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METABOLITE CHARACTERIZATION IN SERUM SAMPLES FROM NORMAL HEALTHY HUMAN SUBJECTS BY ^{1}H AND ^{13}C NMR SPECTROSCOPY

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ABSTRACT. One and two dimensional NMR spectroscopy has been employed to characterize the various metabolites of serum control healthy samples. Two dimensional heteronuclear experiment has been included to take advantage of larger chemical shift spread of ^{13}C resonances allowing a more detailed identification of metabolites not possible in one dimensional spectra. This methodology has successfully allowed the assignment of ninety four resonances of various metabolites. The importance of the work lies in the fact that characteristic fingerprints of various metabolites of serum samples of normal healthy control have been obtained which can identify and distinguish metabolic differences from other diseased specimens or non-diseased/healthy serum samples. This study will help enhance the literature of metabolite identification in serum samples.

KEY WORDS: Metabonomics, Serum, HSQC, CPMG

INTRODUCTION

Proton NMR spectroscopy provides enriched information about identification of metabolites in body fluids such as serum and urine [1]. Metabonomics is a powerful and emerging technology that characterizes the complex time-dependent metabolic profiles within biofluids and tissues. It is a holistic approach for examining the dynamic metabolic changes in organisms and in the study of diseases [2]. Metabonomics offers a distinct advantage over other tests as it can be carried out on standard preparations of tissues, body fluids and even whole bodies, needing no special preparation. NMR spectroscopy is non-invasive and is considered to be the most powerful tool for investigating cellular metabolism and as biomarker identification [3].

The biochemical composition of body fluids is known to reflect the metabolic status of the donor. However, most of the standard techniques require extensive sample preparation together with careful selection of analytical conditions. This can clearly restrict the number of compounds that can be studied at a given time. However, a number of fluids contain a range of molecules with diverse chemical and molecular masses including amino acids, proteins and lipoproteins, all or some of which can be affected in disease. The non-selective nature of NMR spectroscopy makes it an ideal technique for obtaining metabolic profiles of all available body fluids. Furthermore NMR spectroscopy can give useful insights into molecular interactions within intact fluids which are not readily available by standard biochemical techniques. Thus, NMR spectroscopy, although relatively insensitive has been successfully applied to the detection and quantitation of a variety of molecules in intact body fluids, including, blood plasma, serum, urine, and synovial fluid [4].

The HSQC (heteronuclear single quantum coherence) experiment is frequently used in NMR spectroscopy. The resulting spectrum is two dimensional with one axis for ^{1}H and other for a heteronucleus, most often ^{13}C or ^{15}N. The spectrum contains a peak for each unique proton attached to the heteronucleus being considered. Among all stable nuclei, ^{1}H has the highest sensitivity and detects simultaneously a large number of metabolites that may potentially be present. Metabolites, the end products of cellular process reflect the system level biological stress response. Hence, any enzymatic perturbation is directly or indirectly related to the cellular

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behavior and its metabolism. The metabolites contributing to the metabolism provide a precise snapshot of the system biology. Metabolic profiling in heart disease has also been successfully carried out [5].

The NMR method achieves high spectral resolution from body fluids. Partial overlap describes metabolites that resonate so closely together that they cannot be resolved from each other like choline phospholipids whose singlet's are highly overlapped at about 3.2 ppm in one dimensional spectra. The HSQC spectrum contains a peak for each unique proton attached to the hetero nucleus (^{13}C) being considered. Since the ^{13}C isotope is found in very low abundance in nature, so molecules in a mixture that are labelled with ^{13}C will give much stronger NMR signals. Thus, if the chemical shift of a specific proton is known, then the chemical shift of the coupled heteronucleus can be determined. Being a relatively cheap and quick experiment, the HSQC spectra's were found very useful in identification of a large number of metabolites. In principle, the values for scalar couplings between ^{1}H and ^{13}C and the signal multiplicity can give additional information for the structure determination. Correlations between directly bonded ^{1}H and ^{13}C nuclei can be obtained with ^{1}H-^{13}C HSQC experiment.

In this work, two dimensional heteronuclear experiment has been included to take advantage of larger chemical shift spread of ^{13}C resonances allowing a more detailed identification of metabolites. This methodology has been successfully helpful in the assignment of ninety four resonances corresponding to various metabolites.

EXPERIMENTAL

The study was carried out on 26 age/sex matched normal healthy individuals. After 12 hours of fasting 10 mL of blood sample from each individual was taken and was allowed to clot in plastic tube for 2 h at room temperature. The serum was collected by centrifugation. The samples were stored under liquid nitrogen for NMR analysis. Before NMR analysis, 600 μL of the samples were taken in a 5 mm high quality NMR tubes. Inside the NMR tube a capillary containing D_2O with 70 mg/dL of external reference TSP was inserted inside the 5 mm NMR tube prior to NMR measurement.

The NMR measurements were carried out at 400.13 MHz on a Bruker Avance FT NMR spectrometer (Switzerland) operating at 400 MHz frequency, equipped with 5 mm multinuclear inverse probehead with Z shielded gradient. Recording of the NMR spectra was carried out using pulse sequences of NOESY and CPMG with water presaturation at 298 K for evaluation of various metabolites. In some samples 2D COSY (correlation spectroscopy) measurements were performed for the unambiguous assignment of various metabolites. Chemical components were assigned on the basis of data available in literature.

Heteronuclear two dimensional ^{1}H-^{13}C chemical shift correlations were measured using gradient HSQC adiabatic pulses. The experiments were performed in gradient mode with a spectral width of 3.591 kHz in F_2 dimension and 14.340 kHz in F_1 dimension, 256 t_1 increments. For each t_1, 88 transients using 1.8 s relaxation delay were added with 2048 complex data points.

RESULTS AND DISCUSSION

The complete assignments of the various metabolites were carried out by a combination of one dimensional ^{1}H, CPMG (Figure 1-3), two-dimensional HSQC spectral plots along with their expansions (Figure 4-8). Prior to resonance assignments all the spectra's were referenced with respect to the methyl group (CH_3) of lactate at 1.33 ppm for ^{1}H and 22.6 ppm for ^{13}C. Resonances due to lipid moieties were effectively filtered by the use of CPMG experiments. The

one-dimensional proton NMR spectra showed a large number of signals and a high degree of overlap especially in the range of 3.0-4.0 ppm and were significant with their own distinctive pattern as shown in Figure 1-3, respectively. While the CPMG spectra provided detailed resonances of the small metabolites showing fingerprints of serum metabolomic profile (Figure 3). The assignments are based on the comparison of chemical shifts and spin multiplicities with data reported in literature [6-10]. The two-dimensional HSQC spectrum allowed us to assign signals to particular metabolites through the examination of their existing cross peak correlations. A complete list of identified metabolites along with their spin multiplicities and ^{1}H and ^{13}C chemical shifts is given in Table 1.

Table 1. Resonance assignments of most significant metabolites of serum control healthy samples.

S. No	Metabolite	Group	^{1}H shift	^{13}C shift	Multiplicities
1	Fatty acids	CH_3	0.90	19.7	t
2	Isoleucine	δCH_3	0.94	13.8	t
3	Leucine	δCH_3	0.95	23.5	d
4	Leucine	δCH_3	0.96	24.7	d
5	Valine	γCH_3	0.98	19.2	d
6	Isoleucine	γCH_3	1.01	17.4	d
7	Valine	γCH_3	1.04	20.6	d
8	Isoleucine	γCH_2u	1.24	27.2	m
9	Fatty acids (a)	(2) CH_2	1.28	34.6	m
10	Fatty acids (a) (b)	(n) CH_2	1.29	32.5	m
11	Fatty acids (a)	(1) CH_2	1.29	25.5	m
12	Fatty acids (c)	(n) CH_2	1.31	31.8	m
13	Lactate	CH_3	1.33	22.6	d
14	Threonine	γCH_3	1.34	21.9	d
15	Fatty acids (b)	(1) CH_2	1.37	32.3	m
16	Isoleucine	γCH_2d	1.46	27.1	m
17	Lysine	γCH_2	1.46	24.1	d
18	Alanine	βCH_3	1.47	18.9	d
19	Fatty acids (c)	(2) CH_2	1.58	27.6	m
20	Lysine	δCH_2	1.68	29.1	m
21	Leucine	βCH_2	1.71	42.4	m
22	Leucine	γCH	1.71	27.1	m
23	Lysine	βCH_2	1.90	32.5	m
24	Acetate	CH_3	1.91	25.6	s
25	Isoleucine	βCH	1.98	38.5	m
26	Fatty acids (b)	(2) CH_2	2.04	27.3	m
27	Glutamate	βCH_2u	2.04	29.6	m
28	Proline	βCH_2u	2.06	31.6	m
29	Glutamate	βCH_2d	2.12	29.5	dt
30	Glutamine	βCH_2	2.14	28.8	m
31	Acetone	CH_3	2.23		s
32	Fatty acids (c)	(1) CH_2	2.24	36.2	m
33	Valine	βCH	2.28	31.7	m
34	Acetoacetate	CH_3	2.29		s
35	Glutamate	γCH_2	2.34	36.1	dt
36	Proline	βCH_2d	2.35	31.1	m
37	Glutamine	γCH_2	2.44	33.5	m
38	Fatty acids (b)	CH_2	2.81	28.1	m

39	Aspargine	βCH_2u	2.86	37.2	dd
40	Aspargine	βCH_2d	2.96	37.2	dd
41	Creatine	$N(CH_3)_3$	3.03		s
42	Lysine	CH_2	3.05	41.6	t
43	Ethanolamine	CH_2-NH_3	3.12	44.1	t
44	Phenylalanine	βCH_2u	3.12	39.1	dd
45	Choline	$N^+(CH_3)_3$	3.19	56.6	s
46	PCho	$N^+(CH_3)_3$	3.20	56.6	s
47	GPCho	CH_2-NH_3	3.24	56.7	s
48	Myo-inositol	C5H	3.25	76.5	t
49	Phenylalanine	βCH_2d	3.30	39.1	dd
50	Proline	CH_2u	3.34	48.7	t
51	$\bar{\alpha}$Glucose	C4H	3.41	72.3	t
52	$\bar{\beta}$Glucose	C4H	3.42	72.3	t
53	Proline	δCH_2d	3.42	48.7	t
54	$\bar{\beta}$Glucose	C5H	3.46	78.6	t
55	$\bar{\beta}$Glucose	C3H	3.48	78.2	t
56	Myo-Inositol	C1H,C3H	3.53	74.1	dd
57	Choline	βCH_2	3.53	70.1	m
58	Glycerol	$1,3CH_2OHu$	3.56	65.1	dd
59	PCho	βCH_2	3.57	69.3	t
60	Threonine	αCH	3.58	63.1	d
61	Glycogen	C2H	3.61	71.8	dd
62	Valine	αCH	3.61	63.2	d
63	MI	C4H,C6H	3.61	75.1	t
64	Glycerol	$1,3CH_2OH$	3.63	65.1	dd
65	Glycogen	C4H	3.63	77.1	q
66	GPCho	βCH_2	3.67	68.6	m
67	Isoleucine	αCH	3.67	62.1	m
68	α-Glucose	C3H	3.69	75.5	t
69	Glycogen	C5H,C6H	3.71	61.2	dd
70	Leucine	αCH	3.73	56.2	t
71	$\bar{\beta}$Glucose	C6Hd	3.73	63.5	dd
72	Glutamate	αCH	3.75	57.2	t
73	Alanine	αCH	3.76	53.4	q
74	α-Glucose	C6Hu	3.77	63.4	m
75	Lysine	αCH	3.77	56.2	t
76	Glutamine	αCH	3.77	56.9	t
77	Ethanolamine	CH_2-OH	3.78	71.8	t
78	Glycerol	CH (OH)	3.78	74.9	m
79	α-Glucose	C5H	3.82	73.9	m
80	α-Glucose	C6H d	3.83	63.4	m
81	$\bar{\beta}$Glucose	C6H	3.91	63.5	dd
82	Glycogen	C3H	3.96	73.8	dd
83	Phenylalanine	α CH	4.00	58.2	dd
84	Myo-inositol	C2H	4.04	74.8	t
85	Choline	αCH_2	4.05	58.8	m
86	Lactate	CH	4.11	71.1	q
87	Proline	αCH	4.12	63.8	t
88	PCho	αCH_2	4.17	60.7	t
89	Threonine	βCH	4.25	68.7	m

90	GPCho	αCH_2	4.28	56.6	t
91	βGlucose	C1H	4.64	98.7	d
92	α-Glucose	C1H	5.23	94.8	d
93	Fatty acids (b)	(2) CH	5.32	132.5	m
94	Fatty acids (b)	(1) CH	5.33	130.6	m

u, up-field; d, down-field.

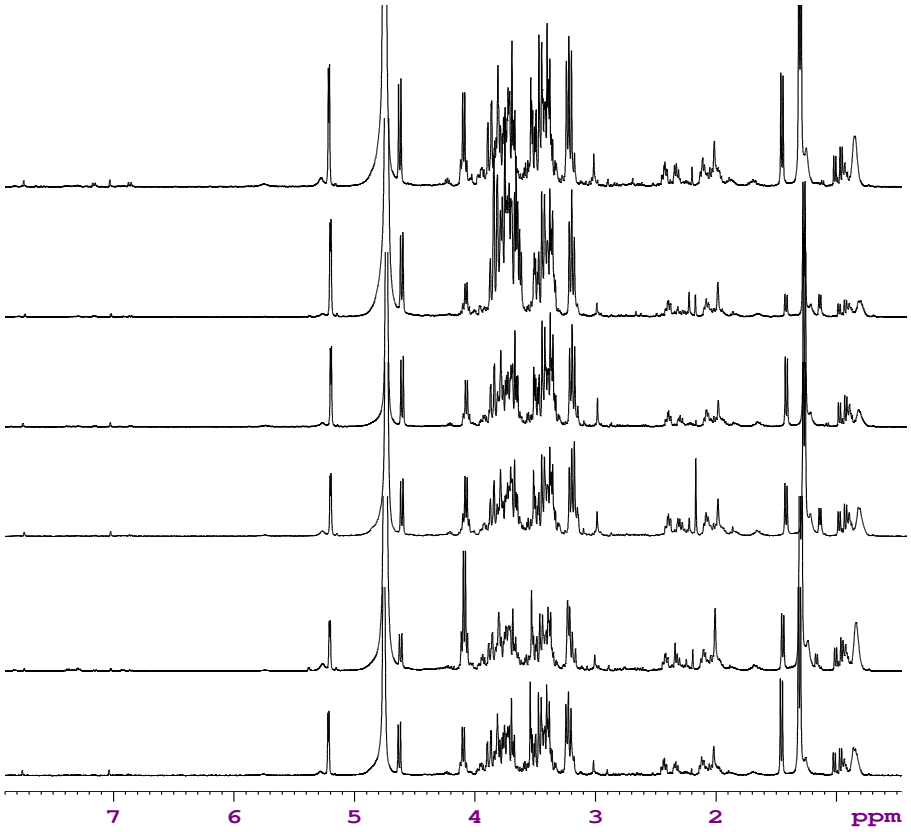

Figure 1. Stack plot of the CPMG spectra of serum control healthy samples.

Spectral assignments

Prior to resonance assignment process, all the spectra's were referenced with respect to methyl group of lactate at 1.33 ppm and 22.6 ppm in ^{1}H and ^{13}C, respectively. One dimensional ^{1}H CPMG spectra of serum control healthy samples showed assignment problems due to overlap of small molecule signals with many broad signals from macromolecules (VLDL, LDL and HDL resonances). By direct inspection of one dimensional plot, the following resonances were readily assigned. At about 0.8 ppm broad singlet due to lipids was observed, this was followed by doublet's due to γCH_3 group of valine and isoleucine at 0.98 ppm and 1.01 ppm. A doublet due to γCH_3 group of valine was observed at about 1.04 ppm followed by a broad signal from CH_2 groups of lipids from lipoproteins in the range of 1.28-1.31 ppm. An overlapped doublet due to

methyl group of lactate and threonine was observed at about 1.33 ppm, followed by a very low intensity doublet due to methyl group of alanine at 1.47 ppm, similarly singlet due to methyl groups of acetate was observed at 2.02 ppm. Once again a multiplet due to CH_2 group of lipids was observed at 2.04 ppm. Whereas, acetone at 2.23 ppm and CH_3 group of acetoacetate was observed at 2.29 ppm. A complex multiplet overlapped by resonances from other metabolites was observed at about 2.35 ppm which was assigned to proline. The singlet due to $-N(CH_3)_3$ group of creatine and creatinine at 3.03 ppm of small intensity was observed followed by a very high intensity clear triplet from the CH_2 group of lysine.

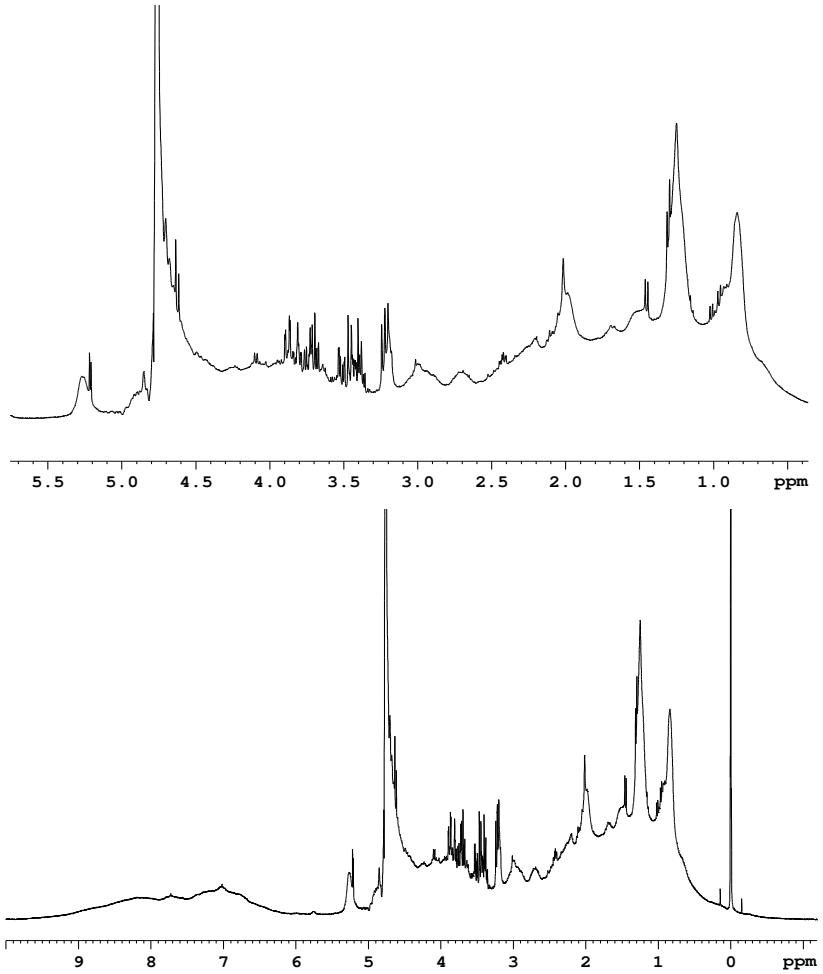

Figure 2. NOESY PRESAT of serum control healthy samples.

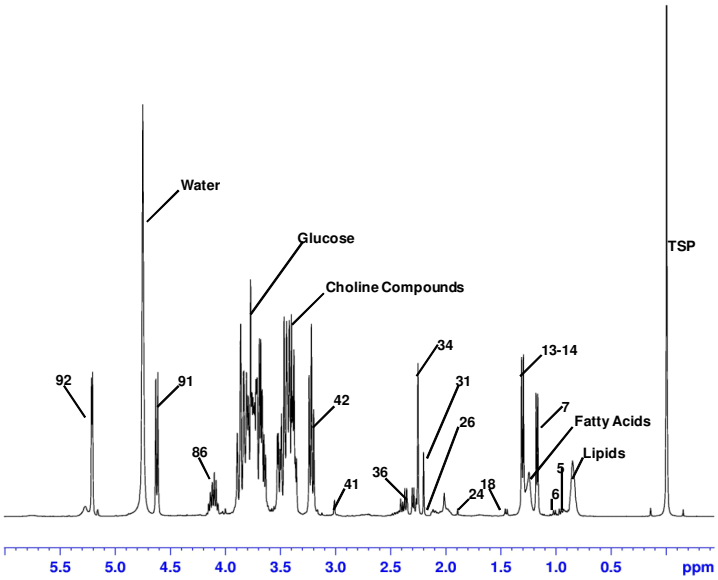

Figure 3. The typical ^{1}H one dimensional CPMG NMR spectra (0-6 ppm) of serum control healthy samples highlighting the assignments of the small molecule metabolites as given in Table 1.

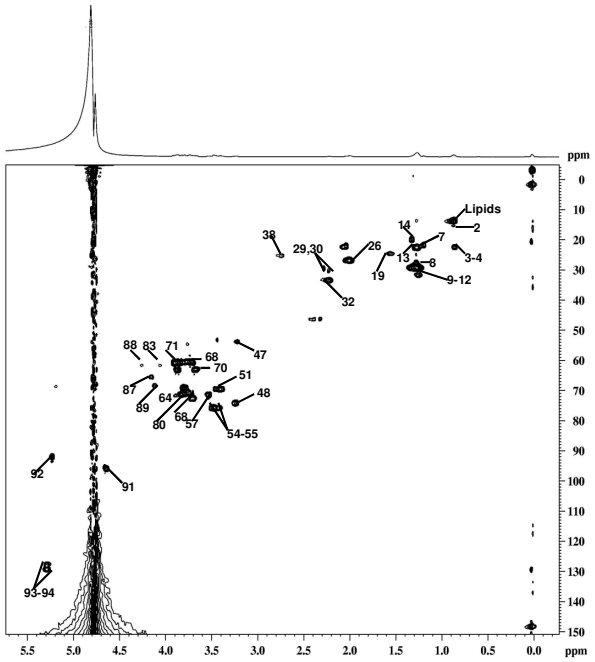

Figure 4. The ^{1}H-^{13}C HSQC spectra (0-6 ppm) of serum control healthy samples labelled assignments are as per Table 1.

In the region between 3.2-3.5 ppm highly overlapped signals were observed. They consisted of overlapped signals due to choline phospholipids and other metabolites. The complex and highly overlapped multiplet in the range of 3.3-3.9 ppm was well resolved in the two dimensional HSQC spectra. The quartet due to CH group of lactate was observed at 4.11 ppm followed by very clearly observable intense doublet's due to C1H group of β-α glucose at 4.64 ppm and 5.23 ppm. Also an intense singlet due to water at 4.79 ppm was also observed.

Highly intense ^{1}H-^{13}C peak below 1 ppm was observed for lipids (Figure 5), followed by medium intensity ^{1}H-^{13}C cross peak due to methyl CH_3 group's of isoleucine and leucine at 0.94-13.8 ppm and 0.95-23.5 ppm. While cross peak due to methyl group of valine and isoleucine were observed at 1.04-20.6 ppm, 1.01-17.4 ppm. A broad intense and highly overlapped peak due to methine groups of fatty acids in the range 1.28-1.31 ppm on proton axis and 25 to 34 ppm on the carbon axis were observed.

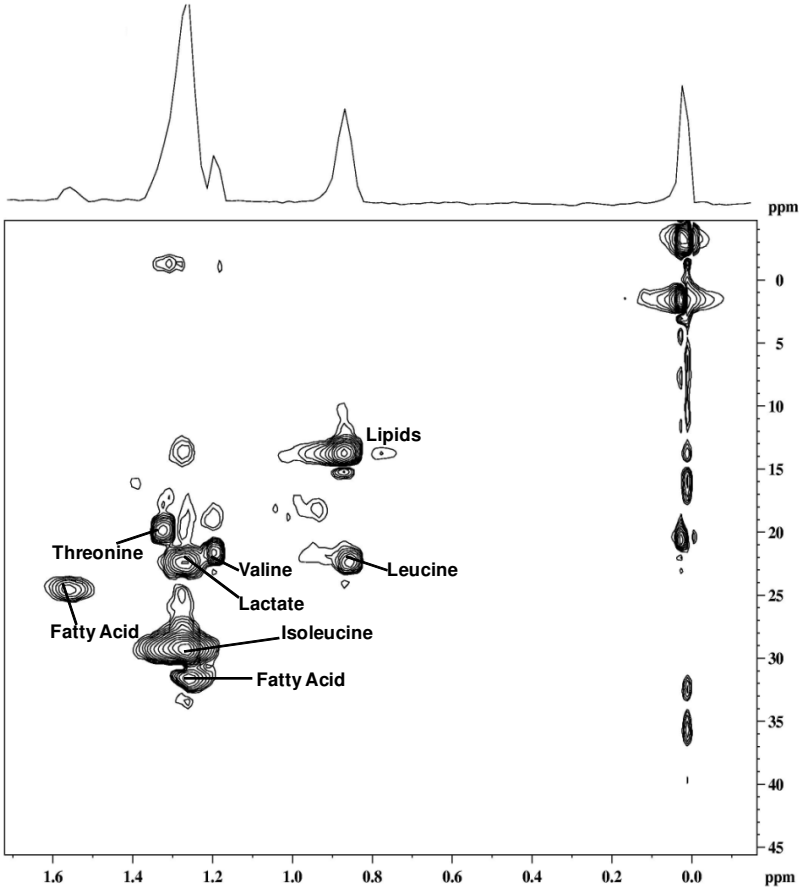

Figure 5. Expansion of ^{1}H-^{13}C HSQC spectra of serum control healthy samples highlighting the resonance assignments in the region 0-1.7 ppm.

The overlapped doublet due to methyl group of lactate and threonine in one dimensional plot was clearly resolved in the HSQC spectra, giving their confirmed identification with the ^{1}H-^{13}C cross peak of lactate at 1.33-22.6 ppm and that due to threonine at 1.34-21.9 ppm. As observed in Figure 6 a broad peak due to methine group's of lipids was observed at 1.58-27.6 ppm. Followed by another intense broad peak due to CH_2 group of lipids at 2.04-27.3 ppm. CH_2 group of fatty acids showed another ^{1}H-^{13}C cross-peak at 2.81-28.1 ppm. The one dimensional spectra showed a very complex and highly overlapped resonance pattern in the range of 3 to 4 ppm. Taking advantage of the large spread of ^{13}C chemical shift, we were successful in assigning large number of metabolites in this range, which was not possible in the one dimensional plot.

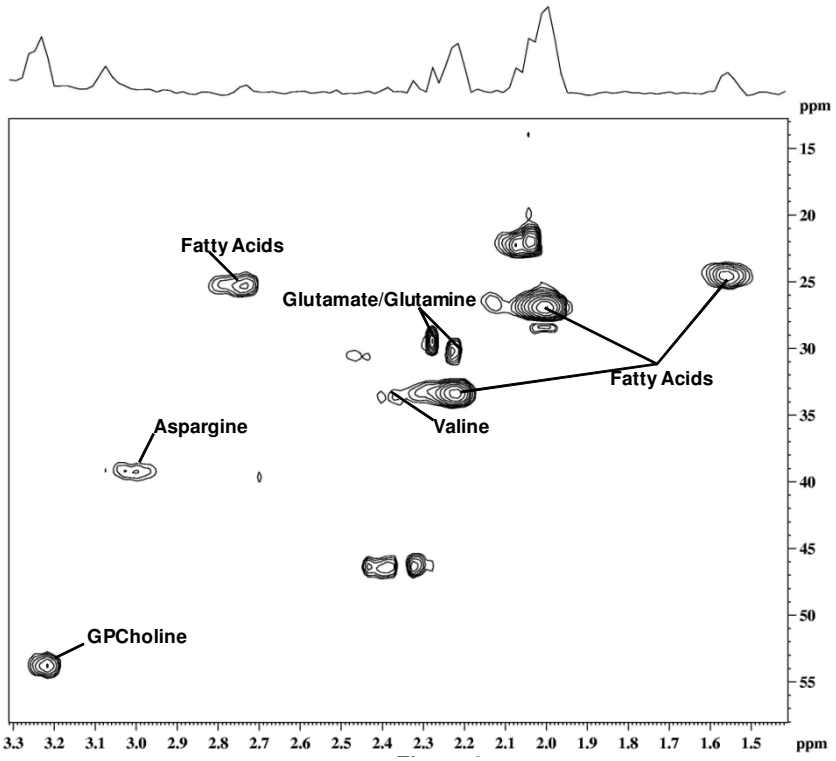

Figure 6. Expansion of ^{1}H-^{13}C HSQC spectra of serum control healthy samples highlighting the resonance assignments in the region 1-3 ppm.

The choline phospholipids were easily assigned in the HSQC plot Figure 7 with the CH_2-NH_3 peak of glycerophosphocholine, being observed at 3.24-56.7 ppm. While βCH_2 group of choline and phosphocholine were observed at 3.53-70.1 ppm, 3.57-69.3 ppm, respectively. HSQC spectra also helped in the confirmed identification of myo-inositol with its CH cross-peaks at 3.25-76.5 ppm. Confirmed identification was also possible for ethanolamine, glycogen, phenylalanine, proline, arginine. Almost all the ^{1}H-^{13}C cross-peaks of α-β glucose were observed. Highly intense cross-peaks due to CH groups of lipids at 5.32-132.5 ppm and 5.33-130.6 ppm were observed as shown in Figure 8.

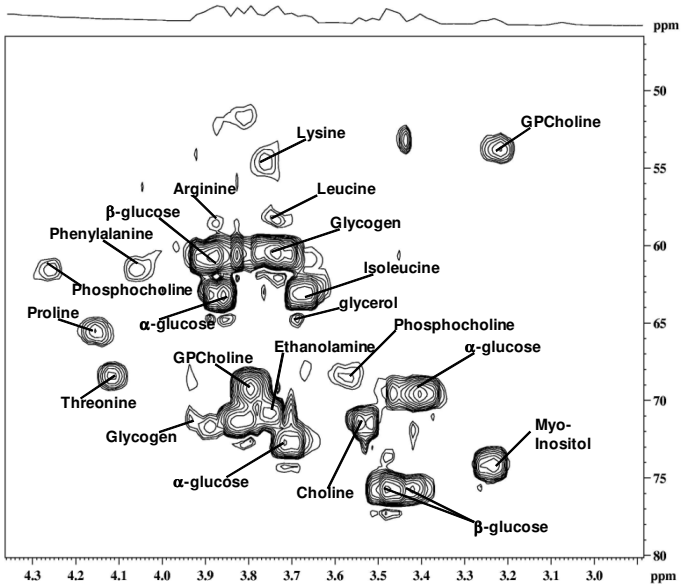

Figure 7. Expansion of ^{1}H-^{13}C HSQC spectra of serum control healthy samples highlighting the resonance assignments in the region 2.5-4.4 ppm.

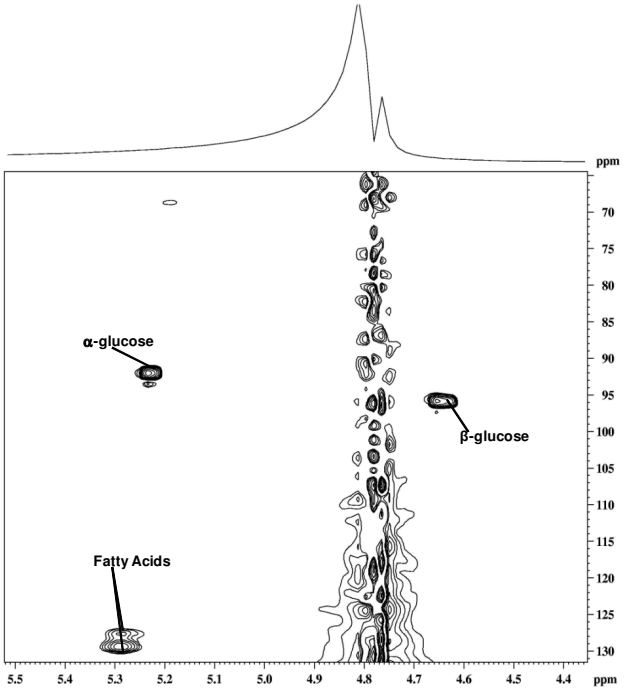

Figure 8. Expansion of ^{1}H-^{13}C HSQC spectra of serum control healthy samples highlighting the resonance assignments in the region 4-5 ppm.

In the one dimensional CPMG plot clear splitting pattern was observed for doublet due to CH_3 group of alanine, multiplet due to β-CH_2 group of proline, quartet due to CH group of lactate, and α-β glucose monomers and short polymers located at 5.23 ppm and 4.64 ppm, respectively. Confirmed assignments of the rest of the metabolites were only possible with the help of HSQC spectra, taking advantage of the large chemical shift window of ^{13}C resonances. Hence, HSQC spectra helped in the confirmed identification of isoleucine, leucine, valine, lactate, threonine, lysine, glutamate, proline, glutamine, aspargine, ethanolamine, phenylalanine, glycerol, glycogen, α-β glucose and lipids.

The ^{1}H-^{13}C HSQC spectra has been very useful and in some cases crucial for a unique identification of overlapped peaks, such as for example the whole spin system of α-β glucose, the choline phospholipids, etc. The identification of these metabolites by NMR spectroscopy may provide very interesting information about serum control healthy subjects can be correlated to any disease state in relationship of different organs in any human system which can make the study very interesting.

CONCLUSIONS

The importance of the work lies in the fact that characteristic fingerprints of various metabolites of serum samples of healthy human tissue specimen's have been obtained which may provide distinguishing differences between metabolites from other diseased serum samples. This preliminary approach has shown how it is possible to obtain information of a large number of metabolites in serum samples by means of a simple procedure.

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REFERENCES

1. Tripathi, S.; Somashekar, B.S.; Mahdi, A.A.; Gupta, A.; Mahdi, F.; Hasan, H.; Roy, R.; Khetrapal, C.L. *J. Biomoecular Toxicology* **2008**, 22, 119.
2. Makinen, V.P.; Soininen, P.; Forsblom, C.; Parkkonen, M.; Ingman, P.; Groop, H.; Ala-Korrela, M. *Molecular Systems Biology* **2008**, 4, 167.
3. Wang, Y.; Holmes, E.; Nicholson, J.K.; Cloarec, O.; Chollet, J.; Tanner, M.; Singer, B.H.; Utzinger, T. *Proceedings of the National Academy of Sciences (PNAS)* **2004**, 101, 12676.
4. Nicholson, J.K.; Flynn, M.P.; Sadler, P.J.; Macleod, A.F.; Sonksen, P.H. *J. Biochem.* **1984**, 217, 365.
5. Grainger, D.L. *Heart Metabolism* **2006**, 32, 22.
6. Cheng, L.L; Wen, C.I.; Louis, D.N.; Gilberto, R. *Cancer Research* **1998**, 58, 1825.
7. Govindraju, V.; Young, K.; Mauolsley, M. *NMR Biomed*. **2000**, 13, 129.
8. Sitter, B.; Sonnewald, U.; Spraul, M.; Fjosne, E.H.; Gribbestad, I.S. *NMR Biomed.* **2002**, 15, 327.
9. Martinez, B.; Monleon, D.; Martinez, M.; Rodrigo, J.M.; Olmo, J.; Lluch, P.; Ferrandez, A.; Bonmati, M.; Celda, B. *NMR Biomed.* **2006**, 19, 90.
10. Burtscher, I.M.; Holtas, S. *Neuroradiology* **2001**, 43, 345.