	L I V	1 2 8 3	B	Dispatch: 14.4.06 Journal: LIV	CE: Shalini
	Journal Name	Manuscript No.		Author Received: No. of pages: 5	Saravan A/Anand Kumar

Liver International 2006: 26: 1–5

Liver International
DOI: 10.1111/j.1478-3231.2006.01283.x

Detection of new amino acid markers of liver trauma by proton nuclear magnetic resonance spectroscopy

Ranjan P, Gupta A, Kumar S, Gowda GAN, Ranjan A, Sonker AA, Chandra A. Detection of new amino acid markers of liver trauma by proton nuclear magnetic resonance spectroscopy.
Liver International 2006: 26: 1–5.
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Abstract: *Objective:* We examined serum in patients of liver injury to explore the possible clinical application of abnormal micrometabolites as a marker of liver injury and severity in cases of traumatic liver damage. *Methods:* Serum were screened by proton nuclear magnetic resonance spectroscopy in 96 patients with varying degree of liver injury and compared with concentrations in healthy control volunteers. *Results:* Large quantities of phenylalanine and tyrosine were detected by spectroscopic analysis in patients with liver injury but not in those without liver injury ($P<0.001$). Proton nuclear magnetic resonance spectroscopy revealed two unique amino acids, phenylalanine and tyrosine, in the sera of the subjects with liver injury, irrespective of the extent and type of injury gauged by radiology or laparotomy. Phenylalanine spectrum was obtained in all 84 patients with liver injury (100% sensitivity) whereas tyrosine spectrum was present in 83 out of 84 patients (98.8% sensitivity) suggesting that these amino acids were specifically released in the patients of liver injury. Significant correlations were observed between phenylalanine and tyrosine concentrations and total bilirubin levels and albumin levels. Serum phenylalanine and tyrosine concentrations correlated well with imaging and laparotomy findings of liver injury. *Conclusion:* Phenylanaline and tyrosine appear to be specific and new markers of liver injury.

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Keywords: liver – NMR spectroscopy – serum markers – trauma

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Received ■ ■ ■,
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Despite its relatively protected position in the rib cage, liver is the most commonly injured organ following blunt abdominal trauma (1). Imaging techniques like ultrasonography, computed tomography scan and magnetic resonance imaging have certain limitations in diagnosing solid visceral abdominal injury in the emergency setting. Unlike spleen and kidney, liver injury exudes enzymes in sera after hepatocyte damage. Routine assessment of liver function is done by monitoring serum bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase.

Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) is a new evolving technique to analyze biofluids, including sera, for micro-metabolites. It has been applied in non-selective detection of a number of molecular markers in biofluids (2). Liver graft dysfunction and subsequent rejection in the post-operative period was predicted through urea and glutamine markers in biofluids by this technique, by investigators at Sanjay Gandhi Postgraduate Institute (3).

The above observations and detection of micro-metabolites by ^{1}H NMR spectroscopic analysis prompted investigators to search for new markers in cases of liver injury. The present preliminary work was on a cross-section of patients of blunt abdominal trauma. Our hypothesis was that the physiological changes arising from the trauma to hepatocytes (liver injury) will lead to release or accumulation of metabolites unique

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to liver injury, not found in other abdominal visceral injury, principally in form of amino acids.

Patients and methods

All patients hospitalized with blunt abdominal injury from July 2003 to December 2004 to our emergency department were included in this study. After initial resuscitation, 2 ml of each blood specimen was obtained by venipuncture aseptically. The blood was then placed in a sterile stoppered test tube and was allowed to coagulate for 30 min. The separated serum was pipetted out and was placed in a sterile eppendorf tube. The separated sera were transported in dry ice to the Center of Biomedical Magnetic Resonance (CBMR), Sanjay Gandhi Postgraduate Institute on Medical Sciences, Lucknow to be analyzed by ^{1}H NMR one-dimension spectroscopy at 22 °C (Bruker Avance 400) on the same day.

A total of 96 sera were analyzed from 58 patients of isolated liver injury, 26 patients of liver along with another visceral injury, 12 patients of other solid viscera (spleen – 6, kidney – 4 and pancreas – 2) injury and 38 subjects with no significant intraabdominal injury. The final diagnosis was achieved after consideration of ultrasonography, computed tomography and laparotomy that was performed in 52 subjects. The sera were coded and the investigators were blinded to the clinical diagnosis.

The NMR experiments on the untreated sera samples were performed on a Bruker Biospin Avance 400 MHz spectrometer using 5 mm Broad Band Inverse probe at 22 °C. Samples of 450 µl sera were taken in 5 mm NMR tubes. A sealed capillary containing precalibrated 35 µl (0.14 mg/dl) of 0.375% trimethyl silyl propionic acid sodium salt (TSP) deuterated at CH_2 groups dissolved in deuterium oxide was inserted into the NMR tube containing the sera samples for recording the spectra. While TSP served as a chemical shift reference as well as the standard signal for absolute quantitative estimation of the metabolites, deuterium oxide served as the 'field-frequency-locking' solvent. For all the sera samples, one-dimensional ^{1}H NMR experiments were performed at 22 °C by suppression of water resonance by presaturation and Carr–Purcell Meiboom–Gill (CPMG) sequence with a total mixing time of 269 ms to remove the broad resonances arising from macromolecules. Parameters used were: spectral width: 8000 Hz; time domain points: 32 K; relaxation delay: 3 s; pulse angle: 45°, number of scans: 64; spectrum size: 32 K and line broadening: 0.3 Hz. For confirmation of assignment of metabolites, one-dimensional spectra of standard phenylalanine and tyrosine dissolved in deuterium oxide solvent were also recorded separately. The quantification of the metabolites in all sera samples was carried out using the software program NMRQUANT, available on the spectrometer, with respect to a known concentration of TSP (0.14 mg/dl) serving as an external reference.

Results

A total of 134 sera (84 subjects had liver injury and 50 had either other solid visceral injury or no significant visceral injury) were analyzed. The subjects were distributed in four groups: A – isolated liver injury ($n = 58$); B – liver injury along with other solid visceral injury ($n = 26$); C – other solid visceral injury ($n = 12$) (spleen – 6, kidney – 4, pancreas – 2) and D – subjects with history of abdominal trauma but no significant abdominal visceral injury ($n = 38$). Thus groups A and B constituted subjects with liver injury and groups C and D served as control (Table 1).

^{1}H NMR spectroscopy revealed two unique amino acids, phenylalanine and tyrosine, in the sera of the subjects with liver injury, irrespective of the extent and type of injury gauged by radiology or laparotomy (Fig. 1). Phenylalanine spectrum was obtained in all 84 patients with liver injury (100% sensitivity) whereas tyrosine spectrum was present in 83 out of 84 patients (98.8%

Table 1. Mean values and standard deviation of the biochemical and ^{1}H NMR parameters, compared with conventional measures of liver enzymes and bilirubin

		Mean (range)			Mean, SD	
Group	Type of injury	SGOT (IU/l)	SGPT (IU/l)	Serum bilirubin (mg/dl)	Phenylalanine (mg/dl)	Tyrosine (mg/dl)
A ($N = 58$)	Liver injury (isolated)	535 (88–986)	792(148–1705)	3.46(1.4–5.7)	17.56, 23.18	20.37, 27
B ($N = 26$)	Liver injury (associated with other organ injury)	320 (76–853)	347(48–1075)	3.05(1.4–4.2)	20.61, 26.62	21.58, 35.4
C ($N = 12$)	Other organ injury	30 (25–38)	37(24–55)	0.8(0.6–0.9)	ND	3.87, 1.02
D ($N = 38$)	Blunt abdominal trauma – no visceral injury	33 (24–40)	31(23–45)	0.88(0.6–1.1)	ND	1.18, 0.18

ND, not detectable (assumed to 0.1 for the purpose of calculation); SD, standard deviation.

sensitivity) suggesting that these amino acids were specifically released in the patients of liver injury. The sera of 50 control subjects showed short and insignificant peaks of amino acid tyrosine, which was statistically much lower compared with patients with liver trauma. The presence of these amino acids correlated well with serum bilirubin levels. A correlation of these metabolites with routine biochemical measures of liver injury is presented in Table 1.

All other metabolites were similar in both the groups and there was no statistically significant difference in the quantity/spectra of other metabolites.

The data were analyzed using non-parametric Kruskal–Wallis test for the multiple groups and showed statistically highly significant differences between the groups with liver injury and no liver injury (Kruskal–Wallis statistics: phenylalanine: KW = 34.407, $P < 0.0001$; tyrosine: KW = 21.667, $P < 0.0001$; Table 1).

A complete illustration of the serological spectra of a typical patient with liver injury is provided (Fig. 2).

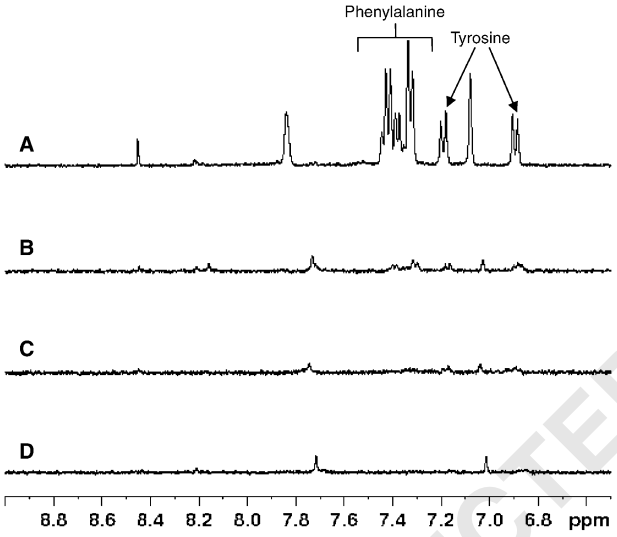

Fig. 1. Typical proton nuclear magnetic resonance spectroscopy (^{1}H NMR) spectra of the serum shows: (A) spectra of a patient with isolated liver injury; (B) spectra of a patient with injured liver coexisting with another intraabdominal visceral injury; (C) spectra of a patient with other visceral injury; (D) spectra of a patient with no significant intra abdominal injury.

Discussion

Blunt abdominal injury is commonly found in road traffic accidents. Liver injury is not uncommon in these patients. In patients with blunt abdominal trauma with strong clinical suspicion of liver damage, specific monitoring is traditionally done by repeated clinical observations, sonography and/or computed tomography. Not infrequently the liver injury is left undetected or diagnosed late because of limitations of emergency imaging (4). Also the CT suite is often unsuitable for acutely ill patients. In advance liver disease, aromatic amino acids viz phenylalanine, tyrosine and tryptophan accumulate in the blood then branched chain amino acids due to defective hepatic catabolism (5). A serum marker, which is sensitive and specific for liver injury besides its quantitative and serial estimations, and can predict the extent and outcome of liver trauma patients, is desirable. Phenylalanine and tyrosine in serum may fulfill this very important need of a marker of liver injury. Thorough hand and computer-assisted med-line search of medical literature has not shown phenylalanine and tyrosine reported as markers of hepatic injury although they have been described in several studies relating liver function with plasma amino acids.

Alterations in serum phenylalanine and tyrosine levels have also been described either individually or in combination in various other pathological conditions including infections,

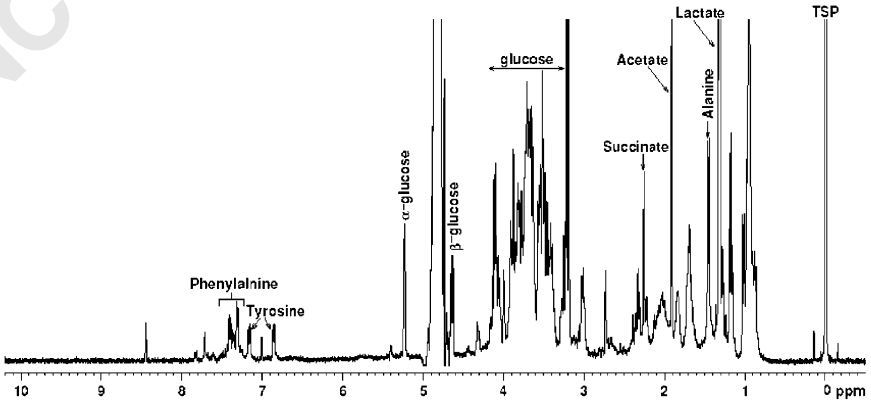

Fig. 2. Typical proton nuclear magnetic resonance spectroscopy (^{1}H NMR) spectra of the serum of a patient with liver injury.

severe burns, liver diseases and encephalopathy along with states of surgical stress (6–10). This association of elevated levels of phenylalanine and tyrosine is not new. Rosen et al. (6) analyzed the plasma amino acid patterns in hepatic encephalopathy of differing etiology. They found that plasma amino grams tended to group into two distinct patterns depending on the etiology of the patients' hepatic pathology. Patients with chronic liver disease with superimposed acute insults, i.e., gastrointestinal bleeding, infection, alcoholic hepatitis, had elevated levels of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, as well as methionine, glutamate, and aspartate, whereas levels of the branched chain amino acids, valine, leucine, and isoleucine, were consistently depressed. Those patients with previously normal livers and acute hepatic necrosis, i.e., 'fulminant hepatitis,' had grossly elevated levels of all amino acids except the branched chain amino acids, which were normal. In yet another preclinical study correlating the alterations in the level of plasma phenylalanine and its catabolism in the liver of stressed rats, Mori et al. (7) observed elevated blood phenylalanine concentrations after trauma or infected rats with altered phenylalanine catabolism. They showed that catalytic activity of phenylalanine hydroxylase decreased to 60% of the control values and thus concluded that a reduction in enzyme levels occurs during conditions of hepatic stress. They also concluded that inadequate activation of native phenylalanine hydroxylase by regulatory mechanisms involving phenylalanine *in vivo* was also associated the accumulation of plasma phenylalanine in infected rats during massive mobilization of amino acids from muscles under conditions of enhanced and sustained catabolism. Stinnett et al. (8) also presented a preclinical data after analyzing the sequential changes in plasma and skeletal muscle free amino acids following severe burn injury. Their preclinical data revealed that the amount of total free amino acids in the plasma fell following burns and suggested relative protein deficiency. However they found that plasma phenylalanine was consistently elevated in those patients having transient hepatic dysfunction evidenced by elevated plasma live enzymes. Dale et al. (9) studied the effect of abdominal operation on plasma amino acid levels and reported that increased postoperative levels of phenylalanine, tyrosine and methionine could be due to transient liver dysfunction. The ratio of phenylalanine and tyrosine usually is a constant (10). although direct liver involvement has not been established several experimental and preclinical data have reported that this ratio gets increased during inflammatory conditions probably due to enhanced muscle catabolism. Similarly in their cross-sectional study of plasma amino acids in alcoholic cirrhosis, Shiota et al. (11) concluded that alcoholic liver disease presented a deranged plasma amino acid pattern. Pruim et al. (12) have also described the use of phenylalanine and tyrosine ratio as a measure of metabolic function of liver grafts studied in early reperfusion phase of liver transplant patients. Thus there is ample evidence in the form of preclinical and experimental data, which has established the importance of phenylalanine and tyrosine in states of liver dysfunction. The same data is extended in our study in patients of liver trauma. Thus these biomarkers detected by NMR spectroscopy could be of immense clinical importance in establishing liver injury in patients of blunt abdominal trauma.

Further studies on serial estimations of these markers two-dimensional ^{1}H NMR spectroscopic analysis – a method of sophisticated metabonomics, need to be undertaken. Falling titers of these metabolites should save unwarranted laparotomy while rising levels may alert the clinician to undertake surgery early despite hemo-dynamically stable status. In selected patients, non-operative management of liver laceration has gained acceptance. Further work is also needed to study their role as predictors of the extent of liver injury, outcome and their ability to effectively select between operative and conservative management of liver injury.

The advantage of measuring the amino acids, phenylalanine and tyrosine, by NMR spectroscopy is that they can be quantified specifically and separately with well-resolved spectra. By other means, e.g., by calorimetric method, the aromatic amino acids can not be quantified individually as all aromatic amino acids are assessed simultaneously with color formation reaction. Moreover, other methods are time consuming and labor intensive with a small but significant probability of error. The NMR spectroscopy is rapid non-invasive approach, which can be used to significantly improve the therapeutic interventions of critical care patients, once established.

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