Journal of Magnetic Resonance 206 (2010) 157–160

Contents lists available at ScienceDirect

Journal of Magnetic Resonance

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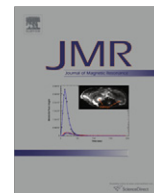

Communication

^{1}H–^{14}N HSQC detection of choline-containing compounds in solutions

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ARTICLE INFO

Article history:
Received 7 May 2010
Available online 9 June 2010

Keywords:
^{1}H–^{14}N HSQC
Choline
Solution
Milk

ABSTRACT

Choline nitrogen (^{14}N) has a long relaxation time (seconds) which is due to the highly symmetric chemical environments. ^{14}N in choline also has coupling constants with protons (0.6 Hz to methyl protons, 2.7 Hz to CH_2O protons and 0.2 Hz to NCH_2 protons). Based on these properties, we introduce a two-dimensional NMR method to detect choline and its derivatives in solutions. This method is the ^{1}H–^{14}N hetero-nuclear single-quantum correlation (HSQC) experiment which has been developed in solid-state NMR in recent years. Experiments have demonstrated that the ^{1}H–^{14}N HSQC technique is a sensitive method for detection of choline-containing compounds in solutions. From 1 mM choline solution in 16 min on a 500 MHz NMR spectrometer, a ^{1}H–^{14}N HSQC spectrum has been recorded with a signal-to-noise ratio of 1700. Free choline, phosphocholine and glycerophosphocholine in milk can be well separated in ^{1}H–^{14}N HSQC spectra. This technique would become a promising analytical approach to mixture analyses where choline-containing compounds are of interest, such as tissue extracts, body fluids and food solutions.

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In recent years, the nitrogen isotope ^{14}N has shown renewed interest in solid-state NMR [1], since ^{14}N has a natural abundance of 99.6% and nitrogen is one of the most important atoms in biomolecules. Gan et al. [2–4] and Cavadini et al. [5–8] have developed indirect methods for detecting ^{14}N in amino acids and small peptides based on the one-bond couplings between ^{14}N and ^{13}C and between ^{14}N and ^{1}H. These methods include hetero-nuclear multiple- and single-quantum correlation (HMQC and HSQC) techniques, which have shown promising applications in biomolecular studies. Unfortunately, so far these techniques have not been applied to solution NMR. The dominant reason should be the fast relaxation of ^{14}N in common biomolecules in solutions. However, ^{14}N in choline stands out, since the nitrogen atom sits at an environment with high symmetry. It has been shown that the relaxation time of ^{14}N in choline is as long as 3.88 s [9].

Choline is one of the most important small molecules in biology. It is an essential nutrient for human health. It is the basic construction unit of cell membranes. It is also the precursor of the messenger molecule acetyl choline. There has been clear need for fast and accurate detection of choline and its metabolites in solutions, as they can foretell biochemical changes in human body, which are believed to be related to cancer development [10,11]. Therefore, advanced NMR techniques such as dynamical nuclear polarization (DNP) has been proposed for choline detection [12–14]. In this communication, we introduce the newly developed spin-1/2–spin-1 HSQC method [1–8,15] for choline detection. We demonstrate that, by applying this experiment to the long-range coupling (^2J and ^3J) networks between ^{1}H and ^{14}N in choline, choline can be detected in minutes from solutions with the concentration of 10^{-3} molar and lower.

The ^{1}H–^{14}N HSQC experiment used in this study is somewhat different from those used in solid-state NMR [3,8]. Since the spin–spin interactions in solutions are much simpler than in solid states, the conventional spin-1/2–spin-1/2 HSQC pulse program [16] can be adopted directly without any modifications. However, theoretically the INEPT [17] evolution time τ should be $1/8J$ instead of $1/4J$, where J is the coupling constant. The principle of this experiment has been well explained [1–8], but is briefly mentioned here. Due to the coupling to ^{14}N, the magnetization vector of proton is split into three components: a centre component (affiliated to the $|0>$ spin state of ^{14}N) and two side ones (affiliated to the $|\pm1>$ spin states of ^{14}N). After excited by a ^{1}H $\pi/2$ pulse, in the proton rotating frame with the rotating frequency of the centre component, the two side components rotate in opposite directions, while the centre component remains still. After the INEPT 2τ evolution, the two side components become antiphase in the xy plane. At this moment, another ^{1}H $\pi/2$ pulse is applied in the direction of the centre component and turns the two side components into the z-direction. In this way the magnetization of proton can be successfully transferred to ^{14}N by a ^{14}N excitation pulse. By neglecting

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doi:10.1016/j.jmr.2010.06.003

the centre component, the theory of spin-1/2–spin-1 HSQC is exactly the same as the theory of spin-1/2–spin-1/2 HSQC [16].

It is known that ^{14}N in choline is coupled to all of the three groups of protons [18,19]: to the CH_2O methylene protons with ^3J = 2.7 Hz, to the methyl protons with ^2J = 0.6 Hz and to the NCH_2 methylene protons with ^2J = 0.2 Hz. With the very long relaxation time (T_1 = 3.88 s) [9], in NMR experiments ^{14}N in choline should behave as if it were a spin-1/2 nucleus. Thus, the ^{1}H–^{14}N HSQC experiments on choline would be much like the HSQC experiments on long range (or multiband) ^{1}H–^{13}C coupling pairs. It should be feasible and its sensitivity should be comparable to the ^{1}H–^{13}C hetero-nuclear multi-bond connectivity (HMBC) experiments on ^{13}C enriched samples [20].

In the first test of the ^{1}H–^{14}N HSQC experiment we used a rather concentrated (20 mM) choline solution, fearing that the sensitivity of the experiment might be low, because the coupling constants are so small. However, it turned out that the experiment on 20 mM sample was very sensitive. With the adjustment of the INEPT evolution time τ, all three groups of protons (CH_3, NCH_2 and CH_2O) were easily detected, as can be demonstrated by the first slices of the experiments shown in Fig. 1.

From Fig. 1 we can see how the signal intensities varied with the adjustment of τ. The maximum of the CH_2O signal at 4.051 ppm appeared when τ was between 31.3 and 41.7 ms. The maximum of the methyl signal at 3.19 ppm appeared when τ was around 167 ms. The NCH_2 signal at 3.507 ppm appeared when τ was beyond 250 ms and the signal was too weak to call for attention. The optimal τ values for maxima of the signals were apparently shorter than the theoretical values of 1/8J, which are 46 ms for the CH_2O signal (^3J = 2.7 Hz) and 208 ms for the CH_3 (^2J = 0.6 Hz). This effect should be due to the transverse relaxation decay during the INEPT evolution, which has been well known in protein NMR [21].

Almost with all τ values tested, the nine methyl protons, though with smaller coupling constant with ^{14}N (^2J = 0.6 Hz) than the CH_2O protons (^3J = 2.7 Hz), were more easily detected than the CH_2O protons. There are two reasons for CH_3 being more sensitive: (1) the methyl protons have the outnumbering advantage; (2) the methyl signal has a much narrower line width than the CH_2O signal, since the CH_2O signal is split into multiplets from homonuclear coupling with the NCH_2 protons (7 Hz) and between the two protons in CH_2O (3 Hz) [18,19].

We then performed a ^{1}H–^{14}N HSQC experiment on a 1 mM choline sample with τ = 167 ms. The spectrum recorded with 16 min measurement time is presented in Fig. 2, which shows very high signal-to-noise ratio (SNR = 1700:1). ^{14}N is a low-γ spin. Although ^{14}N NMR is accessible to hundreds of μM choline [9], the indirect detection of ^{14}N through ^{1}H can enhance the detection sensitivity by a factor of 712, since $\gamma(^1\text{H})/\gamma(^{14}\text{N})$=13.84 and NMR sensitivity is dependent on $\gamma^{5/2}$. The ^{1}H–^{14}N HSQC experiment is basically a ^{1}H NMR experiment and the detection limit can go down as low as nanomolar concentrations. Furthermore, the detection sensitivity using this method is effectively enhanced by the outstanding filtering effect of the INEPT pulse sequence and the pulsed field gradients, which cleanly suppress unwanted signals including the water signal. The very high sensitivity of the ^{1}H–^{14}N HSQC as demonstrated in Fig. 2 suggests that this technique can find wide applications.

As an example of application, we present in Fig. 3 the ^{1}H–^{14}N HSQC spectra on a milk sample, which was prepared by diluting 1 part of saturated solution of milk powder (Nestle, Switzerland, purchased locally in Wuhan, China) into seven parts of D_2O, so that the D_2O content was about 90% in volume. In Fig. 3, A was recorded with τ = 83 ms for optimizing the detection of the methyl signal, and B was recorded with τ = 18 ms to favor for the methylene signal. The τ values for this milk sample were much smaller than the τ values for the pure choline samples, meaning that the proton relaxation times in milk were shorter than those in pure choline solutions. While the conventional ^{1}H spectrum of milk is dominated by lactose signals and the choline signals are hardly distinguished [22] (also see the ^{1}H spectrum in the projection of Fig. 3), with ^{1}H–^{14}N HSQC we were able to selectively detect choline in a clean manner.

In Fig. 3A, two methyl-^{14}N correlation peaks can be clearly distinguished, which are slightly different in both ^{1}H (differing by 0.028 ppm) and ^{14}N (differing by 0.2 ppm) dimensions. Assignment of these two peaks can be achieved with the help of the methylene-optimized ^{1}H–^{14}N HSQC spectrum in Fig. 3B and the reported chemical shifts of choline (Cho), phosphocholine (PCho) and glycerol-phosphocholine (GPCho) [18,23–25]. Since the ^{1}H signal at

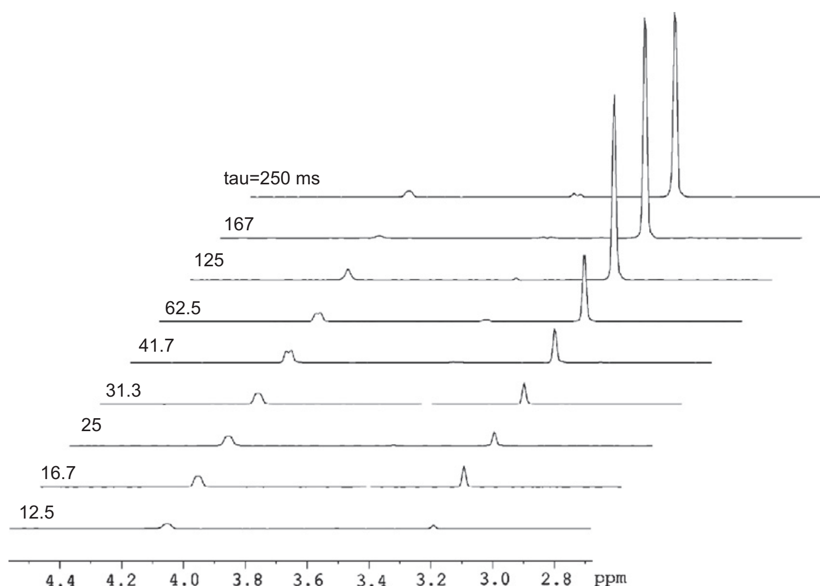

Fig. 1. The first slices (with two scans for each) of ^{1}H–^{14}N HSQC spectra of 20 mM choline with varied τ changing from 12.5 to 250 ms. The maximum of the CH_2O signal at 4.051 ppm appeared when τ was between 31.3 and 41.7 ms. The maximum of the methyl signal at 3.19 ppm appeared when τ = 167 ms. The NCH_2 signal at 3.507 ppm appeared when τ was beyond 250 ms.

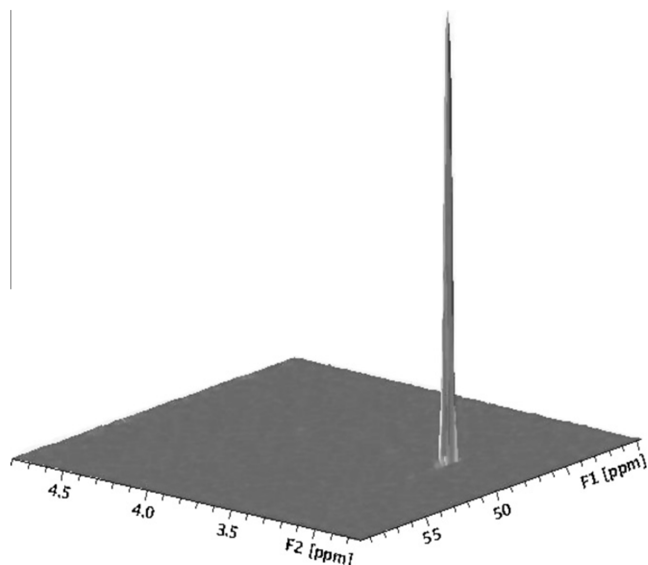

Fig. 2. $^1H–^{14}N$ HSQC spectrum (SNR = 1700:1) of 1 mM choline solution recorded with τ = 167 ms and 8 scans for each FID on a Bruker Avance 500 spectrometer. The total experimental time was 16 min.

4.051 ppm, which should stem from CH_2O in free choline, correlates with the ^{14}N resonance at 48.1 ppm in Fig. 3B, the chemical shift of ^{14}N resonance of free choline in milk should be 48.1 ppm. In Fig. 3B two other ^1H signals at 4.165 and 4.317 ppm simultaneously correlate with the ^{14}N resonance at 48.3 ppm. The ^1H signal at 4.165 ppm is the typical CH_2O signal of PCho [23–25] and the ^1H signal at 4.317 is the typical CH_2O signal of GPCho [18,23,25]. Therefore, the ^{14}N peak at 48.3 ppm should be assigned to the overlapping of PCho with GPCho. From Fig. 3B it can also be seen that the PCho signal has slightly large chemical shift in ^{14}N than the GPCho signal, but the complete separation in the ^{14}N dimension requires more t_1 increments.

In cancer studies, the methyl signals of PCho and GPCho need to be resolved as their intensity change can be used for assessing tumor progression [11,23]. While separation of the methyl signals of PCho and GPCho requires very high resolution (their ^1H resonances differ only by 0.009 ppm, being prone to overlap), the CH_2O signals of PCho and GPCho are well separated (their ^1H resonances differ by 0.15 ppm, see discussion above). Therefore, $^1H–^{14}N$ HSQC experiments with shorter τ is particularly useful for distinguishing the PCho and GPCho signals. However, relating the volume of the CH_2O signals to the concentration needs caution, because the CH_2O signal volume is not only affected by the transverse relaxation decay, but also affected by the interspin *J*-modulation ($^1H–^1H$ coupling and $^1H–^{31}P$ coupling) during the INEPT evolution. Discussion about the quantitative analyses needs more space and is not reported here.

The spectra in Fig. 3 were from a 90% D_2O solution. There would be a concern if the $^1H–^{14}N$ HSQC technique is feasible for real milk samples, where D_2O can hardly reach 90% and water suppression would be difficult. Then we test a real milk sample of the 2% fat or the fat-reduced milk (Friendly Farms, USA, purchased locally in Cleveland, USA) without D_2O added. The $^1H–^{14}N$ HSQC spectra are displayed in Fig. 4, where the plot region includes the water resonance so that the quality of water suppression can be appreciated. It is seen that even without lock substance in the solution, there is no problem of water suppression, and the quality of the spectra does not look worse than the spectra of the "artificial milk" in Fig. 3.

In all milk samples tested, we did not find the $^1H–^{14}N$ correlation signals of phosphatidylcholine (PDCho) and sphingo-myelin (SM), which should be contained in milk. This is understandable, because PDCho and SM have much larger molecular size than PCho and GPCho, and ^{14}N relaxation in PDCho and SM must be so fast as to impair their HSQC detection. It has been shown [9] that ^{14}N relaxation time is very sensitive to molecular size; the ^{14}N relaxation time decreases quick as the molecular size increases (for Cho, T_1 = 3.88 s, for PCho, T_1 = 1.14 s and for GPCho T_1 = 0.49 s). When the relaxation times are shorter than the INEPT evolution time τ, the $^1H–^{14}N$ HSQC technique will no longer be feasible.

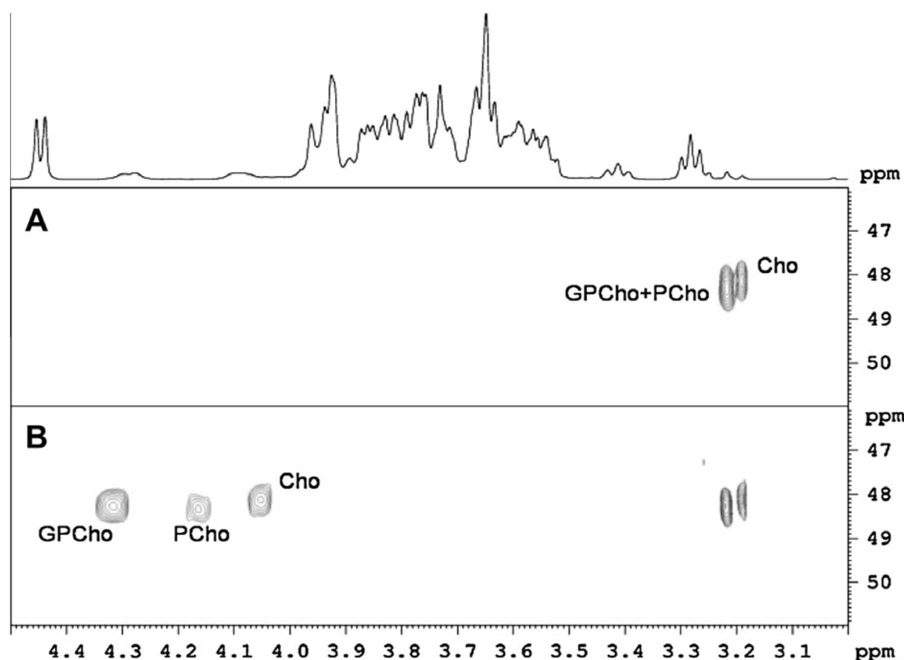

Fig. 3. $^1H–^{14}N$ HSQC spectra of some milk powder in 90% D_2O recorded with τ = 83 ms for A (8 scans, 13 min in total) and τ = 18 ms for B (48 scans, 66 min in total). While the spectrum in (A) shows two methyl peaks, the spectrum in (B) shows two methyl peaks and three methylene peaks. These peaks can be assigned to free choline (Cho), phosphocholine (PCho) and glycerophosphocholine (GPCho). Experiments were run on a Bruker Avance 500 spectromter.

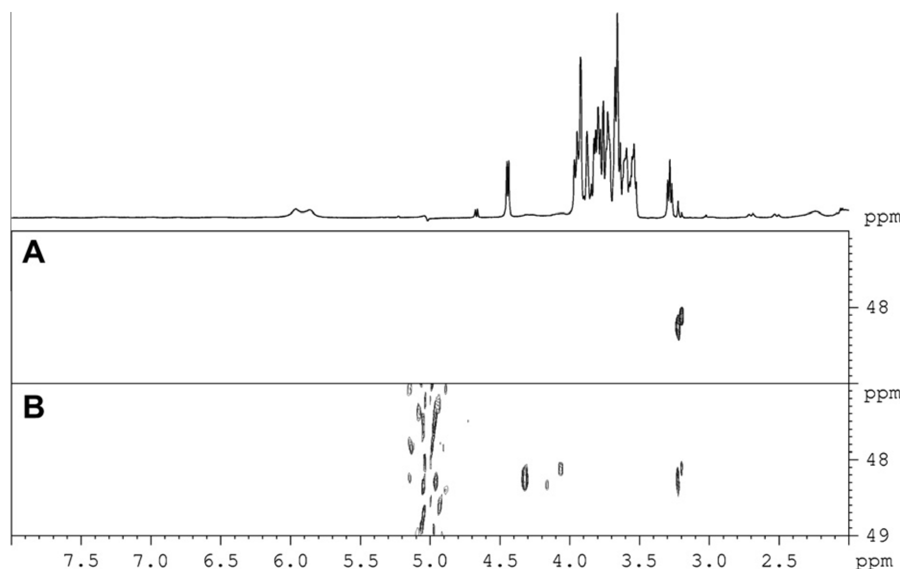

Fig. 4. ^1H–^{14}N HSQC spectra of fat-reduced milk with τ = 89 ms for A (8 scans, 13 min in total) and τ = 10 ms for B (16 scans, 21 min in total). The sample was shimmed by monitoring the water resonance. The information from these spectra is principally the same as the information from Fig. 3. Experiments were run on a Bruker Avance 600 spectrometer.

In conclusion, ^1H–^{14}N HSQC experiment is introduced to solution NMR. It has been shown that ^1H–^{14}N HSQC is a sensitive detection method for choline, phosphocholine and glycerophosphocholine in solutions despite the small ^2J(0.6 Hz) and ^3J(2.7 Hz) ^1H–^{14}N coupling constants in these compounds. Meanwhile, the ^1H–^{14}N experiment is versatile for methyl and methylene detections. Longer τ favors for the methyl detection, while shorter τ favors for the methylene detection. Milk was used for testing example in this work. However, the application of ^1H–^{14}N HSQC is not limited to milk analyses. Because of its sensitivity and high selection, this technique should be able to be developed to a useful analytical tool for mixture analyses where choline-containing compounds are of interest, particularly biological mixtures such as body fluids, extracts of tissues including cancer tissues and food solutions.

Acknowledgments

The authors thank National Natural Science Foundation of China (#20921004 and #90813017) and National Major Basic Research Program of China (#2009CB 918603) for financial support.

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