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ORIGINAL ARTICLE

Quantitative metabolic profiling of NMR spectral signatures of branched chain amino acids in blood serum

Soumita Ghosh[1,2] · **Arjun Sengupta**[1,3] · **Kousik Chandra**[1,4,5]

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Abstract Branched Chain Amino Acids (BCAAs) are related to different aspects of diseases like pathogenesis, diagnosis and even prognosis. While in some diseases, levels of all the BCAAs are perturbed; in some cases, perturbation occurs in one or two while the rest remain unaltered. In case of ischemic heart disease, there is an enhanced level of plasma leucine and isoleucine but valine level remains unaltered. In 'Hypervalinemia', valine is elevated in serum and urine, but not leucine and isoleucine. Therefore, identification of these metabolites and profiling of individual BCAA in a quantitative manner in body-fluid like blood plasma/serum have long been in demand. ^{1}H NMR resonances of the BCAAs overlap with each other which complicates quantification of individual BCAAs. Further, the situation is limited by the overlap of broad resonances of lipoprotein with the resonances of BCAAs. The widely used commercially available kits cannot differentially estimate the BCAAs. Here, we have achieved proper identification and characterization of these BCAAs in serum in a quantitative manner employing a Nuclear Magnetic Resonance-based technique namely T_2-edited Correlation Spectroscopy (COSY). This approach can easily be extended to other body fluids like bile, follicular fluids, saliva, etc.

Keywords BCAA · NMR · Metabolites · COSY · CPMG

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S. Ghosh and A. Sengupta are equally contributed to this work.

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✉ Kousik Chandra
kousikchandra@gmail.com; kchandra@sif.iisc.ernet.in; kousik.chandra@jku.at

[1] Department of Chemical Sciences, Tata Institute of Fundamental Research, Homi Bhabha Road, Colaba, Mumbai 400005, India

[2] Laboratory of Clinical Investigation, National Institute on Aging, 251 Bayview Boulevard, Baltimore, MD 21224, USA

[3] Department of Systems Pharmacology and Translational Sciences, University of Pennlsylvania, 3400 Civic Center Boulevard, Philadelphia, PA 19104, USA

[4] Indian Institute of Science, Bangalore 560012, India

[5] Present Address: Institute of Organic Chemistry, Johannes Kepler Universität Linz, Altenberger Straße 69, TNF Turm, 3rd floor, 4040 Linz, Austria

Introduction

The field of metabolomics has emerged as an efficient tool for studying response to environmental and/or genetic stimuli (Nicholson et al. 2008), and toxicological studies (Robertson 2005) in vivo. Metabolomics involves generation of high-throughput data from bio-fluids followed by several multivariate data analysis techniques. This process establishes particular changes in metabolite composition of the bio-fluid followed by further experiments to confirm the identity of the metabolites contributing to the changes.

The analytical techniques which are most widely used in the field of metabolomics include NMR (Wishart 2008) and Mass spectrometry, coupled with several pre-separation techniques (Dettmer et al. 2007). The inherent advantages of NMR spectroscopy, which includes ease of sample preparation, unbiased approach towards sample, appreciably less time requirement for data acquisition and easy methods for quantification of the spectral peaks, made

NMR an obvious method of choice for detailed analysis. One of the key steps in the NMR metabolomics workflow is the identification of the several metabolites in a single experiment. This enables one to monitor them in different sample in a systematic manner. Metabolite quantification of serum sample using ^{1}H NMR spectra is straightforward except when there is peak overlapping. In this context, it is important to note that the quantification of overlapping NMR peaks arising from valine, leucine and isoleucine (resonating at 0.92–0.99 ppm) is difficult. ^{1}H NMR quantification for three individual metabolites is thus erroneous. The broad lipid peak resonating at 0.89 ppm (CH_3 group) further complicates the situation. Although Traditional 2D homonuclear experiments like COSY, TOCSY and/or hetero-nuclear ^{1}H–^{13}C correlation spectroscopy HSQC are known to resolve overlapping peaks and are used for the identification of small molecular weight metabolites in various metabolomics studies (Ludwig and Viant 2010), it does not solve the present issue as one of the cross peaks of Branched Chain Amino Acid (BCAA) has some component of lipids and hence is not useful for quantitation. A similar situation prevails for quantification of lactic acid in serum which resonates at 1.33 ppm and is overlapped by a large and broad lipid peak. Therefore, in order to fully exploit the advantages of 2D spectroscopy, it is important to use certain spectral editing technique to attenuate the broad resonances. In earlier studies, variant of COSY has been used to extract the different relaxation properties from tissue metabolites. Nevertheless, the relaxation properties could be used to specifically observe fast or slowly relaxing components in bio-fluid. Such an approach was presented earlier using relaxation filter for bio-fluid analysis (Tang et al. 2004; Williams et al. 1988). In this article, a similar strategy is used. We used a variant of COSY pulse program in which a CPMG spin echo sequence is attached directly to the COSY sequence for the purpose of the T_2 editing of large bio-macromolecules. Applying a CPMG spin echo sequence $(\tau\text{–}180^\circ\text{–}\tau)_n$, and manipulating spin echo time (τ, half of the delay between two successive 180° pulses) and number of loops (n) help in partial or complete elimination of the resonances from large macromolecules and small molecules bound to them owing to large differences in their respective tumbling times. The parameters, namely, τ and n, are extensively optimized for the optimal attenuation of the different bio-macromolecules present mainly in the overlap region of branched chain amino acids (BCAAs) in sera. In addition to that, calibration curves are prepared for metabolites (lactic acid, glutamine and BCAAs) of known concentration to aid in the determination of concentration of individual metabolites. Therefore, this approach promises to be of utility in the field of metabolomics.

Materials and methods

Animal handling

The animal was treated in accordance with the guidelines set forth by the Institutional Animal Ethics Committee of TIFR (IEAC Approval No: TIFR/IEAC/2010-3). Female C57BL/6 mice aged 6–8 weeks and weighing 20–25 g were used for the study. It was kept under 12 h day and night cycle.

Sample preparation for ^{1}H NMR of sera

Blood was collected from the mouse by retro-orbital bleeding. The blood sample was incubated at 37 °C for 10 min and was centrifuged for 10 min at 13100g at 4 °C. The supernatant was collected and frozen immediately in liquid N_2 and was further stored at −80 °C till NMR experiment. For NMR experiments, 300 µl of the sample was mixed with 300 µl of phosphate buffer (0.075 M $Na_2HPO_4{\cdot}7H_2O$, 12 µl of 4 % NaN_3, 0.02 % DSS, pH 7.4). The buffer recipe was provided by Bruker Biospin, metabonomics unit. The pH of the samples was checked before experiments.

Optimization of parameters in T_2-edited COSY

The magnitude mode variant of the COSY experiment with presaturation was used for all the relevant experiments and spectral editing (Bruker Pulseprogram COSYPRQF). The T_2-edited COSY was generated by introducing the CPMG spin echo block $(\tau\text{–}180\text{–}\tau\text{–})_n$ before the last 90° pulse in COSY. The pulse program is shown in Figure S1. This pulse sequence was previously used for understanding slowly relaxing species in malignant tissues. In this study, we have optimized the CPMG block parameters, namely the loop count, 'n', while fixing the echo time, 'τ', so that the attenuation of the broad signals especially in the region of BCAA is achieved. The relaxation delay is kept as 1 s. We used the echo time (τ) of 300 µs and recorded 2D spectra with various loop counts like 200, 400, 550, 700 and 1000. All the spectra are processed in the similar way as that of COSY. All the experiments were recorded with ^{1}H carrier placed at on water (4.7 ppm). The number of complex points was 2048 and 200 and corresponding acquisition time was 142 and 13.8 ms in direct and indirect dimensions, respectively. Each 2D experiment was performed with 32 scans which resulted in total measurement time of 3 h 19 min. All data were processed with sine window function. All data were zero-filled to 512 and 2048 complex points along t_1 and t_2, respectively.

Preparation of solutions for calibration experiment

Stock solutions of (0.1 M) of leucine isoleucine, valine, glutamine and lactate (SISCO Pvt Ltd.) were prepared. The dilutions of the stock leucine, valine, isoleucine, lactate and glutamine were done to obtain six known concentrations for the calibration curve (shown in supplementary Figure S2). The linearity of the individual components confirms the validity of NMR for accurate quantification. The concentration of the same set of samples was measured with BCAA assay kit (purchased from Sigma Aldrich) mixture to show the correlation between two methods. For BCAA assay, the calibration curve was achieved using a known concentration of Leu standard sample supplied with the kit. The correlation coefficient was obtained using R 3.1.1. BCAA assay results were plotted against the total concentrations of the mixture using SIGMAPLOT 10. We also made samples of 8 different concentrations of mixture of Leu, Val and Ile in the presence of 300 mg/ml BSA (Bovine Serum Albumin) and sodium formate (100 μM) to focus on the effect of small molecule–macromolecule binding on relaxation times and mimic the cellular condition. The cross-peak intensity was measured in TOPSPIN 3.2 and CARA. In the process of integration, we fix a particular contour level for a particular cross peak and integrate the cross peak across all the spectra at that contour level. The area of the cross peak is fitted against the concentration of the particular metabolite to a straight line in SIGMAPLOT 10.

Spike-in experiment

We have performed concentration measurement using spike-in experiments. In this case, serum solution was divided into four parts, and in one part nothing was added which served as control. In the rest, three samples, known amount of Val, Ile and Leu, were added. In all the samples, total solution volumes were kept identical. Since peak volume is proportional to the sample concentration, the concentration of control sample was obtained independently with the three different spike-in experiments. The average was compared with the concentration obtained from the calibration curve.

Results

Analysis of 1D ^{1}H NMR of whole sera

The peaks from leucine, isoleucine and valine resonate in the region of 0.92–1.02 ppm are overlapped with each other and shown in Fig. 1a, b for serum samples. The resonances consist of doublets and triplets from the methyl groups of the Leu, Val and Ile. This is explained in Fig. 1c. This high degree of overlap prevents these resonances to be estimated in a quantitative manner. Additionally, methyl resonances, belonging to the lipid moieties of serum lipoproteins, result in a broad hump in this region which further complicates the situation. Although incorporation of CPMG spin echo

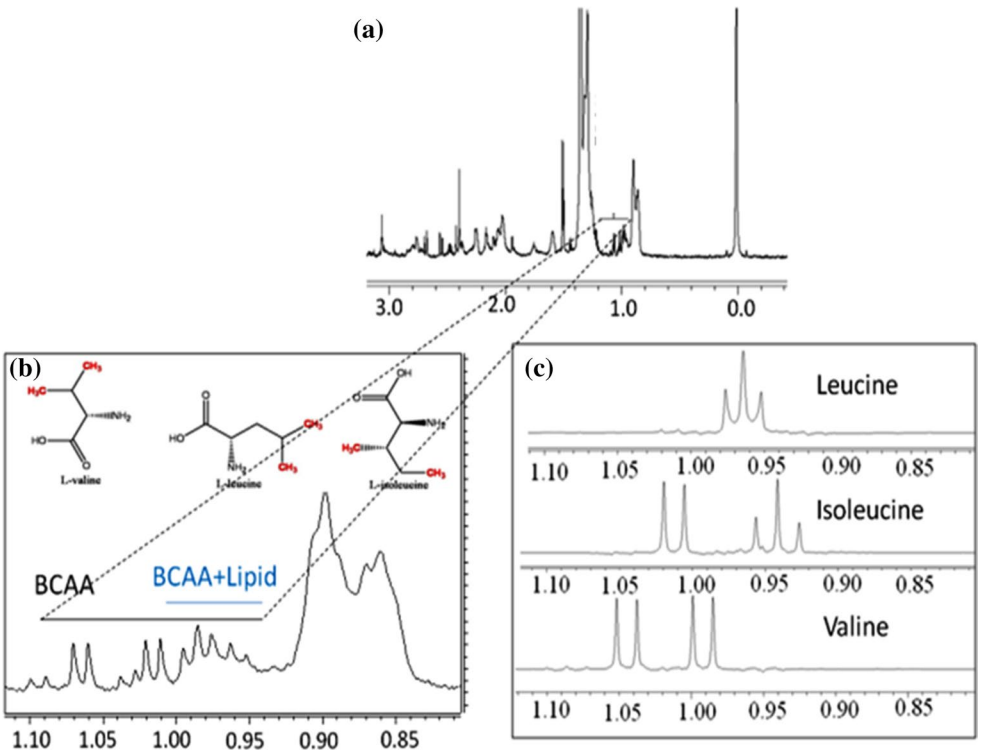

Fig. 1 Typical 700 MHz ^{1}H NMR spectrum of serum sample and equimolar mixture of BCAA. **a** ^{1}H CPMG NMR spectrum of female C57BL/6 mouse (0–3 ppm). **b** Zoomed region of NMR spectrum containing BCAA and lipoprotein. **c** ^{1}H NMR stalked spectra of leucine, isoleucine and valine

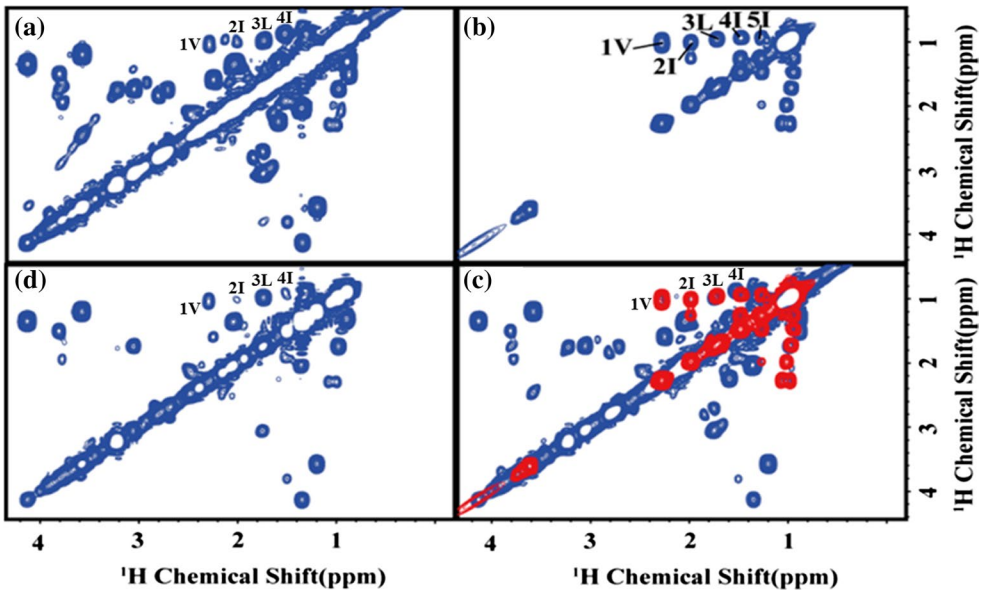

Fig. 2 Typical 700 MHz 2D NMR spectrum of serum sample and BCAA. **a** (^{1}H–^{1}H) COSY NMR spectrum of serum sample. **b** (^{1}H–^{1}H) COSY NMR spectrum of mixture of equimolar mixture of BCAA. The cross peaks of BCAA are assigned 0.1—Valine, 2—Isoleucine, 3—Leucine. **c** Overlap of (**b**) over (**a**). **d** CPMG-COSY of serum sample

helps in attenuating this hump in 1D ^{1}H NMR spectroscopy, a complete attenuation is usually not possible as shown in Fig. 1b. To resolve the peaks from the branched chain amino acids and the lipids, we have recorded 2D COSY spectrum of the serum sample. This spectrum is shown in Fig. 2a. To further identify the cross peaks of three BCAAs in serum samples, a 2D COSY spectrum of the mixture of BCAAs was acquired and shown in Fig. 2b. Further, these two spectra were overlapped to identify the signature cross peaks of BCAA in serum. The overlapped spectrum is shown in Fig. 2c. However, the cross peaks of BCAA have contributed from lipoproteins. Therefore, there is still scope to resolve the COSY spectrum to obtain the small molecule-specific information. To attenuate the signals from the lipid, a T_2-edited COSY was employed. The pulse program of COSY and T_2-edited COSY is depicted in supplementary Figure S1. To optimize the attenuation from large molecules, the loop count (n) was carefully varied and the spectrum was recorded at each loop count of 200, 400, 550, 700 and 1000.

These T_2-edited spectra were further compared with the COSY spectrum of the same sample and at same contour level to select the optimized attenuations and is depicted in Fig. 3. It can be seen that the peaks 1, 2 and 3 from valine, leucine and isoleucine show a gradual decrease in intensity with the increase in loop counts with respect to COSY. However, the peak 4 shows a drastic decrease in intensity at 200, 400 and 550 loop counts. It is apparent that peak 4 has lipid component, unlike in peaks 1–3 representing valine, leucine and isoleucine, respectively. For peak 4, the drastic decrease in intensity continues till 550 loop count. These peaks (peaks 1–3) are further used for quantification in calibration experiment. Peak 5 is not used for quantification

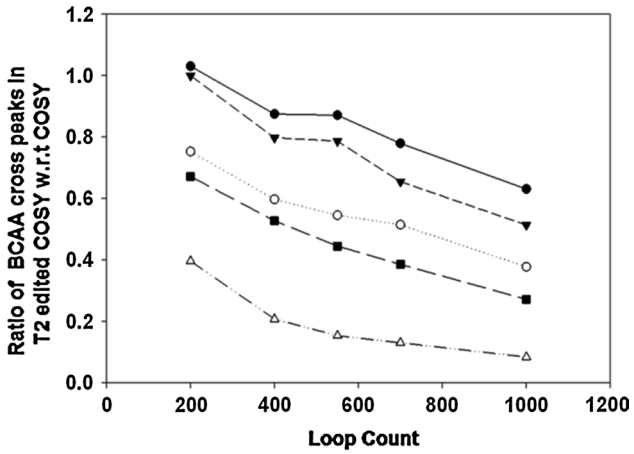

Fig. 3 Ratio of the peak volume of various cross peaks in CPMG-COSY with that of COSY at various loop count. Relative intensity of cross peaks of BCAA at different loop count w.r.t. COSY spectrum the symbols are cross peaks of, *filled circle* (1), *filled inverted delta* (2), *circle* (3), *delta* (4), *filled square* (5). The *numbers* refer to peaks in Fig. 2

because it was very near to diagonal stretch and hence might suffer from the overlapping peak of strong diagonal. All the loop counts from 400 onwards attenuate the broad signals. To avoid train of π pulses which can cause sample heating, the number of loops to be used in the experiment was kept minimal. For the requirement of selectivity with maximum sensitivity, an optimal situation was found at a loop count of 550 where the macromolecular signals were mostly attenuated keeping the small molecular signals intact. With this the acquired CPMG-COSY spectra is depicted in 2D which is much more "clean" compared to normal COSY. Moreover, we could resolve the peaks

of BCAA from that of lipid peak and we derive signature cross peaks to be integrated in a CPMG-COSY spectrum for specific quantification of BCAAs.

Quantification of BCAAs

Another important aspect of small molecular metabolite analysis is the quantification of the metabolite. Quantification of metabolites from 2D spectroscopy is not straight forward as the cross peak volume depends on various experimental parameters like J coupling, spin–spin relaxation time, evolution time, etc. (Gronwald et al. 2008; Hu et al. 2007a, b). Although ^{13}C–^{1}H HSQC is used for quantification of metabolites (Rai et al. 2009), the experimental time required for recording ^{13}C–^{1}H HSQC is very large owing to the fact that natural abundance of ^{13}C is less. Mainly two ways of approaches are considered to obtain quantitative information. The first approach relies on estimation of some samples of known concentration and preparation of calibration curve for it, keeping all experimental parameters fixed. Based on this, unknown concentration of certain compounds in the mixture sample can be determined. The other method depends on the recording of experiments which takes account of the loss of intensity during the pulse sequence and obtaining the intensity information after the first $\pi/2$ pulse which is quantitative as shown recently (Hu et al. 2007a). However, the second approach demands density matrix at the end of the pulse program to be same as that after first $\pi/2$ pulse. This is not the case with (^{1}H–^{1}H) COSY experiments, so we have followed the first strategy. This technique is used in many analytical techniques like spectro-photometry and chromatography.

Calibration curves were created from the cross peaks generated by CPMG-COSY spectra of mixture of metabolites of different known concentrations (mentioned earlier). The cross-peak volumes of individual metabolite were plotted against different concentration of the metabolite to create a calibration curve. Such curves for five metabolites, namely valine, leucine, isoleucine, glutamine and lactate are demonstrated in Fig S2. We also compare our NMR results with conventional method, like using the BCAA Assay Kit for the same set of mixtures. Excellent agreement in determining the total concentration was obtained as shown in Fig. 4a. The correlation coefficient was found to be 0.998 and slope of the curve was found to be 0.900 confirming the robustness of the method. Additionally, the individual concentration of the metabolites can be obtained using NMR as those can be monitored separately. Using BCAA Assay Kit measurement, this is not feasible as absorbance cannot be measured separately for different metabolites. For BCAA assay, calibration curve was built using various diluted concentrations of supplied Leu standard. The standard curve is shown in Fig. 4b. We also assess

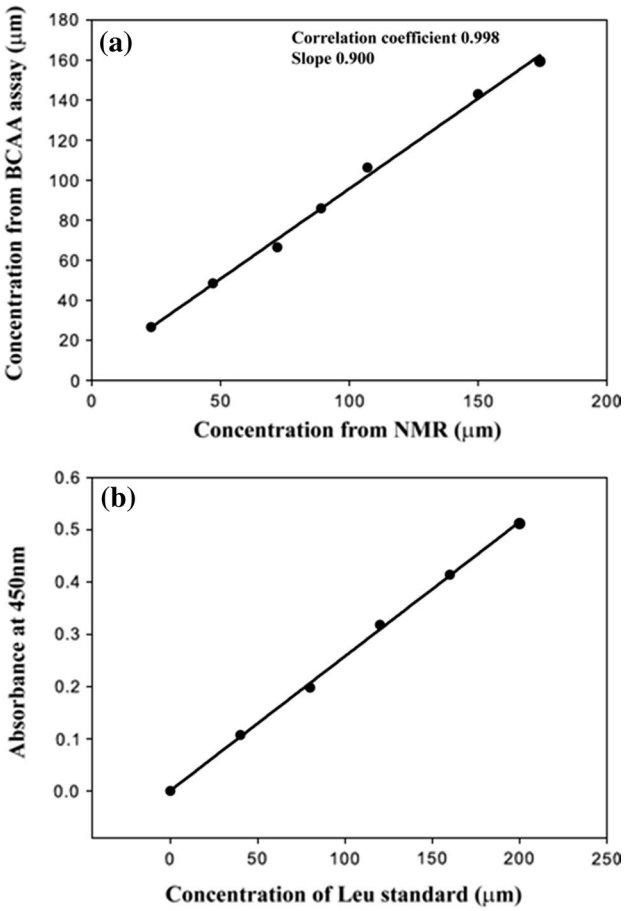

Fig. 4 **a** Correlation plot for total concentration of mixtures of Val, Ile and Leu determined using NMR (plotted on *x* axis) and using BCAA assay kit (plotted along *y* axis). The correlation coefficient was found to be 0.9982 and the slope was 0.900 indicating excellent correlation between two methods of measurement. **b** Calibration curve for BCAA assay determined by measuring absorbance at 450 nm of different known supplied concentrations of standard Leu sample

the role of crowding as macromolecular matrix components can affect the response of individual BCAAs differently in the CPMG experiment. Hence we have followed the known concentration of the mixture of metabolites in the presence of 300 mg/ml BSA and sodium formate (100 μM) which mimics the cellular environment. Here, linear correlation was also obtained as calibration curve, shown in Fig. 5. From the figure, it could be seen that all the individual metabolites show a linear trend which suggests that with the help of a standard calibration curve, the quantification of specific metabolite can be achieved for unknown samples. Apart from validating our result with BCAA Assay Kit, we also have performed spike-in experiments for further validation. We also compare our NMR results with the average of the concentration obtained using three different spike-in experiments for same serum sample. All the

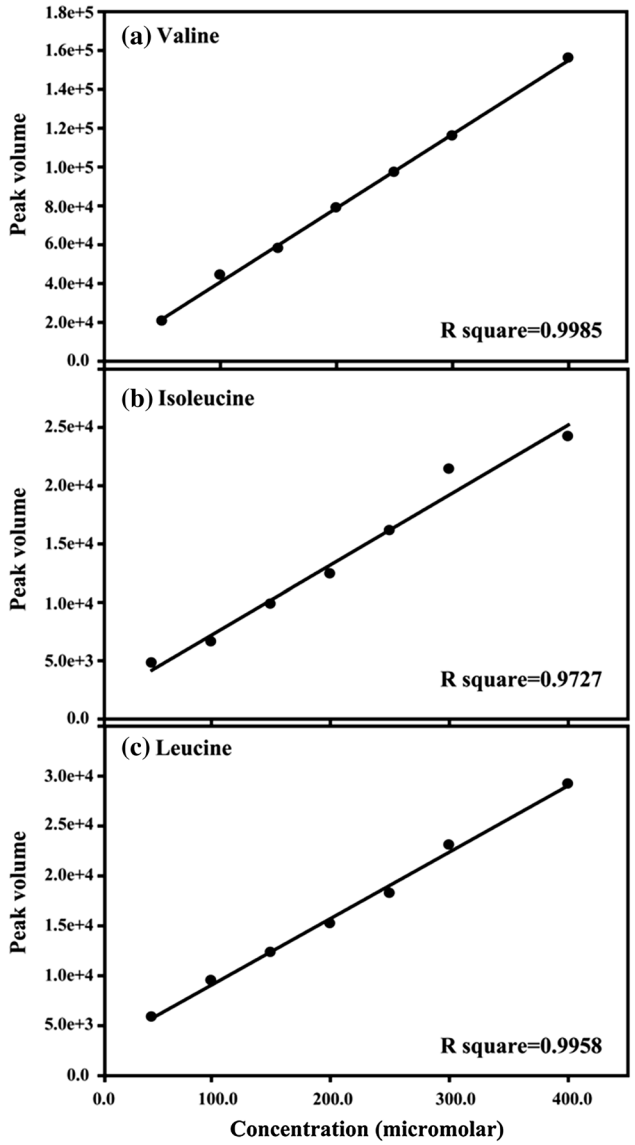

Fig. 5 Calibration graph of various metabolites from the cross peak volume of 2D CPMG-COSY with the concentration of the metabolite in the presence of 300 mg/ml BSA and sodium formate. **a**, **b** and **c** represent valine, isoleucine and leucine, respectively. Peak volume is plotted against the concentration of the mixture and linear fit confirms the validity of NMR as a tool for quantification method

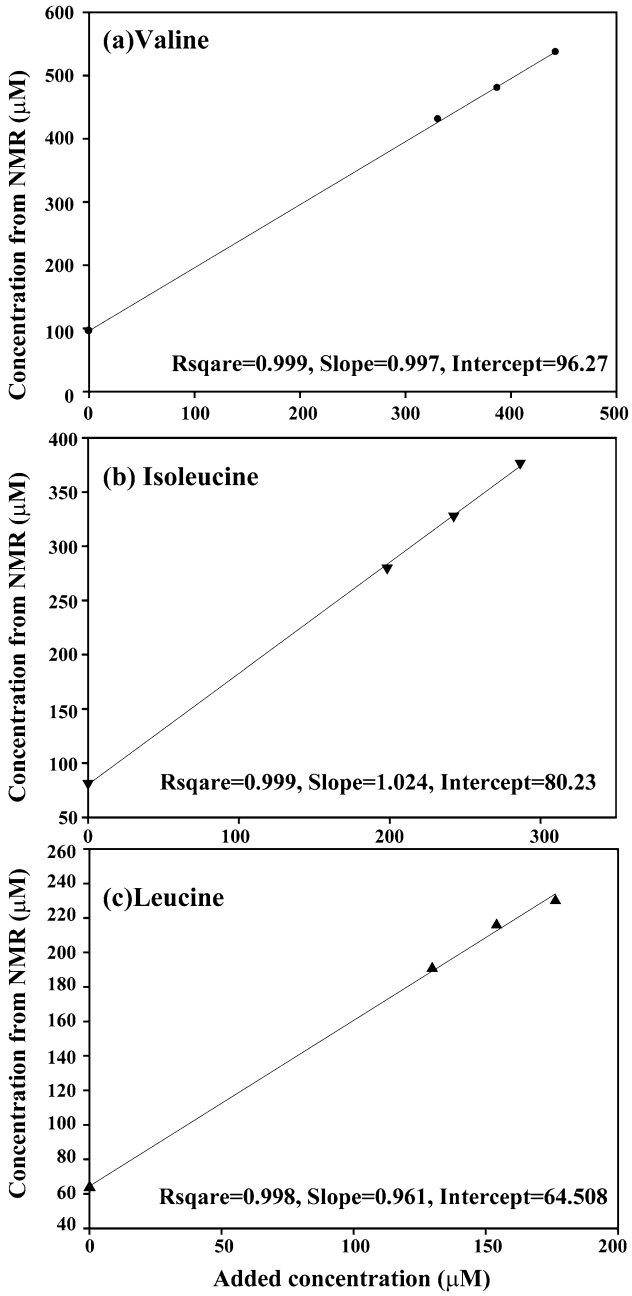

Fig. 6 Correlation graph of concentration of individual amino acid determined using spike-in experiments and using NMR calibration curve. The individual curves for each compound are represented as **a** Val (*filled circle*), **b** Ile (*filled inverted delta*) and **c** Leu (*filled delta*), respectively. The *x* axis represents the added concentration in spike-in experiment and y axis represents concentration determined from NMR. The blank value is also included. The Chi square (R square) value, the axis intercept and the slope have been indicated for each between two methods of measurement

three different amino acids showed excellent agreement as confirmed by Chi square (R square) value and the slope indicated for each case in Fig. 6. The blank value is also included in the figure.

Discussion

Small molecular weight metabolites are implicated in various disease conditions. For example, lactic acid is known to be increased in the blood of patients during the events of severe acidosis (Bakker et al. 1996). Common diseases such as severe malaria are characterized by heavy lactic acidosis and increase in other organic acids, such as beta-hydroxybutyric acid in the blood of the patients (Sasi et al. 2007). Branched chain amino acids such as valine, leucine

and isoleucine are also implicated in different disease conditions. For example, Maple Syrup Urine Disease (MSUD) is characterized by the disorder of BCAA metabolism, specifically by the lack of branched chain alpha-keto acid dehydrogenase complex (Harris et al. 1990). The role of BCAA metabolism in heart disease is also recently reviewed (Huang et al. 2011). It was observed that plasma leucine and isoleucine levels are elevated, while valine levels remain unaltered. On the other hand, in case of 'Hypervalinemia' there is a perturbation in the level of only valine (Tada and Arakawa 1967). In schizophrenic disease, isoleucine levels are perturbed in serum (De Luca et al. 2008). BCAAs are implicated in other situations as well. For example, the supplementation of BCAAs during physical activity and exercise is practiced for decades. For example, an increased tryptophan and decreased BCAA in the blood plasma after a long duration of exercise were indicated to be a cause of the physical and mental fatigue (Blomstrand et al. 1988). Similarly, the ratio between BCAAs and aromatic amino acids is implicated in several physiological conditions owing to the fact that the ratio is often a good predictor of their preferential transport to the brain (Crandall and Fernstrom 1983). Other pathophysiological conditions such as alcohol withdrawal also showed an important effect on this ratio (Saito et al. 1994). Therefore, identification of these metabolites is important in terms of understanding the different disease conditions and management. In several cases, the overall concentrations of the BCAAs are required, while in some conditions concentration of the individual BCAAs is required. Thus, the quantitative signatures of individual BCAAs are extremely important in the serum or plasma.

It is already an established fact that NMR spectroscopy is an unbiased and rapid technique to identify and quantify metabolites in complex biological mixtures. However, due to reasons discussed earlier, proper identification of BCAAs in serum/plasma remains a challenge. The approach demonstrated here will find utility in this regard because the disturbances in the cross peaks due to the lipid/lipoprotein peaks could be omitted almost completely.

Conclusion

In this report, we achieved accurate identification of small metabolites like BCAAs employing CPMG COSY although the peaks of BCAAs overlap with lipid/lipoproteins. This report also demonstrates that the cross peaks arising out of the small molecules in T_2-edited COSY can be quantified with the help of a standard calibration curve. This approach will find utility in the area of metabolic profiling for other small molecules in various bio-fluids which are rich in protein/macromolecule mixtures or bio-fluids such as blood plasma.

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Conflict of interest The authors declare no conflict of interest.

References

Bakker J, Gris P, Coffernils M, Kahn RJ, Vincent JL (1996) Serial blood lactate levels can predict the development of multiple organ failure following septic shock. Am J Surg 171:221–226

Blomstrand E, Celsing F, Newsholme EA (1988) Changes in plasma concentrations of aromatic and branched-chain amino acids during sustained exercise in man and their possible role in fatigue Acta physiologica. Scandinavica 133:115–121

Crandall EA, Fernstrom JD (1983) Effect of experimental diabetes on the levels of aromatic and branched-chain amino acids in rat blood and brain. Diabetes 32:222–230

De Luca V, Viggiano E, Messina G, Viggiano A, Borlido C, Viggiano A, Monda M (2008) Peripheral amino acid levels in schizophrenia and antipsychotic treatment. Psychiatry Investig 5:203–208

Dettmer K, Aronov PA, Hammock BD (2007) Mass spectrometry-based metabolomics Mass spectrometry reviews 26:51–78

Gronwald W et al (2008) Urinary metabolite quantification employing 2D NMR spectroscopy. Anal Chem 80:9288–9297

Harris RA et al (1990) Regulation of the branched-chain alpha-ketoacid dehydrogenase and elucidation of a molecular basis for maple syrup urine disease. Adv Enzyme Regul 30:245–263

Hu K, Westler WM, Markley JL (2007a) Simultaneous quantification and identification of individual chemicals in metabolite mixtures by two-dimensional extrapolated time-zero (1)H-(13)C HSQC (HSQC(0)). J Am Chem Soc 133:1662-1665

Hu F, Furihata K, Kato Y, Tanokura M (2007b) Nondestructive quantification of organic compounds in whole milk without pretreatment by two-dimensional NMR spectroscopy. J Agric Food Chem 55:4307–4311

Huang Y, Zhou M, Sun H, Wang Y (2011) Branched-chain amino acid metabolism in heart disease: an epiphenomenon or a real culprit? Cardiovasc Res. doi:10.1093/cvr/cvr070

Ludwig C, Viant MR (2010) Two-dimensional J-resolved NMR spectroscopy: review of a key methodology in the metabolomics toolbox. Phytochem Anal 21:22–32. doi:10.1002/pca.1186

Nicholson JK, Holmes E, Elliott P (2008) The metabolome-wide association study: a new look at human disease risk factors. J Proteome Res 7:3637–3638

Rai RK, Tripathi P, Sinha N (2009) Quantification of metabolites from two-dimensional nuclear magnetic resonance spectroscopy: application to human urine samples. Anal Chem 81:10232–10238

Robertson DG (2005) Metabonomics in toxicology: a review. Toxicol Sci 85:809–822

Saito T, Kobatake K, Ozawa H, Ogata M (1994) Aromatic and branched-chain amino acid levels in alcoholics. Alcohol and alcoholism (Oxford, Oxfordshire) 29:133–135

Sasi P et al (2007) Metabolic acidosis and other determinants of hemoglobin-oxygen dissociation in severe childhood Plasmodium falciparum malaria. Am J Trop Med Hyg 77:256–260

Tada KWY, Arakawa T (1967) Hypervalinemia. Its metabolic lesion and therapeutic approach. Am J Dis Child 113:64–67

Tang H, Wang Y, Nicholson JK, Lindon JC (2004) Use of relaxation-edited one-dimensional and two dimensional nuclear magnetic resonance spectroscopy to improve detection of small metabolites in blood plasma. Anal Biochem 325:260–272

Williams PG, Saunders JK, Dyne M, Mountford CE, Holmes KT (1988) Application of a T2-filtered COSY experiment to identify the origin of slowly relaxing species in normal and malignant tissue. Magn Reson Med 7:463–471

Wishart DS (2008) Applications of metabolomics in drug discovery and development. Drugs R&D 9:307–322