Optimum Conditions for the ^{13}C-Phenylalanine Breath Test

Toshihiro ISHII,[a] Kazuhiko TAKATORI,[a] Katsumi IIDA,[a] Teruhiko HIGUCHI,[b] Akihiko OHSHIMA,[b] Hiroshi NARUSE,[c] and Masahiro KAJIWARA*,[a]

Department of Medicinal Chemistry, Meiji College of Pharmacy,[a] 1–22–1 Yato-cho, Tanashi, Tokyo 188–0001, Japan, Neuropsychiatry Department, Showa University Fujigaoka Hospital,[b] 1–30 Fujigaoka Aoba-ku, Yokohama 227–0043, Japan, and Tokyo Institute of Medical Science, Kyorin University,[c] 6–20–2 Shinkawa, Mitaka, Tokyo 181–0004, Japan. Received January 13, 1998; accepted April 27, 1998

We have conducted optimization studies to develop a superior ^{13}C-phenylalanine breath test for the diagnosis of liver disease. First, we examined the optimum ^{13}C-labeling position in phenylalanine for use in a breath test based on infrared spectroscopic detection of $^{13}CO_2$ in exhaled air. L-[1-^{13}C]Phenylalanine gave the best result. Next, a suitable dosage to give a short peak time (the time expressed in minutes at which $^{13}CO_2$ excretion is maximal) after administration was determined. The $^{13}CO_2/^{12}CO_2$ ratio in exhaled air after administration of 100 mg/body of L-[1-^{13}C]phenylalanine peaked sharply at 15 min. We also examined the effect of food on the hepatic metabolism of L-[1-^{13}C]phenylalanine. We found that a fasting period of over 7 h before the test resulted in a higher $^{13}CO_2$ peak excretion. The peak appeared sooner than that in the ^{13}C-phenacetin breath test and, therefore, the ^{13}C-phenylalanine breath test appears preferable for the rapid evaluation of hepatic function.

Key words ^{13}C-phenylalanine; ^{13}C-phenacetin; breath test; liver disease; diagnosis

Evaluation of the hepatic detoxification capacity in humans is of great importance in medical diagnostics and non-invasive methods which do not require blood sampling are preferable. The breath test, in which a ^{13}C-labeled compound is administered and the metabolized $^{13}CO_2$ is measured in exhaled air, is a valuable clinical test[1a–c] and we previously employed ^{13}C-phenacetin for this purpose.[1b,c] However, in liver dysfunction, plasma phenylalanine levels are known to be raised[2a–d] and Burke *et al.* have reported a breath test involving the oral administration of L-[1-^{13}C]phenylalanine[3]; the liver first-pass effect results in conversion of phenylalanine to tyrosine, which is subsequently metabolized in the cytosol with loss of CO_2. The extent of metabolism is detected by measurement of labeled CO_2 exhaled. The altered metabolism of phenylalanine in liver cirrhosis involves impairment at several steps in the metabolic pathway of this amino acid. Thus, $^{13}CO_2$ production reflects cumulative changes, and may be greatly influenced by the procedures and conditions employed for the test. Therefore, in order to optimize the test, we have compared the levels of $^{13}CO_2$ production from L-[1-^{13}C]phenylalanine, L-[2-^{13}C]phenylalanine and L-[3-^{13}C]phenylalanine, to identify the best substrate. Next, we employed various dosages to find which would give a sharp $^{13}CO_2$ peak as soon as possible after administration. We then compared the results obtained by using different fasting periods before oral administration of the substrate to investigate the effect of food on the hepatic metabolism of ^{13}C-phenylalanine. Finally, the optimized L-^{13}C-phenylalanine breath test (^{13}C-PheBT) was compared with the ^{13}C-phenacetin breath test[1b,c] (^{13}C-PBT).

Results and Discussion

L-[1-^{13}C]Phenylalanine (**1a**) and L-[2-^{13}C]phenylalanine (**1b**) were purchased commercially, and L-[3-^{13}C]-phenylalanine (**1c**) was synthesized from [carbonyl-^{13}C]-benzoic acid using the cyclic glycine enolate template method reported by Dellaria and Santarsiero.[4] Three form of ^{13}C-phenylalanine (**1a**—**c** 100 mg/body) were administered to healthy volunteers and the $^{13}CO_2$ exhaled was measured ($^{13}CO_2/^{12}CO_2$) as the Δ ^{13}C permil (‰) using infrared (IR) spectroscopy.[5] The Δ ^{13}C permil (‰) was calculated from the IR absorption intensities of $^{13}CO_2$ ($2280 \pm 10\,cm^{-1}$) and $^{12}CO_2$ ($2380 \pm 10\,cm^{-1}$).[1c] The results of these breath tests are shown in Fig. 1. To avoid the effects of interindividual differences, the same subjects were used for all substrates. L-[1-^{13}C]Phenylalanine (**1a**)

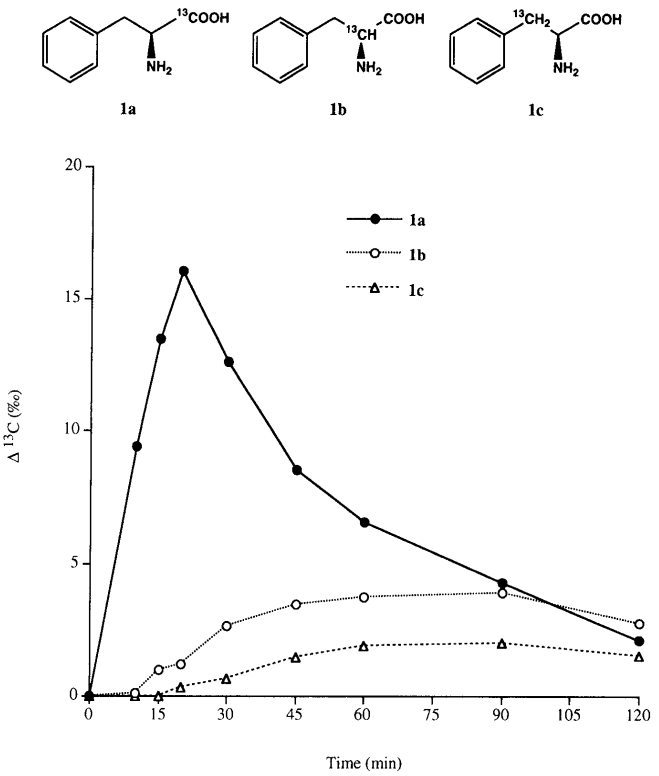

Fig. 1. Time-Courses of $^{13}CO_2$ Excretion in Expired Air Measured by Breath Test after Ingestion of L-[1-^{13}C]Phenylalanine (**1a**), L-[2-^{13}C]Phenylalanine (**1b**), or L-[3-^{13}C]Phenylalanine (**1c**)

Values are expressed as means ($n=2$).

* To whom correspondence should be addressed.

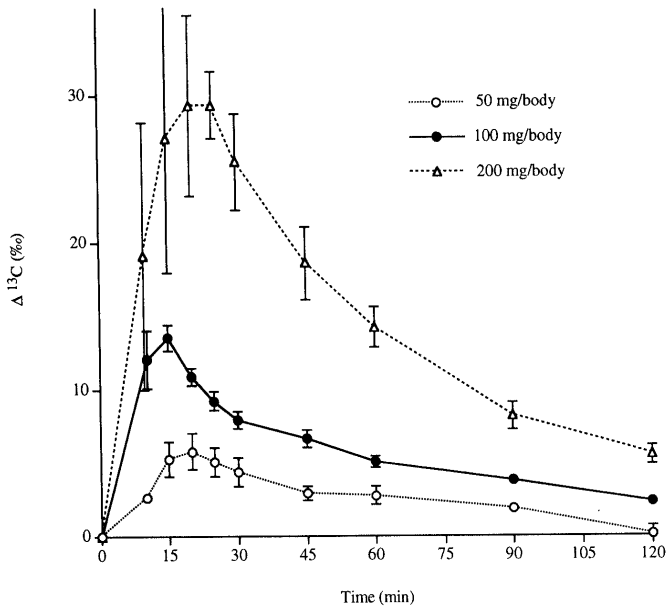

Fig. 2. Results of L-[1-^{13}C]Phenylalanine (**1a**) Breath Test ($n=4$) after Administration of 50, 100, and 200 mg/body

Values are expressed as means ± S.E.

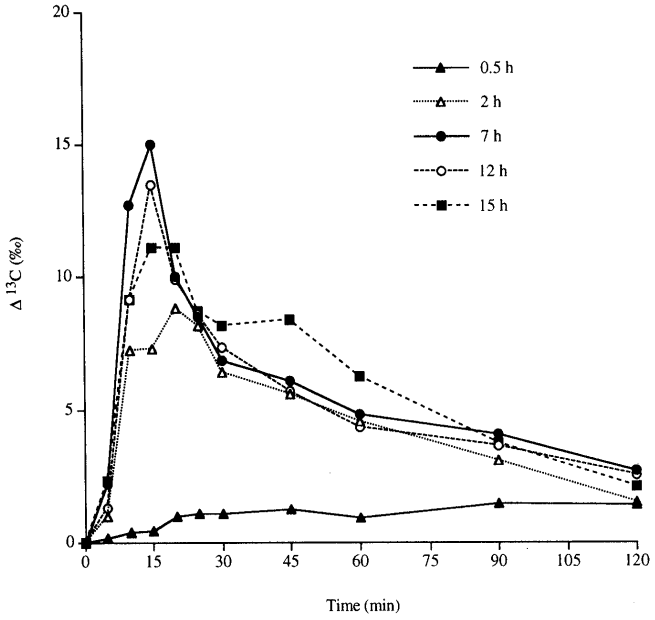

Fig. 3. Effect of Prior Fasting Time on the L-[1-^{13}C]Phenylalanine (**1a**) Breath Test ($n=1$)

gave the best result, producing by far the most $^{13}CO_2$ in the shortest time. We therefore used it in all subsequent tests. Phenylalanine participates in a number of metabolic pathways and the major one is irreversible hydroxylation to form tyrosine. Three other pathways of phenylalanine metabolism, normally of minor quantitative importance, are transamination to phenylpyruvic acid (PPA), decarboxylation to form β-phenylethylamine, and acetylation of the amino group. PPA may be reduced to phenyllactic acid (PLA) or may undergo oxidative decarboxylation to phenylacetic acid (PAA) which may then be converted to benzoic acid (BA). PAA may also be obtained from β-phenylethylamine by oxidation. Tyrosine may be hydroxylated to form dihydroxyphenylalanine (DOPA), iodinated to form triiodothyronine (T_3) or decarboxylated to form tyramine. The major metabolic pathway for tyrosine, however, is transamination to *p*-hydroxyphenylpyruvic acid (PHPPA). Most PHPPA is converted to homogentisic acid and, at this step, the label of L-[1-^{13}C]phenylalanine (**1a**) is released as $^{13}CO_2$. Then, the aromatic ring of homogentisic acid is cleaved to give fumaric and acetoacetic acids. Fumaric and acetoacetic acids are metabolized in the tricarboxylic acid (TCA) cycle and, at this stage, the label of L-[2-^{13}C]phenylalanine (**1b**) or L-[3-^{13}C]phenylalanine (**1c**) is released as $^{13}CO_2$.[6a] The hepatic enzyme activities may be altered in liver disease, and the major metabolic pathway of phenylalanine may change; this is why determination of the optimum ^{13}C-labeling position for the PheBT is necessary.

We next studied the effect of the dosage of **1a**. The time-courses of $^{13}CO_2$ excretion after administration of 50, 100, and 200 mg/body of **1a** to healthy subjects are illustrated in Fig. 2. Again, to avoid the influence of interindividual differences, the same subjects were used in all experiments. The $^{13}CO_2/^{12}CO_2$ ratio in exhaled air after administration of 200 mg/body of **1a** peaked at 25 min. A dose of 100 mg/body produced a reasonably sharp peak in the shortest time (15 min) after administration. At 50 mg/body, the peak of $^{13}CO_2$ excretion was broad and poorly defined. Therefore, in all subsequent tests, we used a dose of 100 mg/body.

Figure 3 shows the effect of feeding on hepatic metabolism of **1a**. All data were obtained from one individual. The peak times were compared for healthy volunteers who had fasted for various times before oral administration of **1a**. ^{13}C-PheBT after a fast of over 2 h gave clear peak times. ^{13}C-PheBT after a fast of over 7 h gave the best results, producing the most $^{13}CO_2$ in the shortest time. When the fasting period was shorter, the peak time was delayed and the excretion volume of $^{13}CO_2$ was lower. We concluded that ^{13}C-PheBT should be given after a fast of at least 7 h.

The results for ^{13}C-PheBT and ^{13}C-PBT are compared in Fig. 4. ^{13}C-PBT is a breath test involving the oral administration of [ethyl-1-^{13}C]phenacetin (**2**, 100 mg/body), which was prepared as reported previously[1c] by alkylation of *N*-acetyl-*p*-aminophenol with [1-^{13}C]iodoethane. The ^{13}C-PheBT peak time was 15 min, while that of ^{13}C-PBT was 90—120 min after administration; thus, ^{13}C-PheBT produced a higher and earlier peak than ^{13}C-PBT. This means that the ^{13}C-PheBT requires less time to complete than the ^{13}C-PBT, which would reduce patient stress.

In this study, we used L-phenylalanine, a physiologic amino acid, as the substrate for the ^{13}C-PheBT. Glaubitt and Siafarikas have reported a breath test using DL-[1-^{13}C]phenylalanine in healthy subjects and in children with cystic fibrosis.[7] We, therefore, compared the L-[1-^{13}C]phenylalanine (**1a**) breath test with their DL-[1-^{13}C]phenylalanine breath test. The L-[1-^{13}C]phenylalanine breath test produced a higher and earlier peak than the DL-[1-^{13}C]phenylalanine breath test, and the peak of $^{13}CO_2$ excretion after administration of DL-[1-^{13}C]phenylalanine was broad and poorly defined. Thus, the breath test using L-[1-^{13}C]phenylalanine (**1a**)

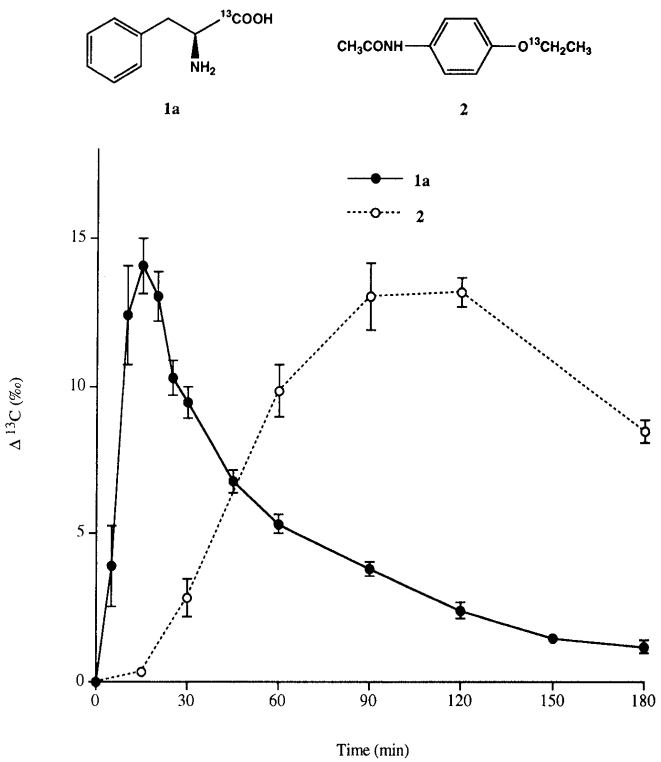

Fig. 4. Time-Courses of $^{13}CO_2$ Excretion in Exhaled Air Measured by Breath Tests with L-[1-^{13}C]Phenylalanine (**1a**, $n=15$) and [Ethyl-1-^{13}C]Phenacetin (**2**, $n=5$)

Values are expressed as means±S.E.

seems to be superior to other available breath tests.

The liver is the main site of phenylalanine metabolism, so that the plasma concentration of this amino acid is largely dependent upon liver function.[2b] Phenylalanine is mostly converted into tyrosine by phenylalanine hydroxylase in the liver. Because this hydroxylation is the rate-limiting step in mammalian phenylalanine metabolism,[8] phenylalanine should be superior to tyrosine for use in a breath test to diagnose liver disease. As mentioned above, tyrosine is mostly transaminated in the liver to PHPPA, which is further metabolized with loss of CO_2. This tyrosine metabolism is inhibited in patients with severe hepatocellular lesions, leading to increased concentrations of aromatic amino acids in plasma, and increased elimination of aromatic amino acids in urine.[9] Therefore, it seems reasonable to conclude that we can evaluate liver function by observation of the time-course of $^{13}CO_2$ excretion after administration of L-[1-^{13}C]-phenylalanine (**1a**). Phenylalanine hydroxylase activity is known to be reduced in patients with chronic renal insufficiency.[6a,b] Also, phenylalanine has been reported to disappear very slowly from the blood after oral administration to dialysis patients as compared with normal subjects.[10] Young and Parsons have reported that phenylalanine hydroxylase activity is present in the kidney tissue of rats, but at a relatively low level, so it is unlikely that loss of kidney tissue would significantly affect the metabolism of phenylalanine.[6b] They suggested that the impairment of phenylalanine hydroxylation in patients with chronic renal insufficiency may be caused by a reduction in liver hydroxylase. This suggests that the ^{13}C-PheBT might have some value for follow-up studies in patients with chronic renal insufficiency, if there is no deterioration in liver function.

In the present study, we determined the optimum ^{13}C-labeling position in phenylalanine for use in a breath test to diagnose liver disease. L-[1-^{13}C]Phenylalanine was found to be the best substrate. In addition, the time-courses of $^{13}CO_2$ excretion in the ^{13}C-PheBT and ^{13}C-PBT were compared. These results support the superior clinical utility of ^{13}C-PheBT. We are now examining the sensitivity of the ^{13}C-PheBT for the detection of hepatic damage in patients with various liver diseases. We believe that the determination of liver function in patients by the ^{13}C-PheBT is potentially of great diagnostic value.

Experimental

Materials L-[1-^{13}C]Phenylalanine (**1a**), L-[2-^{13}C]phenylalanine (**1b**) and [carbonyl-^{13}C]BA were obtained from Cambridge Isotope Laboratories. Aluminized bags were supplied by Shiseido Co., Ltd.

Instruments IR spectra for the $^{12}CO_2/^{13}CO_2$ breath test were measured with a $^{13}CO_2$ analyzer (EX-130, JASCO, Tokyo, Japan). The Δ $^{13}CO_2$ permil (‰) was calculated from the IR absorption intensities of $^{13}CO_2$ ($2280\pm10\,cm^{-1}$) and $^{12}CO_2$ ($2380\pm10\,cm^{-1}$).

^{13}C-PheBT by IR Spectroscopy Informed consent was obtained from all subjects, 13 healthy males and 2 healthy females, before the start of the study. They had no history of liver disease and normal values in the standard liver function and blood tests (*i.e.*, serum albumin, serum total bilirubin, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, serum choline esterase activity, serum total cholesterol, Fisher's amino acid molar ratio and hepaplastine test). A dose of 50, 100 or 200 mg/body of ^{13}C-labeled L-phenylalanine in 100 ml water was administered orally after the subjects had fasted for 0.5, 2, 7, 12 or 15 h. Breath samples were collected in 250 ml aluminized bags at 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 min after ingestion of ^{13}C-phenylalanine. The content of $^{13}CO_2$ in exhaled air was determined by IR spectroscopy.[1c,5]

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