J|A|C|S COMMUNICATIONS

Published on Web 11/19/2010

A Selective NMR Method for Detecting Choline Containing Compounds in Liver Tissue: The $^{1}H-^{14}N$ HSQC Experiment

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Abstract: The feasibility of a $^{1}H-^{14}N$ HSQC experiment on tissues is demonstrated with a mouse liver based on the *J* couplings between the protons and the quadrupolar nucleus ^{14}N in choline. Free choline, phosphocholine, and glycerolphosphocholine $^{1}H-^{14}N$ HSQC signals were selectively observed with all unwanted signals cleanly suppressed. The CH_2O signals were well resolved in the two-dimensional spectrum, which can be used for quantitative analyses.

Based on *in vitro*, *ex vivo* NMR and *in vivo* MRS on tissues, choline (Cho) and its derivatives, phosphocholine (PCho) and glycerophosphocholine (GPCho), have been suggested as biomarkers for cancer diagnoses.[1−3] Much progress has been achieved, particularly in the diagnoses of breast cancer and prostate cancer;[2−7] however, for cancer diagnoses of other tissues, this method has not been well established and investigation is still undergoing. As far as the ^{1}H MRS (which has the highest sensitivity) is concerned, the signals (*i.e.*, the methyl signals) of these choline containing compounds (CCC) are poorly resolved from each other and not even able to be resolved from other metabolite signals. Take human liver for example. The CCC methyl signals (δ3.20−3.23) are closely flanked by the glucose and the trimethyl amine oxide signals (δ3.24−3.27),[8,9] and it is very difficult to quantify the CCC concentrations using ^{1}H MRS. In ^{31}P MRS, which has a much lower sensitivity than that of ^{1}H MRS but has also gained much attention, the CCC (with the choline signal missing) signals are overlapped with other phosphomonoester (PME) and phosphodiester (PDE) signals.[10] These difficulties prevent CCC from becoming biomarkers for liver cancer diagnoses. However, these difficulties can be overcome by a simple NMR method, which is the two-dimensional (2D) $^{1}H-^{14}N$ HSQC (heteronuclear single quantum correlation) experiment developed in solid state NMR[11,12] and introduced to solution NMR just recently.[13] In the $^{1}H-^{14}N$ HSQC spectra, Cho, PCho, and GPCho signals are well resolved and quantification for each species is reliable. In this communication we demonstrate this method with a mouse liver as an *ex vivo* example, which should be able to be duplicated in clinical (*in vivo*) MRS experiments.

A 2D $^{1}H-^{14}N$ HSQC spectrum with INEPT $\tau = 12.5$ ms of the mouse liver is presented in Figure 1, which was acquired on a Bruker Avance 600 spectrometer with 64 Δt_1 for $\Delta\delta$6 in the indirect dimension using the pulse sequence as mentioned in Supporting Information Part I. In order to prevent possible biochemical reactions, the experiments were performed at 4 °C. For each t_1 32 transients were averaged. The assignments for the peaks are straightforward.[13] In the water presaturated 1D spectrum, which is also shown in Figure 1, these peaks are poorly distinguished. However, in the 2D map these peaks are well resolved, particularly the CH_2O methylene signals. All other unwanted signals have been cleanly removed from the spectrum. Even the water peak residues are observed only at much lower levels.

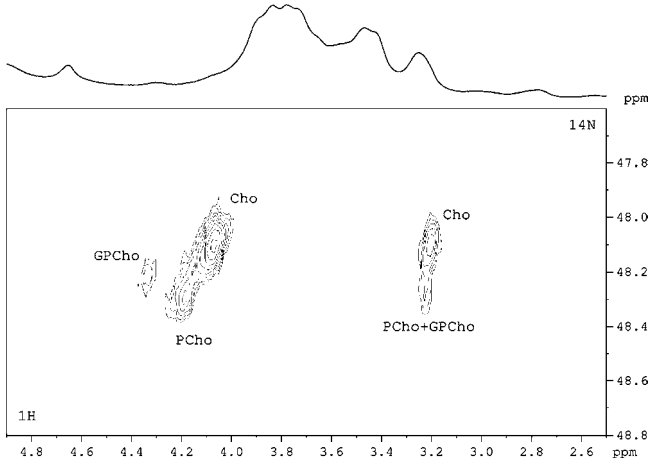

Figure 1. Two-dimensional $^{1}H-^{14}N$ HSQC spectrum recorded with INEPT $\tau = 12.5$ ms, along with the water suppressed 1D ^{1}H spectrum, of a mouse liver at 4 °C. Peaks are assigned to methyl and methylene (CH_2O) signals of Cho, PCho, and GPCho. The CH_2O signals are more separated than the methyl signals. The signal-to-noise ratio (SNR) for the choline CH_2O signal is 33:1.

Each CCC has nine methyl protons in the trimethylamine head, and the nine magnetically equivalent methyl protons can be more easily detected than the CH_2O methylene protons in solutions.[13] However, the methyl signals of CCC are not well separated in the 2D HSQC spectrum in both dimensions (see Figure 1). Therefore, the less sensitive but better separated CH_2O signals were detected for quantitative purpose.

The sensitivity of the $^{1}H-^{14}N$ HSQC experiment strongly depends on the INEPT evolution time τ (each INEPT period consists of two τ's; see Supporting Information Part I). In order to optimize the detection of the CH_2O signals, a series of 1D HSQC spectra with varied τ were recorded (see Supporting Information Part II), from which we decided to choose $\tau = 12.5$ ms for recording the spectrum in Figure 1. However, even when τ was optimized and 32 transients were averaged, the signal-to-noise ratio was still not high (SNR = 33:1 for the CH_2O signal of Cho). Important reasons include homonuclear coupling in the AA′BB′ system, very short transverse relaxation times at 4 °C, and inhomogeneity of the field inside the tissue.

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We performed measurements of the relaxation times for the CH_2O HSQC signals. Although NMR/MRS have been widely applied to metabolite studies and relaxation times have been measured for metabolites in liver,[8,9] T_1 and T_2 data of protons in choline and its esters are far from complete, because the CCC signals are poorly resolved, even under magic angle spinning conditions.[8,9,14] The pulse sequences for measuring T_1 and T_2 are presented in Supporting Information Part I, which are combinations of saturation recovery and spin echo with HSQC, respectively. The saturation recovery HSQC spectra and the spin echo HSQC spectra are presented in Supporting Information Part III. Fitting of the intensities gave T_1 and T_2 data as listed in Table 1 with a rather small standard deviation (SD of ~0.02 for T_1 fitting and ~0.05 for T_2 fitting), which suggests high precision. Unfortunately, the CH_2O signal of GPCho was too weak to yield any valuable data.

Table 1. Quantitative Result of the Mouse Liver As Studied by $^1H-^{14}N$ HSQC Technique on the Methylene CH_2O Signals

	Cho	Pcho	GPCho
T_1 (ms)	881(±44)	752(±38)	-----
T_2 (ms)	28(±3)	29(±3)	-----
Volume ratio	1	0.45	0.21
Concentration (mM)	3.14(±0.3)	1.4(±2)	0.65(±0.1)

While the T_1 data were helpful in the setup of the HSQC experiments, T_2 data are essential for evaluation of the concentration for CCC in liver. The HSQC signal volume should follow the equation

$$V = V_0[\sin(4\pi J\tau)\exp(-2\tau/T_2)]^2 \quad (1)$$

where both the J ($^1H-^{14}N$ coupling) modulation effect and the T_2 decay effect are taken into account. In eq 1 V_0 is the signal volume when $\tau = 1/8J$ and $T_2 = \infty$; the square in the equation accounts for both of the forward and backward INEPT evolution periods. Under the experimental conditions, Cho and PCho happened to have similar T_2 values (28 and 29 ms; see Table 1). Since the Cho and PCho signals were acquired with the same τ, the volume ratio (see Table 1) measured from the spectrum in Figure 1 can represent the concentration ratio.

For the reference sample (0.3 mM choline), T_1 of the CH_2O signal was evaluated to be 2.04 s in an inversion recovery relaxation experiment with the water signal properly suppressed. Then 2D HSQC experiments were conducted with d1 = 10 s, and the other parameters were kept the same as those for the tissue HSQC experiment. The proton slice at ^{14}N δ48.1 was compared with the slices from the tissue spectrum as shown in Figure 2. The integration ratio between the tissue choline CH_2O signal and the CH_2O signal of the standard sample was 1.84:1. According to eq 1, the correction factor for the tissue sample is $\exp(4\tau/T_2) = \exp(4 \times 0.0125/0.0285) = 5.78$, while the correction factor for the standard sample was $\exp(4 \times 0.0125/2.04) = 1.025$ (here $T_2 = T_1$ is assumed). As a result, the concentrations of free choline and PCho in the mouse liver were determined to be 3.14 and 1.4 mM, respectively, as listed in Table 1. It is reasonable to assume that the CH_2O signal of GPCho had similar T_2 values as in the case of the other two CCC. Then the concentration of GPCho in the mouse liver can be estimated to be 0.65 mM, also listed in Table 1.

Since Cho, PCho, and GPCho are involved in choline phospholipid synthesis and metabolism,[2] the concentration of CCC can vary in animal liver. The choline concentration in mouse liver has been measured to be 2.5 nmol/mg protein.[15] This equals 3.13 mM if we reasonably assume a volume of 0.8 mL for every gram of protein. Then a good agreement is found with our result (3.14 mM).

In most NMR/MRS studies of CCC concentrations, the overlapped methyl peaks at δ3.20−3.23 are analyzed and the concentration of total choline (tCho) is measured.[2] With the $^1H-^{14}N$ HSQC technique, however, the concentrations of the three compounds can be separately measured. This method is expected to bring deeper insight into the molecular mechanism of carcinogens than the concentration of tCho.

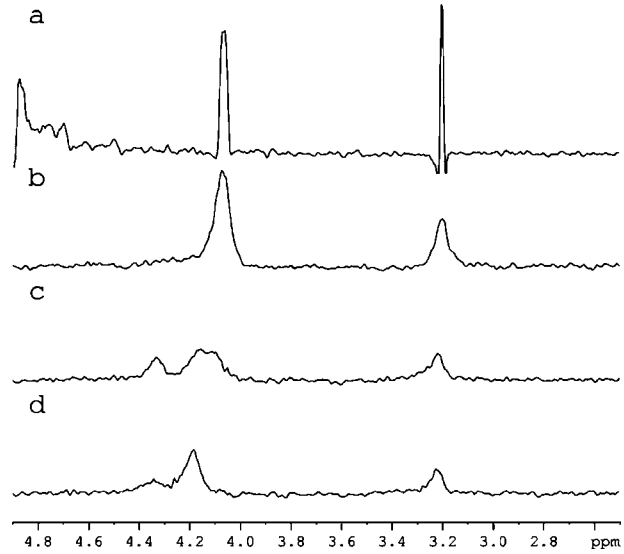

Figure 2. Proton slices from the CH_2O peaks in the $^1H-^{14}N$ HSQC spectra of the reference sample (a) and the mouse liver sample (b−d). The integration of the peak at δ4.065 in (a) is taken as a reference with the integral equaling 1. The integrations of the Cho peak at δ4.07 in (b), the GPCho peak at δ4.33 in (c), and the PCho peak at δ4.19 in (d) represent the relative concentration of Cho, GPCho, and PCho whose ratio is 1.84: 0.39:0.83 or 1:0.21:0.45.

The $^1H-^{14}N$ HSQC experiment should be able to be applied to *in vivo* MRS studies on human liver when a ^{14}N surface coil is available. There are at least two reasons for an *in vivo* $^1H-^{14}N$ HSQC experiment being feasible in clinics. First, human liver is thousands of times larger than mouse liver, leaving no question about the detection sensitivity. Second, at physiological temperature, the protons should have much longer transverse relaxation times (around 80 ms)[8] and much of the magnetization should be saved from losses. We are hopeful that challenges like inhomogeneity in *in vivo* MRS can be overcome in the future.

In summary, we have for the first time conducted a $^1H-^{14}N$ HSQC experiment on animal tissue. With this technique, concentrations of choline, phosphocholine, and glycerylphosphocholine in liver can be separately measured. As far as clinical application is concerned, this technique is much simpler than DNP (dynamical nuclear polarization) which aims at choline detection.[16−18]

Acknowledgment. The authors thank the National Natural Science Foundation of China (#20635040, #20921004, and #90813017) and National Major Basic Research Program of China (#2009CB918603) for financial support.

Supporting Information Available: The Experimental Section (Part I, II, and III). The information is free of charge via the Internet at http://pubs.acs.org.

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JA107745G