, since the former are observed in the chemical shift range consistent with carbohydrates; the excipients also have a significantly longer T_1 relaxation in this instance. [17] Signal to noise ratios (SNR, shown as average $\pm$ standard deviation) are 34 ± 22 (or 37 ± 22 without linear prediction) for all ibuprofen peaks and 46 ± 19 (or 48 ± 18 without linear prediction in the indirect dimension) for one- and two-bond correlation peaks.

This HETCOR spectrum facilitates straightforward assignments of all carbon and proton nuclei in ibuprofen, in detail as follows. The peak at (185.2, 12.7) ppm is assigned to the carboxylic group (labeled *a*). Proton frequencies of *b* and *c* are 2.3 and 0.7 ppm, respectively, corresponding to the nearby protons attached to the neighboring methine and methyl groups. Methine carbon *b* can then be located at 46.0 ppm. The peak at 47.8 ppm seems to have similar proton frequency, but close inspection of the data reveals that it consists of two broad components (roughly at 1.3 and 2.7 ppm), consistent with the strongly coupled CH_2 group *j*. Upfield in the ^1H dimension from the strong peak *b*, a correlation to proton *c* resonance is also observed. Peaks with ω_1 at 17.3 and 23.9 ppm correlate also to methyl ^1H resonances at ~ 0.7 ppm; the $\omega_1 = 17.3$ ppm resonance is also coupled to $\omega_2 = 2.3$ ppm (^1H frequency *b*), confirming that the peak at (17.3, 0.7) ppm is due to methyl group *c*. The two remaining methyl groups *l* and *m* are assigned to peaks (26.9, 0.9) and (23.9, 0.7) ppm, and the methine group *k* is to (35.6, 1.8) ppm according to the typical tertiary carbon chemical shifts. The aromatic quaternary carbon *i* at 144.1 ppm is identified with connectivity to *j* proton frequencies; carbon *i* also correlates to neighboring aromatic protons *g* and *h* as a single unresolved peak at (144.1, 7.0) ppm. Carbons *g* and *h* are then located at 134.4 and 131.0 ppm based on the resemblance of their proton resonance position and lineshape to those of peak *i*. The other aromatic quaternary carbon *d* at 139.1 ppm is identified from the two correlation peaks with *b* and *c* proton frequencies; carbon *d* also correlates to neighboring aromatic protons *e* and *f* as two equally strong and partially resolved peaks at (139.1, 7.7) and (139.1, 6.9) ppm. Carbons *e* and *f* are located at 132.7 and 128.7 ppm according to the same proton resonance doublet lineshape as with carbon *d*.

Important dynamics information can also be extracted from the HETCOR spectrum. The aromatic ring carbons *e* (*g*) and *f* (*h*) are magnetically distinct, consistent with a rigid ring on the chemical shift timescale (~ 1 ms). Furthermore, carbon *e* (or *f*) correlates to both *e* and *f* proton frequencies with nearly equal peak intensities, consistent with an exchange process

which is slow on the chemical shift timescale, but faster than the timescale of longitudinal storage (270 ms) of ^{13}C magnetization during MISSISSIPPI suppression.[13,18] The possibility of millisecond exchange that may occur during evolution periods can be excluded by the lack of any significant motional broadening to the ^{13}C and ^1H resonances.[18] It is also interesting to notice that methyl groups *l* and *m* have much broader linewidths than methyl *c* (1300 versus 700 Hz, including 200 Hz applied broadening), indicating that rotation around the three-fold symmetry axis is more restricted for *l* and *m* than *c*.

Another benefit of proton NMR is the direct insight into hydrogen bonding information derived from the proton chemical shift.[1] For O-H $\cdots$ O type hydrogen bonds, a linear correlation between the hydrogen bond distance $r_{\text{H}\cdots\text{O}}$ (Å) and the isotropic chemical shift δ was demonstrated using crystallographic and NMR data assembled by Jeffrey and Yeon,[19]

$$r_{\text{H}\cdots\text{O}} = 2.210 (\pm 0.005) - 0.044 (\pm 0.004) * \delta \quad \text{Equation 1}$$

where δ is the ^1H chemical shift in ppm. From this equation, $r(\text{H}\cdots\text{O}) = 1.65 \pm 0.06$ Å is calculated for the 12.7 ± 0.15 ppm proton chemical shift, agreeing with the 1.664 ± 0.010 Å hydrogen bond across the carboxylic acid dimer by neutron diffraction (Figure 1b).[14]

Among the three polymorphs of acetaminophen (i.e. paracetamol), the monoclinic form is chosen for drug formulation due to its thermodynamic stability.[20] Between monoclinic and orthorhombic forms, the Raman spectra are identical, but differences are observed in the infrared and ^{13}C NMR spectra.[21] Nevertheless, the one-dimensional ^{13}C spectra did not allow aromatic carbons to be specifically assigned,[21] and ^{13}C -detected HETCOR spectra were time prohibitive due to the very long proton T_1 (63 s) in this tablet formulation (in contrast with 1.7 s for the ibuprofen sample above). With ^1H detection, we acquired a HETCOR spectrum of adequate SNR (20 ± 8 excluding 177 for the strong methyl peak; or 21 ± 7 excluding 153 for the methyl without linear prediction in the indirect dimension) with only two scans per t_1 increment and a recycle delay of 35 s (Figure 2a). In the scenario of multiple samples with long T_1 values, we envision combinations of the high-sensitivity proton detection with multiple MAS module probe design.[22]

Assignments of all carbon and proton nuclei in acetaminophen can also be readily made with the HETCOR spectrum. In the spectrum, the (25.6, 1.1) ppm peak is readily assigned to the methyl group *a*. The resonance at 171.5 ppm is assigned to carbonyl carbon *b*, which can be confirmed by a cross peak with the methyl proton. Amide proton is then assigned with 9.0 ppm from the cross peak with the carbonyl carbon. The aromatic quaternary carbon *c* is found at 134.9 ppm with correlation to the amide proton as well as *d* and *e* protons at 8.0 and 6.7 ppm. Carbons *d* and *e* are found at 134.9 and 122.3 ppm. Then the peak (154.1, 9.5) ppm with low field proton shift is assigned to the correlation between the other quaternary carbon *h* and the hydroxyl proton. Carbon *h* also correlates to two other protons *f* and *g* at 6.7 and 5.6 ppm. Carbons *f* and *g* are then assigned to 117.5 and 118.2 ppm. Peaks at (122.3, 6.7) and (117.5, 6.7) ppm have degenerate proton chemical shifts, therefore, an assumption has been implied above that carbons *f/g* resonate at higher field than *d/e* since the former are second neighbors to the hydroxyl group. The observation that carbon *d* (*f*) does not correlate with proton *e* (*g*) indicates that ring flipping is much slower than in ibuprofen.

Hydrogen bonds play a significant role in acetaminophen polymorphism.[23] The hydrogen bond distance 1.79 ± 0.05 Å predicted using Eq. 1 from the 9.5 ppm chemical shift agrees very well with a single-crystal X-ray diffraction structure, where 1.77 Å is measured between the hydroxyl proton and the carbonyl oxygen of a neighboring molecule (Figure 3).[23] In a neutron diffraction structure this hydrogen bond is 1.693 Å,[24] but the sample differences

(different unit cell parameters and space group from the X-ray study) may also be responsible, in addition to accuracy difference of these techniques. For comparison, in the orthorhombic form the hydrogen bond is 1.84 Å (Cambridge Structural Database code HXACAN08).[23] Other intermolecular contacts can be revealed by further signal accumulation (Figure 2b). Proton *a* also correlates to *c* and *h* carbons (Figure 2b), where distance of 3.2 and 3.4 Å, respectively, are found in the structure (Figure 3). In addition, a three-bond intra-molecular correlation is observed at (25.6, 9.1) ppm between carbon *a* and the amide proton (2.5 Å apart).

Notably, under fast MAS the majority of correlations arise from one-bond dipolar transfers, sharing the selectivity offered by the J-coupling mediated MAS-J-HMQC spectra with a short evolution period.[6,7] Fast MAS dramatically reduces the effective ^1H - ^1H dipolar couplings, separating CH_n groups from other protons during cross polarization (without the need for homonuclear decoupling).[25] Meanwhile, quaternary carbons correlate to protons two and three bonds away in the same spectrum. This feature has been essential in assigning aromatic resonances. In contrast, a separate MAS-J-HMQC experiment is necessary to establish multiple-bond correlations by using a very long evolution period, leading to significant signal loss.[6,7] Longer-range correlations are very weak in fast-MAS HETCOR and can only be observed with additional signal accumulation. When long range contacts are sought, for instance, for the determination of atomic resolution structure,[26] they can be readily reestablished by inserting a ^1H - ^1H dipolar recoupling element in the pulse sequence.[13]

As an alternative to fast-MAS HETCOR, proton double-quantum homonuclear correlation spectroscopy has recently been used to distinguish anhydrous and hydrous forms of a certain drug ingredient.[27] However, the CRAMPS technique (combined rotation and multiple-pulse spectroscopy) used there to narrow the proton linewidths also compromises signal-to-noise ratio as a result of the large receiver bandwidth and low probe quality factor. Moreover, CRAMPS experiments are difficult to calibrate and often show substantial variations in quality among samples. For these reasons, CRAMPS-based proton-detected HETCOR spectra of natural abundance samples have not been demonstrated, to the best of our knowledge. Meanwhile, the proton homonuclear spectra (e.g. 2 hours on 30 mg samples, recycle delay 2 s)[27] correlating abundant protons are comparable in sensitivity to the HETCOR spectra shown here, correlating protons with rare heteronuclei (^{13}C and ^{15}N), which have the benefit of very large chemical shift range. We expect the fast-MAS HETCOR experiments to have adequate resolution for molecules up to 500 Da, for which experiments can be conducted in 3 hours given a favorable proton T_1 . For even larger molecules (e.g. 1 kDa), we envision 3D experiments such as ^{13}C - ^1H - ^1H to provide additional resolution; such an experiment could be completed in a day, and the indirect proton dimension could be further improved in resolution by application of homonuclear decoupling, while the direct proton dimension would rely on fast MAS alone for high sensitivity.

Experimental Section

Sample Preparation

A 310 mg ibuprofen tablet (Major Pharmaceuticals, Livonia, Michigan) containing 200 mg API was crushed and 6 mg was packed in a 1.6 mm NMR sample rotor. A 350 mg acetaminophen tablet (Ivax Pharmaceuticals Inc., Miami, Florida) containing 325 mg API was crushed and 6 mg was packed.

NMR Experiments

The SSNMR experiments were performed with 40 kHz MAS rate on a 750 MHz Varian INOVA spectrometer (Varian, Inc., Palo Alto, CA) with a FastMASTM ^1H - ^{13}C - ^{15}N probe (Varian, Inc., Fort Collins, CO). Sample temperature was regulated at 11 °C; details of variable

temperature setup and calibration have been reported earlier.[13] The heteronuclear correlation pulse sequence employed MISSISSIPPI technique to filter out background proton signals. [13] Contact times of 1.6 and 0.5 ms were used for the ^1H to ^{13}C and ^{13}C to ^1H cross polarization steps, respectively. Other experimental details are listed in figure captions. Chemical shifts were referenced indirectly to 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS) by setting the downfield ^{13}C resonance of adamantane to 40.48 ppm.[28] Note that the ^{13}C chemical shifts in DSS scale are 2.00 ppm larger than in the tetramethylsilane (TMS) scale, while the ^1H shifts are identical in both scales.[28]

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Figure 1.

HETCOR experiment on dosage form ibuprofen containing 3.8 mg active pharmaceutical ingredient (API). (a) ^{13}C - ^1H 2D spectrum acquired in 33 min, 2 scans per row, recycle delay 3 s, $t_{1\text{max}}(^{13}\text{C}) = 3.85$ ms, with forward linear prediction to 7.7 ms, $t_{2\text{max}}(^1\text{H}) = 5$ ms. Lorentzian-to-Gaussian apodization was applied (200-to-400 Hz for ^1H , 20-to-40 Hz for ^{13}C) followed by sine bell apodization (with a 54° shift) for each dimension. Contours are drawn starting at 6 times the noise level, with a spacing factor 1.4. (b) Ibuprofen molecules showing labels and hydrogen bond length across the dimer (Cambridge Structural Database code IBPRAC02).[14]

Figure 2.

HETCOR experiment on dosage form acetaminophen containing 5.5 mg API. (a) ^{13}C - ^1H 2D spectrum acquired in 6 h with 2 scans per row, recycle delay 35 s. (b) ^{13}C - ^1H 2D spectrum acquired in 18 h with 6 scans per row. Other parameters are the same as in Figure 1. The inset of (a) shows the molecule with labels.

Figure 3. Monoclinic acetaminophen in a unit cell. Viewed along the *a* axis; *b*-axis is horizontal, *c*-axis is vertical (Cambridge Structural Database code HXACAN07).[23]