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Analysis of *in vitro* 2D-COSY on Human Brain Metabolites for Molecular Stereochemistry

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Abstract : To investigate the 3-bond connectivity of human brain metabolites by scalar coupling interaction through 2D-correlation spectroscopy (COSY) techniques using high field NMR spectroscopy. All NMR experiments were performed at 298K on Unity Inova 500 or 600 (Varian Inc.) equipped with a triple resonance probe head with z-shield gradient. Human brain metabolites were prepared with 10% D_2O. Two dimensional 2D COSY spectra were acquired with 4096 complex data points in t_2 and 128 or 256 increments in t_1 dimension. The spectral width was 9615.4 Hz and solvent suppression was achieved using presaturation using low power irradiation of the water resonance during 2s of relaxation delay. NMR data were processed using VNMRJ (Varian Instrument) software and all the chemical shifts were referenced to the methyl resonance of N-acetyl aspartate (NAA) peak at 2.0 ppm. Total 10 metabolites such as N-acetyl aspartate (NAA), creatine (Cr), choline (Cho), glutamine (Gln), glutamate (Glu), myo-inositol (Ins), lactate (Lac), taurine (Tau), γ-aminobutyricacid (GABA), alanine (Ala) were included for major target metabolites. Symmetrical 2D-COSY spectra were successfully acquired. Total 14 COSY cross peaks were observed even though there were parallel/orthogonal noisy peaks induced by water suppression. Except for Cr, all of human brain metabolites produced COSY cross peaks. The spectra of NAA methyl proton at 2.02 ppm and Glu methylene proton (CH_2(3)) at 2.11 ppm and Gln methylene proton (CH_2(3)) at 2.14 ppm were overlapped in the similar resonance frequency between 2.00 ppm and 2.15 ppm. The present study demonstrated that *in vitro* 2D-COSY represented the 3-bond connectivity of human brain metabolites by scalar coupling interaction. This study could aid in better understanding the interactions between human brain metabolites *in vivo* 2D-COSY study. Also it would be helpful to determine the molecular stereochemistry *in vivo* by using two-dimensional MR spectroscopy.

Keywords : Magnetic resonance spectroscopy (MRS), 2D-MR, COSY, ROESY

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INTRODUCTION

Until a recent date, magnetic resonance spectroscopy (MRS) has been used not only to investigate human brain metabolite *in vivo*, but also to diagnose many diseases based upon variation of metabolites without performing biopsy. And it has been evaluated the most efficient method to determine molecular stereochemistry. In addition, MRS has been used to evaluate the efficiency for the treatment of various diseases.[1-4]

X-ray crystallography also can be used to determine molecular stereochemistry but the sample must be provided with crystal. However, MRS can determine molecular stereochemistry non-invasively to exist with liquid state on physiological condition even though the sample is not provided with crystal. The limitation of MRS may may be difficult to analyze, and required very long time to obtain the data in case of heavy molecular weight (> 10K Dalton).

The most important information to determine stereochemistry by MRS is the distance information in three-dimension and dihedral angle between the protons. The information of the distance is acquired by nuclear Overhauser effect / enhancement (NOE) which arises between two spins that possess dipolar coupling (i.e. through-space coupling). The signal intensity of NOE depends on distance between the protons. But many processes lead to reduced NOE including spin-lattice relaxation, temperature, increased solvent viscosity, increased molecular weight, and dissolved paramagnetic impurities including oxygen. Therefore, the absence of a NOE peak between protons does not necessarily mean that they are not within 5 Å (Angstroms) since other factors as mentioned above can reduce a NOE peak even if the protons are close in space. Also dihedral angle can be acquired by vicinal coupling constant called three-bond coupling or ^3J. The vicinal coupling constants provide information of spatial relationship between two protons through Karplus relationship whose dihedral angle is the minimum at 90°, but is the maximum at 0° and 180°.[5-6] Therefore molecular stereochemistry can be determined based upon the information of three-dimensional distance and dihedral angle. So, the assignment of resonance peaks of each proton which is very important thing to determine molecular structure.

So far, two-dimensional MR spectroscopy has played a important role in determining three-dimensional structure of new drugs including physiological products (ex, DNA, RNA,

protein, carbohydrate)[7]. And it has contributed to bio-innovation. Two-dimensional MR spectroscopy is divided by through-bond connectivity and through-space connectivity. To analyze through-bond connectivity, there is chemical shift correlation spectroscopy (COSY),[8] relay COSY,[9] total COSY (TOCSY)[10] or homonuclear Hartman-Hahm (HOHAHA) spectroscopy.[11] To analyze through-space connectivity, there is nuclear Overhauser enhancement spectroscopy (NOESY), rotating frame NOESY (ROESY).[12]

Recently, 2-dimensional MR has been used to apply *in vivo*, and reported remarkable growth on these studies.[13-17] Most of these studies were about the *in vivo* brain metabolites,[14, 15, 19-24] and some of them were performed about muscles and breast.[16-18] The objective of this study is to investigate the 3-bond connectivity of human brain metabolites by scalar coupling interaction through 2D-correlation spectroscopy (COSY) technique.

MATERIALS AND METHODS

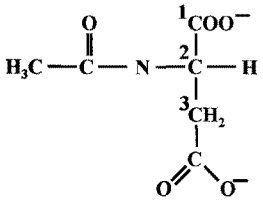
Phantom Solution

We made a diluted solution similar to brain metabolites. GE brain MRS phantom was used as a reference. The concentrations of a brain phantom with ten metabolites were:[1] 12.5 mM N-acetylaspartate (NAA);[2] 12.5 mM glutamate (Glu);[3] 12.5 mM glutamine (Gln);[4] 10.0 mM creatine (Cr);[5] 3.0 mM choline (Cho);[6] 7.5 mM myo-inositol (mI);[7] 5 mM lactate (Lac);[8] 10 mM alanine (Ala);[9] 10 mM γ-aminobutyricacid (GABA);[10] 6 mM taurine (Tau) (Table 1). The structures of main brain metabolites which can be detected by proton MR spectroscopy are shown in Fig. 1.

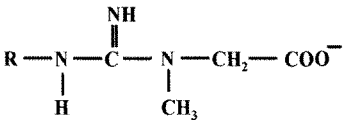
MRS Acquisition

All NMR experiments were performed at 298K on Unity Inova 500 or 600 (Varian Inc.) equipped with a triple resonance probe head with z-shield gradient. Human brain metabolites were prepared with 10% D_2O. Two dimensional (2D) NMR spectra of COSY were acquired with 4096 complex data points in t_2 and 128 or 256 increments in t_1 dimension. The spectral width was 9615.4 Hz and solvent suppression was achieved using presaturation using low power irradiation of the water resonance during 2s of relaxation

delay. NMR data were processed using VNMRJ (Varian Instrument) software and all the chemical shifts were referenced to the methyl resonance of N-acetyl aspartate (NAA) peak at 2.0 ppm.



N-acetyl aspartate (NAA)



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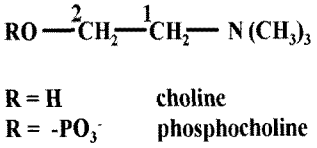
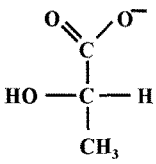


choline (Cho)



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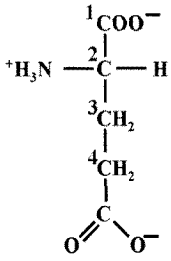

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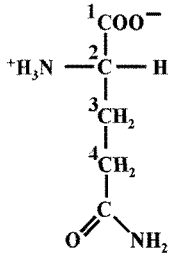
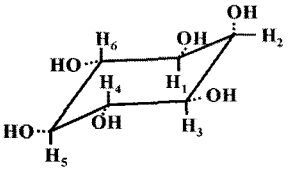
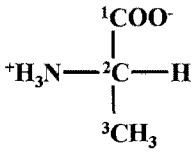


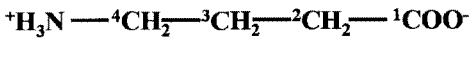
glutamine (Gln)



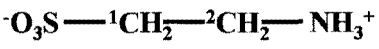
myo-Inositol (Ins)



Alanine (Ala)



γ-aminobutyric acid (GABA)



Taurine (Tau)

Fig. 1. The chemical structures of major human brain metabolites. The number of proton is assigned with respect to carbon one.

Table 1. Phantom ingredients of the various metabolites in the brain mimicking solution

Compound	Abbreviation	CAS no.	Concentration
Potassium phosphate monobasic	KH_2PO_4	[7779-77-0]	50.0 mM
Soduim hydroxide	NaOH	[1310-73-2]	56.0 mM
Sodium azide		[26628-22-8]	0.01 %
L-Alanine	Ala	[56-41-7]	10 mM
Taurine	Tau	[107-35-7]	6 mM
4-Aminobutyric acid	GABA	[56-12-2]	10 mM
L-Glutamic acid	Glu	[6106-04-3]	12.5 mM
L-Glutamine	Gln	[6106-04-2]	12.5 mM
Creatine hydrate	Cr	[6020-87-7]	10.0 mM
Choline chloride	Cho	[67-48-1]	3.0 mM
N-Acetyl-L-aspartatic acid	NAA	[997-55-7]	12.5 mM
Myo-inositol	Ins	[87-89-8]	7.5 mM
DL-Lactic acid	Lac	[16891-53-5]	5.0 mM
Magnevist	Gd-DPTA		1 ml/l

RESULTS

The spectrum of all metabolites such as NAA, Cr, Cho, Gln, Glu, Ins, Lac GABA, Tau, Ala for major targets was successfully acquired. Also 2D-COSY spectrum was acquired from brain metabolites *in vitro* (Fig. 2). The 2D-COSY spectrum was symmetrical with respect to diagonal, a fact which can be used by symmetrization algorithms to distinguish low intensity resonances from noise and improve the suppression of water signals. Also, the cross peaks in a two-dimensional spectroscopy eliminate any ambiguity concerning resonance assignment. As a homonuclear 2D technology, transverse / longitudinal chemical shift was within proton chemical shift range. After we identified NAA resonance peak at 2.02 ppm as a reference point, the resonance assignment of other metabolites was performed.

The results were that resonance peaks were assigned at 3.03 ppm, 3.22 ppm, 1.28 ppm corresponding to Cr, Cho, Lac, respectively.

COSY Data

Lactate: Cross peaks between the methyl protons ($CH_3(3)$) at 1.33 ppm and methine proton (CH(2)) at 4.11 ppm were observed (Figure 2-A).

NAA: Cross peaks between the methylene protons (CH_2: 3, Hβ) at 2.70 ppm and methine proton (CH(2)) at 4.39 ppm were observed (Figure 2-B).

Glu: Cross peaks between the methylene protons ($CH_2(3)$) at 2.11 ppm and methine proton (CH(2)) at 3.76 ppm, between methylene protons ($CH_2(3)$) at 2.11 ppm and methylene protons ($CH_2(4)$) at 2.35 ppm were observed (Figure 2-B).

Gln: Cross peaks between the methylene protons ($CH_2(3)$) at 2.14 ppm and methine proton (CH(2)) at 3.79 ppm, between methylene protons ($CH_2(3)$) at 2.14 ppm and methylene protons ($CH_2(4)$) at 2.46 ppm were observed (Figure 2-B).

Ins: Cross peaks between methine proton (CH(5)) at 3.27 ppm and methine proton (CH(4,6)) at 3.59 ppm, between methine proton (CH(1,3)) at 3.53 ppm and methine proton (CH(4,6)) at 3.59 ppm, between methine proton (CH(1,3)) at 3.53 ppm and methine proton (CH(2)) at 4.05 ppm were observed (Figure 2-B, 2-C).

GABA: Cross peaks between methylenes proton ($CH_2(3)$) at 1.91 ppm and methylene protons ($CH_2(4)$) at 3.01 ppm, between methylenes proton ($CH_2(3)$) at 1.91 ppm and methylene protons ($CH_2(2)$) at 2.30 ppm were observed (Figure 2-B).

Ala: Cross peaks between methyl protons ($CH_3(3)$) at 1.48 ppm and methine proton (CH(2)) at 3.79 ppm were observed (Figure 2-B).

Tau: Cross peaks between N-CH_2 protons at 3.27 ppm and S-CH_2 protons at 3.42 ppm were observed (Figure 2-B, 2-C).

And the spectra of NAA methyl proton at 2.02 ppm and Glu methylene proton ($CH_2(3)$) at 2.11 ppm and Gln methylene proton ($CH_2(3)$) at 2.14 ppm were overlapped in the similar resonance frequency between 2.00 ppm and 2.15 ppm as shown Figure 2-d. The chemical shift and COSY connectivities of the typical brain metabolites are shown in Table 2.

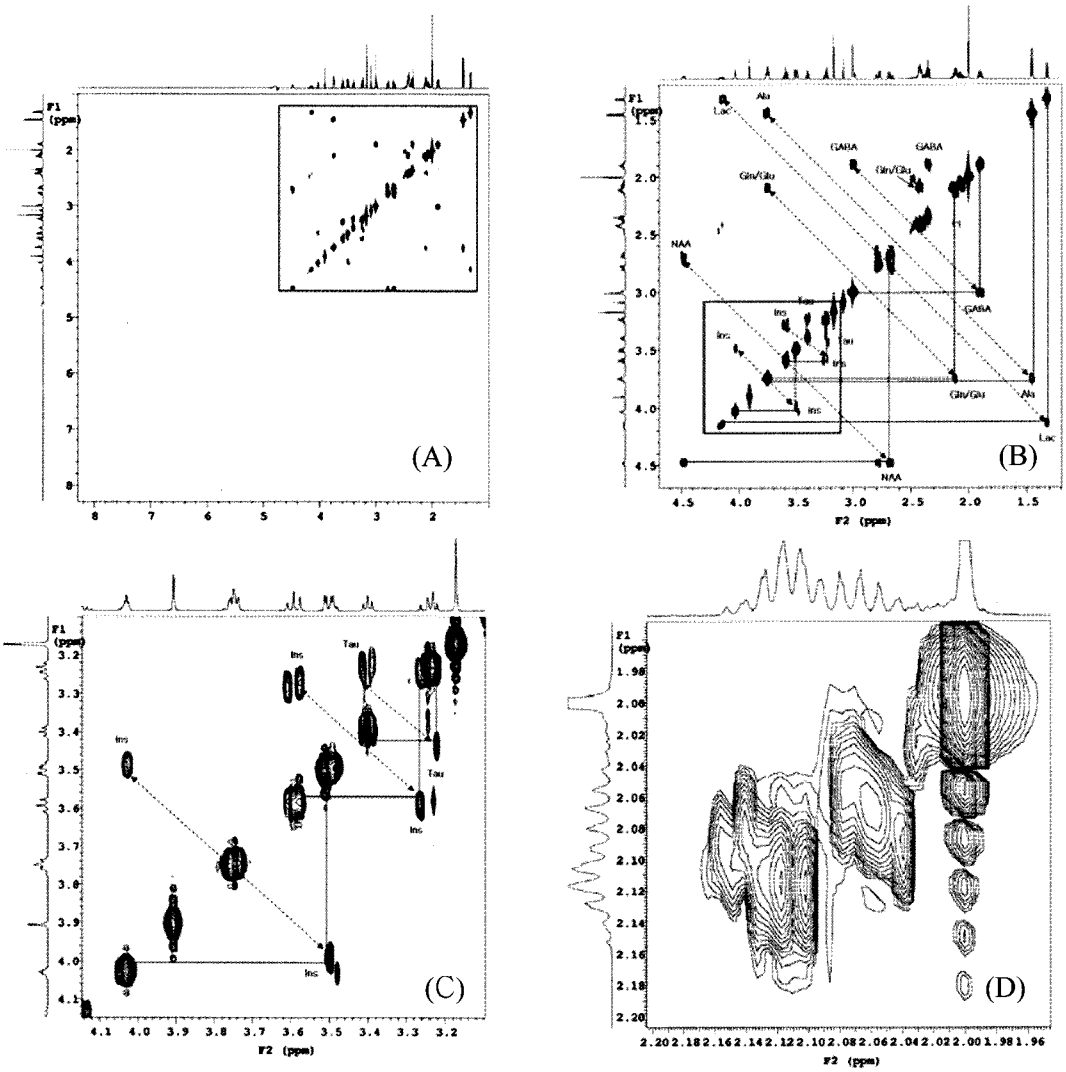


Fig. 2. The 2D-COSY for the brain metabolites. (A) This spectrum shows full scale between 1 ppm and 8.3 ppm. But it may be difficult to distinguish through bond connectivity since it is appeared very small plots. So, the magnified spectrum drawn red box is shown in (A). (B) This spectrum shows detailed through bond connectivity. The scale is between 1.2 ppm and 4.7 ppm. But the cross peaks closed to the diagonal peaks may be difficult to distinguish one another. (C) Through this spectrum, we can distinguish clearly the cross peaks closed to the diagonal peaks. (D) The methylene protons (CH_2(3)) of Glu and Gln were severely overlapped between 2.11 ppm and 2.14 ppm.

DISCUSSIONS

The present study demonstrated that *in vitro* 2D-COSY represented the 3-bond connectivity of human brain metabolites by scalar coupling interaction. All metabolites such as N-acetyl aspartate (NAA), creatine (Cr), choline (Cho), glutamine (Gln), glutamate (Glu), myo-inositol (Ins), lactate (Lac), γ-aminobutyric acid (GABA), alanine (Ala), taurine (Tau) for major target metabolites could be observed in the spectrum. The quantitative analysis was not performed because the concentrations of phantom compounds were determined previously. But the information of the chemical shift and fixed quantity for contrast media (Magnevist) could not be obtained. Since the concentration of Magnevist (Table 1) was 1 ml/l which was a very small amount, it was considered that its spectrum could not be observed.

As expected, 2D-COSY spectrum had a better resolution than 1D-spectrum due to added one dimension. The 2D spectrum can produce the cross peaks which can be used to measure many pathological changes that is connected directly to the change of metabolites. The cross peaks which are positioned under the diagonal are generally used since 2D spectrum may be not precisely symmetrical because of the CHESS pulse for water suppression.[22] The problem can be solved by 2D-post processing using averaging method, but it was excluded due to the potentiality whose artificial artifacts can appear. Through COSY spectrum, we could acquire information about through bond connectivity and interaction. Therefore, if COSY cross peaks are observed, we could identify that three-bond connectivity is made between corresponding to protons. For a protein, a COSY cross peak indicates that the protons corresponding to two correlated diagonal peaks are in the same amino acid residue, where they are separated by at most three chemical bonds.[23]

The COSY data obtained from a phantom containing a mixture of brain metabolites will be used usefully to analyze and compare with COSY data obtained from *in vivo*. As the COSY data of this study were obtained from 11.8 Tesla MR spectrometer, it may provide detailed and accurate information than the data obtained from 1.5 or 3 Tesla MR equipment which is used vigorously in clinic.

Table 2. Chemical shift (δ)[a] and COSY/ROESY connectivities of the typical brain metabolites by proton MRS.[b]

Compound	(ppm)	Resonance	COSY Connectivity	Comments
NAA	2.02	CH_3		
	2.50	CH_2(3,Hα)		
	2.70	CH_2(3,Hβ)	CH_2(3,Hβ) + CH(2)	
	4.49	CH(2)		
Cr	3.03	CH_3		No scalar connectivity
	3.93	CH_2		
Cho	3.24	$N(CH_3)_3$		
	3.56	CH_2 (2)	CH_2 (2) + CH_2 (1)	
	4.07	CH_2 (1)		
Glu	2.11	CH_2 (3)	CH_2 (3) + CH_2 (4)	
	2.35	CH_2 (4)	CH_2 (3) + CH(2)	
	3.76	CH(2)		
Gln	2.14	CH_2 (3)	CH_2 (3) + CH_2 (4)	
	2.46	CH_2 (4)	CH_2 (3) + CH(2)	
	3.79	CH(2)		
Ins	3.27	CH(5)	CH(5)+ CH(4,6)	
	3.53	CH(1,3)	CH(1,3)+ CH(4.6)	
	3.59	CH(4,6)	CH(1,3)+ CH(2)	
	4.05	CH(2)		
Lac	1.33	CH_3(3)	CH_3(3)+ CH(2)	
	4.11	CH(2)		
GABA	1.91	CH_2(3)	CH_2(3) + CH_2(2)	
	2.30	CH_2(2)	CH_2(3) + CH_2(4)	
	3.01	CH_2(4)		
Tau	3.27	CH_2(2)	CH_2(2) + CH_2(1)	
	3.42	CH_2(1)		
Ala	1.48	CH_3(3)	CH_3(3) + CH(2)	
	3.79	CH(2)		

Thomas and his colleague tried to acquire 2D-COSY spectrum at first from *in vivo* human brain using 1.5 T MR spectrometer.[14] Although it was low-resolution, they acquired successfully the results. Later, they reported 2D-COSY from *in vivo* human brain using 3.0 T.[15] But much of noise was included *in vivo* 2D-COSY compared to *in vitro* 2D-COSY. It was thought that it was not enough to acquire 2D-COSY having good SNR in 3T MR equipment. While Welch and his colleague who obtained 2D-COSY *in vivo* rat brain using

7.0 T (Varian 300 MHz) acquired successfully high-resolution spectrum without noise by using ISIS-COSY technique.[25] The ISIS-COSY technique which was proposed by them was excellent to suppress noise by outer-volume suppression (OVS), but had a severe shortcoming to suppress water.

In clinically, there have been reported that 2D-COSY and DQF-COSY which is obtained from tibial bone marrows of the acute leukemia patients using 1.5 T MR equipment was tried to apply in clinic.[26] The results was that relaxation time (T1,T2) was significantly different between normal and patient group.

Generally, the chemical shift assignment of the COSY is carried out prior to obtain NOESY data, because the experiment is performed after ensuring information about through bond interaction. The chemical shift assignment of the macromolecule such as protein, DNA, RNA, carbohydrate in COSY and NOESY may be very difficult and complex, since molecular stereochemistry is not fixed at a liquid state but moving dynamically. Therefore, we would obtain a complete or nearly complete delineation of the connectivity among the protons in molecular stereochemistry using combination with COSY and other related experiment for through space interactions.

CONCLUSION

The present study demonstrated that *in vitro* 2D-COSY represented the 3-bond connectivity of human brain metabolites by scalar coupling interaction. This study could aid in better understanding the interactions between human brain metabolites *in vivo* 2D-COSY study. Also it would be helpful to determine the molecular stereochemistry *in vivo* by using two-dimensional MR spectroscopy.

Acknowledgement

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