Study on parameters of L-[1-^{13}C]phenylalanine breath test for quantitative assessment of liver function in healthy subjects and patients with hepatitis B virus-related liver disease

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Abstract The aims of this study are to investigate the feasibility and validity of the L-[1-^{13}C] phenylalanine breath test (^{13}C-PheBT) which has been used to measure hepatocyte functional capacity in hepatitis B virus-related liver disease patients and to propose validity parameters of the test in 12 healthy volunteer, 8 chronic hepatitis and 26 liver cirrhotic patients. 100mg/body nonradiative L-[1-^{13}C] phenylalanine (^{13}C-Phe) was administered orally to all subjects. Breath samples were taken before and different intervals within 360 min after administration. The $^{13}CO_2/^{12}CO_2$ enrichment was assessed by isotope ratio mass spectrometer. The parameter percentage ^{13}C excretion rate $^{13}CER_t$ (% ^{13}C dose/h) all peaked within 10-30 min after oral ^{13}C-Phe application. The parameters such as maximum value of ^{13}C excretion rate, $^{13}CER_{max}$ (% ^{13}C dose/h) (controls: 18.0±3.3; Child A: 11.0±3.8; Child B: 5.0±0.5; Child C: 3.6±1.2), ^{13}C excretion rate at 30min, $^{13}CER_{30}$ (% dose/h) (controls: 11. 9±2.1; Child A: 8.1±0.4; Child B: 6.1±0.9; Child C: 3.2±1.2), ^{13}C cumulative excretion of first 60 min, $^{13}C_{cum60}$ (% ^{13}C dose) (controls: 9.3±1.4; Child A: 6.6±0.7; Child B: 4.1±0.3; Child C: 2.6±0.9) and half time of ^{13}C excretion rate, $T_{1/2}$ (minutes) (controls: 40.4±4.4; chronic hepatitis: 53.4±4.4; Child A: 59.8±4.5; Child B: 102.0±17.3; Child C: 212.1±87.9) were effective indexes which could be employed to stage hepatocyte impairment and liver functional reserve of advanced HBV-related cirrhotic patients (i.e. healthy subjects, Child A, B, C); $T_{1/2}$ was also useful for distinguishing mild HBV-related liver injure.

Keywords Breath test, Cirrhosis, Hepatitis, Phenylalanine, Parameters

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1 Introduction

Hepatitis B virus-related liver disease is the most common hepatopathy around the world, especially in developing countries like China, which brings more and more increasing healthy and social burden. To measure liver function accurately is critical for monitoring the progression of liver dysfunction; evaluating the effect of treatment; predicting long term prognosis and risk of surgical interventions and optimal timing of liver transplant. But in clinic, there are few routine tests which are sensitive, safe, non-invasive and simple to quantify liver functional capacity.

The liver plays an important role in metabolism of aromatic amino acids such as phenylalanine metabolism. The central plasma concentration of these aromatic amino acids are largely dependent upon liver function and reserve,[1] correlating with surgical outcome.[2] Various dynamic methods are used to assess the liver state and its capacity to metabolize substrates quantitatively.[3,4] One kind of them is breath test using compounds labeled with stable isotope ^{13}C, which is administered and metabolized, then $^{13}CO_2$ is measured in exhaled air. Many kinds of breath test have been used to study series of liver function.[5]

The aims of this study were to investigate the

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feasibility and validity of L-[1-^{13}C] phenylalanine breath test (^{13}C-PheBT) for the detection of L-[1-^{13}C] phenylalanine (^{13}C-Phe) oxidation as a measure of liver function in patients with hepatitis B virus-related liver disease by comparison with the conventional clinical liver functional assessments (standard blood tests and Child-Pugh classification), and to propose validity parameters of the test for quantitative assessment of liver function.

2 Experiments

2.1 Subjects

The study protocol was approved by Ethical Committee of Huashan Hospital (Shanghai City, P. R. China), in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. And an informed written consent was obtained from each subject.

Twelve healthy volunteers (5 females and 7 males, aged from 24 to 52, (167.8±7.1) cm tall, (63.7±10.5) kg body weight) who were in good health as determined by medical history, physical examination and standard blood biochemical profile, and had not been taking any drugs no less than three months; 36 patients with hepatitis B virus (HBV)-related liver disease were recruited for this study and were divided into four groups according to liver biopsy histopathological evaluation and Child-Pugh classification：8 chronic hepatitis patients (CHP) (1 female and 7 males, aged from 27 to 46, (168.6±9.3) cm tall, (63.4±8.6) kg body weight) who were infected virus B no less than eight years, and at the same time, were all diagnosed as G_2S_2 on the basis of histopathological evaluation which was carried out by application of the virus hepatitis diagnosis national criterion (2000, Xi'an, P.R. China); 10 patients group A (Child-Pugh score 5-6) (10 males, aged from 36 to 69, (173.2±6.5) cm tall, (73.4±6.7) kg body weight); 8 patients group B (Child-Pugh score 7-9) (2 female and 6 male, aged from 32 to 51, (171.8±13.2) cm tall, (59.5±14.1) kg body weight)；8 patients group C (Child-Pugh score 10-15) (2 female and 6 male, aged from 50 to 63, (172.3±7.5) cm tall, (79.0±14.3) kg body weight). All patients had positive serum antibodies against HBV and serum HBV RNA was positive using RT-PCR. Patients with other concomitant causes of liver diseases, such as hepatitis A virus infection, hepatitis C virus infection, autoimmunity and alcohol abuse were excluded from the study. None of the patients had previously received interferon therapy and none were taking drugs known to interfere with liver function. None of the subjects was either smoker or alcohol abuse. Normal dietetical habit was maintained before the study.

2.2 Materials

L-[1-^{13}C] phenylalanine (99% ^{13}C atom isoenrichment and chemical purity more than 99%) were obtained from Isotec. Inc. (Miamisburg, Ohio, USA).

2.3 Experimental design and analytical methods

After an overnight fast subjects were administered an oral dose of 100mg/body of the nonradioactive tracer ^{13}C-Phe dissolved in 100mL water and then placed in a resting position and fasted over the test period. Breath samples were recovered by exhaling through a straw into a 10mL vacutainer (Labco Ltd, Buckinghamshire, U.K.), before administration of ^{13}C-Phe, and thereafter at 5 min intervals during the first 45 min, at 15 min intervals from 45 min to 150 min, at 30-min intervals from 150 min to 360 min after administration. The enrichment of ^{13}C in breath was measured by AP 2003 isotope ratio mass spectrometer, from Analytical Precision Products (Northwich, Cheshire, UK). The results were obtained by this technique, which were with respect to the Pee Dee Belemnitella, the international ^{13}C reference. And expressed as $\delta\,^{13}C_{PDB}$ value,[6] it was converted to a percentage of the ^{13}C-administered dose recovered in breath per hour, percentage ^{13}C excretion rate ^{13}CER (% ^{13}C dose/h)[7] where $^{13}CER = {}^{13}CER_{(t)} - {}^{13}CER_{(t=0)}$, where $^{13}CER_{(t=0)}$ was the ^{13}C excretion rate prior to the administration of labeled phenylalanine and $^{13}CER_{(t)}$ was the ^{13}C excretion rate at t min after the test, assuming a ^{13}C production rate of 300 mmol/m^2 body surface area per hour.[8] ^{13}C cumulative excretion at each time interval, $^{13}Ccum_t$, was calculated by the trapezoid method using the area under the $^{13}CER_t = f(t)$ curve (AUC) and the results were expressed as the percentage dose excreted for each time interval (% ^{13}C dose). The pharmacokinetics parameter half time of ^{13}C excretion rate was calculated quantitatively from

the excretion of ^{13}C in the breath, as described by Wang et al.[9] The body surface areas of the subjects were estimated by the formulae of Lam,[10] Gehan,[11] and Haycock.[12]

2.4 Statistical analysis

All data are expressed as mean ± standard deviation (S.D.). Statistical comparisons among the groups were performed using one-way analysis of variance (ANOVA) with Scheffe's F correction. Coefficients of correlation between the parameters of PheBT and Child-Pugh classification scores were analysed by Spearman's rank correlation coefficient. The ^{13}C-PheBT values were compared with the results of standard liver blood tests using the Pearson's correlation coefficient. Differences between the groups at P<0.05 were considered significant. SPSS11.0 (Chicago, Illinois, USA) was used to perform statistical analyses.

3 Results

3.1 Time-course curve of ^{13}C excretion rate (Fig.1)

^{13}C could be detected shortly after oral administration of ^{13}C-Phe. The ^{13}C excretion was a first-order process. A sharp and smooth $^{13}CO_2$ excretion peak was seen within 10-30 min in healthy volunteers, group CHP, group A and group B whereas the response in group C approximated a rugged plateau from 10 to 120 min. For most patients of group C, the time-course curves had not an apparent peak and decline slowly after a slight increase, but there were not statistically significant differences in peak time among these five groups.

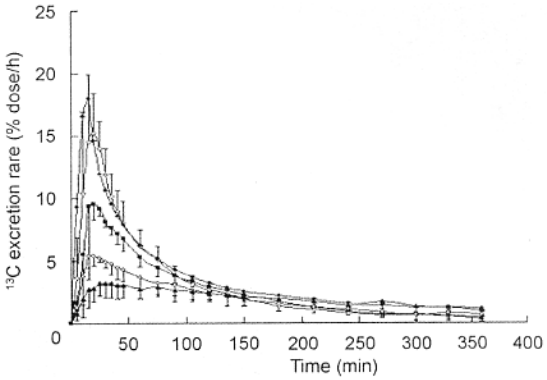

Fig.1 The time-course curve of ^{13}C excretion rate in breath after administration of L-[1-^{13}C] phenylalanine (100mg/body). △ Control (n=12), ● Chronic hepatitis patient (n=8), ■ Child A liver cirrhosis (n=10), ○ Child B liver cirrhosis (n=8), ▲ Child C liver cirrhosis (n=8).

3.2 Maximum value of $^{13}CER_t$, $^{13}CER_{max}$ (Table 1)

The $^{13}CER_{max}$ of ^{13}C-PheBT revealed extremely significant differences (P<0.05) among control, group A, group B and group C while the difference between the control and group CHP was not significant.

Table 1 Results of the L-[1-^{13}C] phenylalanine breath tests in control subjects and patients with liver disease

Parameters	Control subjects (n=12)	Chronic hepatitis patients (CHP) (n=8)	Child A patients (n=10)	Child B patients (n=8)	Child C patients (n=8)
$^{13}CER_{max}$ (% dose/h)	18.0±3.3	17.2±7.0	11.0±3.8$^{△•}$	6.1±0.9$^{△•▲}$	3.6±1.2$^{△▲*•}$
$^{13}CER_{30}$ (% dose/h)	11. 9±2.1	10.6±3.4	8.1±0.4$^{△•}$	5.0±0.5$^{△•▲}$	3.2±1.2$^{△▲*•}$
$^{13}Ccum_{60}$ (% dose)	9.3±1.4	9.0±2.7	6.6±0.7$^{△•}$	4.1±0.3$^{•△▲}$	2.6±0.9$^{△▲*•}$
$^{13}Ccum_{75}$ (% dose)	10.6±1.6	10.3±2.9	7.8±0.7$^{△•}$	5.1±0.3$^{△•▲}$	3.3±1.0$^{△▲*•}$
$^{13}Ccum_{90}$ (% dose)	11.7±1.8	11.3±3.0	8.9±0.7$^{△•}$	6.1±0.3$^{△•▲}$	4.0±1.2$^{△▲*•}$
$T_{1/2}$ (min)	40.4±4.4	53.4±4.4$^{△}$	59.8±4.5$^{△•}$	102.0±17.3$^{△•▲}$	212.1±87.9$^{△▲*•}$

$^{△}$ Compared with control; $^{•}$ Compared with CPH; $^{▲}$ Compared with Child A; * Compared with Child B. P values of all differences were less than 0.5.

3.3 $^{13}CER_t$ value at 30min, $^{13}CER_{30}$ (Table 1)

$^{13}CER_{30}$ differed significantly (P<0.05) among the control group, group A, group B, group C: the values in controls, group A, B, C were decreased in turn while the difference between control and group CHP was not significant yet.

3.4 ^{13}C cumulative excretion at each time interval, $^{13}Ccum_t$ (Table 1, Fig.2)

Analysis of the cumulative excretion from 60

min to 90 min revealed that cumulative excretions at 60 min, 75 min, 90 min: $^{13}Ccum_{60}$, $^{13}Ccum_{75}$, $^{13}Ccum_{90}$ differentiated significantly among control group and group A, B, C, and the values of the four groups decreased ordinally while the difference between the control and group CHP was not significant.

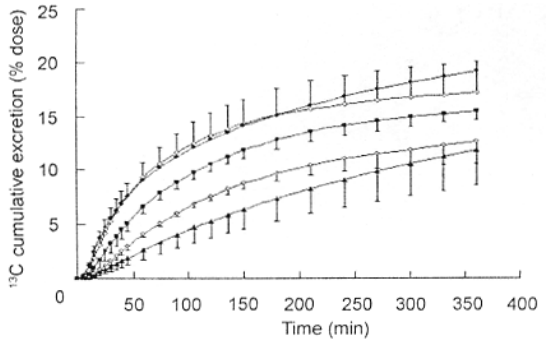

Fig.2 The time-course curve of ^{13}C cumulative excretion in breath after administration of L-[1-^{13}C] phenylalanine (100mg/body). △ Control (n=12), ● Chronic hepatitis patient (n=8), ■ Child A liver cirrhosis (n=10), ○ Child B liver cirrhosis (n=8), ▲ Child C liver cirrhosis (n=8).

3.5 Half time of ^{13}C excretion rate, $T_{1/2}$ (Table 1)

Following ^{13}C-Phe oral administration, phenylalanine transferred, distributed and cleared according to first-order rate dynamics. ^{13}C half excretion time differed significantly (P<0.05) among the five groups and the values in controls, group CHP and group A, B, C were longer ordinally.

3.6 Correlation between the results of ^{13}C-PheBT and Child-Pugh classification and standard biochemical liver function tests (Table 2)

All of test parameters $^{13}CER_{max}$, $^{13}CER_{30}$, $^{13}Ccum_{60}$, $^{13}Ccum_{75}$, $^{13}Ccum_{90}$ had significant positive correlation and $T_{1/2}$ had negative correlation with the serum level of albumin, total protein and negative correlation with total bilirubin, prothrombin time, Child-Pugh classification scores and no statistically significant correlation with serum level of aspartate aminotransferase, alanine aminotranferase.

Table 2 Correlation of L-[1-^{13}C] phenylalanine breath test results with Child Pugh score and standard liver blood tests

	$^{13}CO_2ER_{max}$	$^{13}CO_2ER_{30}$	$^{13}C_{cum60}$	$^{13}C_{cum75}$	$^{13}C_{cum90}$	$T_{1/2}$
ALT	-0.15$^{\#}$	-0.24$^{\#}$	-0.14$^{\#}$	-0.20$^{\#}$	-0.18$^{\#}$	-0.15$^{\#}$
AST	-0.23$^{\#}$	-0.32$^{\#}$	-0.26$^{\#}$	-0.29$^{\#}$	-0.29$^{\#}$	0.03$^{\#}$
TBIL	-0.40**	-0.44**	-0.47**	-0.44**	-0.44**	0.40**
ALB	0.67***	0.56***	0.70***	0.62***	0.70***	-0.74***
PT(%)	-0.68***	-0.73***	-0.79***	-0.72***	-0.74***	0.81***
TP	0.32*	0.34*	0.40**	0.35*	0.39**	-0.49***
Child-Pugh score	-0.80***	-0.90***	-0.91***	-0.80***	-0.88***	0.89***

Note: ALT: Alanine transaminase; AST: Aspartate transaminase; TBIL: Total bilirubin; PT(%): Prothrombin activity; TP: Total protein. $^{\#}$ P>0.05; * P<0.05; ** P<0.01; *** P<0.001.

4 Discussion

In human, phenylalanine is one of eight essential amino acids and it is an intermediate of the protein metabolism, so it has neither pharmacological nor allergic properties. The liver first pass effect results in conversion of phenylalanine to tyrosine, which is subsequently metabolized in the cytosol with direct loss of CO_2 from position 1. The process is illustrated in Fig.3. The first step aided by phenylalanine hydroxylase, which exists exclusively in hepatic cytosol, is the rate-limiting step of phenylalanine metabolism.[13,14] Hepatic phenylalanine hydroxylase activity was decreased in patients with liver cirrhosis and acute hepatitis.[15-17]

Burke et al [18] first used nonradiative ^{13}C-PheBT to measure hepatocyte functional capacity in liver disease, whereafter Kobayashi et al [19] have reported that ^{13}C-PheBT results were correlated with plasma retention rate of indocyanine green (ICG) and scores of the Child Pugh classification, which are widely accepted as a predictor of severity of liver disease. In our study, the correlation between the ^{13}C-PheBT parameters and some standard liver blood test values considered to assess liver synthetic capacity and hepatocyte functional TBIL, ALB and PT as well as Child-Pugh classification was significant. A recent experimental rat study[20] and human study[21] indi-

cated that there is a high significant correlation between results of ^{13}C-PheBT and phenylalanine hydroxylase activity of whole liver.

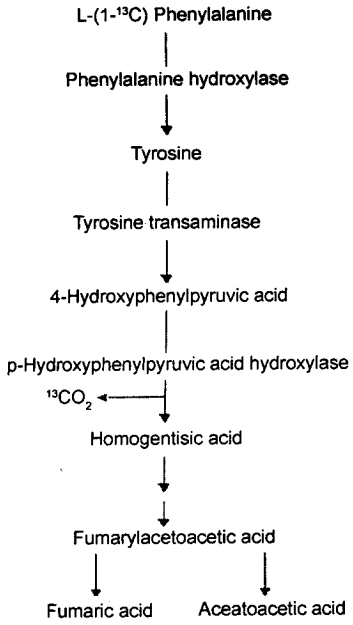

Fig.3 The pathways of L-[1-^{13}C] phenylalanine metabolism in the liver. The conversion phenylalanine to tyrosine catalysed by phenylalanine hydroxylase is a rate-limiting step.

The results of this study indicated an identified role for ^{13}C-PheBT in diagnosing and differentiating patients with hepatitis B virus-related chronic liver disease. The relationships observed between the ^{13}C-PheBT results and the degree of liver disease suggested that it could be used as a diagnostic tool and the ^{13}C-PheBT results were progressively impaired as the severity of disease increases.

The results of ^{13}C-breath tests have been expressed in different ways, the manner of presentation being chosen somewhat arbitrarily and therefore varying between investigators. The percentage of administered dose of ^{13}C recovered per hour $^{13}CER_t$ and the cumulative percentage of administered dose of ^{13}C recovered over time $^{13}Ccum_t$ were the most popular methods of presentation. Our results proved that $^{13}Ccum_{60}$ was considered to be more acceptable for its less time. However, $^{13}CER_t$ and $^{13}Ccum_t$ overlapped among control, chronic hepatitis B virus infection and Child A patients. The half time of ^{13}C excretion, $T_{1/2}$, is rarely utilized in previous breath tests because it has been considered more influenced by gastric emptying and absorption of L-phenylalanine in small intestine. Our study found that following more than 12 hours fasting the $T_{1/2}$, which reflects the dynamics of studied process, is sensitive in discriminating either advanced or mild liver disease, e.g. chronic hepatitis patients and Child A cirrhotic patients. The two clinical entities are often difficult to distinguish on clinical-biochemical basis, thus requiring histological or instrumental evaluation to refine the diagnosis.

Although liver biopsy remains the gold standard technique for the evaluation of patients with hepatitis B virus-related chronic liver disease, phenylalanine absorbed can reach almost all functional hepatocyte and metabolize immediately, so theoretically the ^{13}C-PheBT have the merit that the real-time condition of whole liver can be obtained and the method is non-invasive. ^{13}C-PheBT might be used as a complement to initial biopsy and during the follow-up of patients who cannot, or do not want to, undergo repeated liver biopsy.

In conclusion, ^{13}C-PheBT could be a valuable diagnostic tool for its simplicity, safety and validity to monitor the evolution of hepatitis B virus-related chronic liver disease and stage chronic liver disease. The parameters $^{13}CER_{max,}$ $^{13}CER_{30}$, $^{13}Ccum_{60}$ and $T_{1/2}$ are effective indexes which can be employed to stage hepatocyte impairment and liver functional reserve of advanced HBV-related cirrhotic patients (i.e. healthy subjects, Child A, B, C); $T_{1/2}$ was also useful for distinguishing mild HBV-related liver injure (i.e. healthy subjects, chronic hepatitis, Child A).

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