Simultaneous Assessments of Exocrine Pancreatic Function by Cholesteryl-[\(^{14}\)C]Octanoate Breath Test and Measurement of Plasma \(p\)-Aminobenzoic Acid

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Two noninvasive tests for assessing pancreatic exocrine function, the cholesteryl-[\(^{14}\)C]octanoate breath test and the HPLC N-benzoyl-tyrosyl-\(p\)-aminobenzoic acid/\(p\)-aminosalicylic acid (NBT-PABA/PAS) test, were simultaneously performed in nine patients with pancreatic exocrine insufficiency due to chronic pancreatitis and in nine healthy volunteers. \(^{14}\)C\(_2\)O\(_2\) output in breath and plasma PABA concentration rose slowly in patients but increased rapidly in healthy subjects. The measurement time giving the best discrimination between both groups was 120 min for the cholesteryl-[\(^{14}\)C]octanoate breath test and 90 min for the plasma PABA test. At these points, both single-sample tests had essentially identical diagnostic sensitivity. The diagnostic sensitivities of the two single-sample tests were equal to that of the cumulative 6-h urinary PABA recovery and the cumulative 6-h urinary PABA/PAS ratio. We conclude that, for both the cholesteryl-[\(^{14}\)C]octanoate breath test and the plasma PABA test, a single test sample is sufficient for rapid detection of impaired exocrine pancreatic function.

Indexing Terms: pancreatic function/intermethod comparison/bentiromide/\(p\)-aminosalicylic acid/radioassay/chromatography, liquid

Many tests have been developed to assess exocrine pancreatic function (1). Tests in which the pancreatic enzyme secretion is measured directly by duodenal sampling during stimulation by cholecystokinin or after a test meal are regarded as the gold standard. However, such procedures are expensive as well as time-consuming for medical personnel and are uncomfortable for the patient. Thus, continuing efforts have been made to develop noninvasive methods for quantifying exocrine pancreatic function.

One test that has been licensed in many countries is the \(p\)-aminobenzoic acid (PABA; bentiromide)\(^6\) test, which is based on hydrolysis of the peptide N-benzoyltyrosyl-\(p\)-aminobenzoic acid (NBT-PABA) by pancreatic chymotrypsin (2, 3). The esterolytic activity of chymotrypsin is the rate-limiting step for the absorption of the PABA. PABA, often referred to as “free PABA,” is rapidly absorbed by the small intestinal mucosa, metabolized in the liver, and excreted in urine. Assessments of plasma PABA concentration or urinary PABA recovery after ingestion of NBT-PABA provide an indirect assessment of chymotrypsin activity. Nonetheless, a false-positive test result may occur despite normal hydrolysis of the peptide by chymotrypsin. For example, PABA may be poorly absorbed because of intrinsic intestinal disease, its hepatic metabolism may be impaired because of parenchymal cell disease, or its urinary excretion may be deficient because of renal impairment or incomplete collection of urine. Diabetic gastroparesis with delayed gastric emptying may also give false-positive test results.

To correct for these defects in the absorption of PABA or for its altered postabsorptive metabolism, free PABA can be readministered separately, so that the ratio of PABA recovery after NBT-PABA administration to that after free PABA administration, referred to as the PABA excretion index, can be determined (4). Alternatively, \([^{14}\text{C}]\text{PABA}\), as a marker for uptake of free PABA, can be administered simultaneously with NBT-PABA (5, 6), which provides values for the PABA excretion index after only a single test procedure. In another modification of the procedure, \(p\)-aminosalicylic acid (PAS), an analog of free PABA with a similar chemical structure and identical pharmacokinetic properties, is administered with the bentiromide (7). The combined administration of PAS with NBT-PABA allows calculation of the PABA/PAS index, an index similar to the PABA excretion index, after a single test procedure and no use of radioactive label. In yet another modification, PABA recovery is measured in serum or plasma (8–15). Plasma or serum measurements may offer a shorter test procedure than urinary measurements by obviating the need for an accurate collection of urine.

A relatively new test for noninvasive assessment of exocrine pancreatic function is the cholesteryl-[\(^{14}\)C]octanoate breath test (16–18), which is based on the intraluminal hydrolysis of cholesteryl-[\(^{14}\)C]octanoate by cholesterol esterase (EC 3.1.1.13). \([^{14}\text{C}]\text{Octanoic acid}\) is rapidly (and passively) absorbed by the intestinal mucosa, transported to the liver via the portal vein, and rapidly oxidized to \(^{14}\text{CO}_2\). Because the lipolytic activity of cholesterol esterase is the rate-limiting step for the absorption of \([^{14}\text{C}]\text{Octanoate}\), the rate at which \(^{14}\text{CO}_2\) appears in breath is proportional to the rate of

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\(^{5}\) Nonstandard abbreviations: NBT-PABA, N-benzoyl-tyrosyl-\(p\)-aminobenzoic acid; PAS, \(p\)-aminosalicylic acid; EPI, exocrine pancreatic insufficiency; and AUC, area under the curve.


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hydrolysis. Hence, measurement of $^{14}$CO$_2$ output in breath after ingestion of cholesteryl-$[^{14}$C]octanoate provides a noninvasive way to assess the rate of hydrolysis of the ester substrate and thereby assess exocrine pancreatic function.

To date, a direct comparison between the cholesteryl-$[^{14}$C]octanoate breath test and the NBT-PABA test has not been performed in humans. We report here the results of a study comparing the efficacy of these two tests in patients with exocrine pancreatic insufficiency (EPI) caused by chronic pancreatitis. We performed the same tests in healthy volunteers to define the rate of hydrolysis of the two substrates when pancreatic function is unimpaired.

**Materials and Methods**

**Subjects**

The group of patients (one woman and eight men; ages 37 to 76 years) had well-documented EPI caused by chronic pancreatitis and attended our outpatient clinic regularly. The diagnosis of chronic pancreatitis was based on clinical presentation and characteristic abnormalities detected during endoscopic retrograde cholangiopancreatography and abdominal ultrasound. The etiologies of chronic pancreatitis were alcohol abuse (5), trauma (1), and idiopathic (3). All patients had a fecal fat excretion (without enzyme supplementation) of at least 15 g/day and abnormal recovery of PABA in urine (<50%) by the bentiromide test in previous assessments. None of the patients had undergone gastric, intestinal, or pancreatic surgery.

The control group consisted of nine healthy men (ages 21 to 30 years) with no history of gastrointestinal, hepatic, or renal disease, and with a normal physical examination. The study was approved by the Medical Ethics Committee. All participants gave written informed consent.

**Reagents**

Cholesteryl octanoate (unlabeled) and PAS were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. NBT-PABA, sodium salt, was purchased from Fluka Chemie (Buchs, Germany). Cholesteryl-[1-$^{14}$C]octanoate was synthesized at the University of California, San Diego, by Huong-Tu Ton-Nu from [1-$^{14}$C]octanoic acid (New England Nuclear, Boston MA) via the acyl chloride (prepared with oxaly chloride). The ester was formed with use of pyridine as a catalyst in benzene (19). The radioactive product was purified by silicic acid column chromatography.

**Test Procedure**

Participants were fasted overnight. Before the test, fasting-state breath and blood samples were obtained for baseline determinations of $^{14}$CO$_2$ output in breath and plasma PABA concentration. An untimed urine sample was also obtained for baseline urinary PABA and PAS excretion. After ingestion of the test meal, breath and blood samples were collected at 30-min intervals for 4 h and then hourly for an additional 2 h; urines were collected for 6 h.

Breath samples were collected by blowing alveolar air into a solution of 2 mL of 96% ethanol and 2 mL of hyamine hydroxide, 1 mol/L in methanol (Packard Instrument BV Chemical Operations, Groningen, The Netherlands). Thymolphthalein (Fluka Chemie) indicator was used to detect completion of the titration of hyamine hydroxide by 2 mmol of expired CO$_2$ (20).

Blood samples were collected in tubes containing heparin. After centrifugation, the plasmas were aspirated and transferred to tubes for storage at −20°C until analysis.

The total volumes of the fasting-state urines and the 6-h urine collections were recorded, and aliquots of each were stored at −20°C until analysis.

The test meal consisted of a pancake made from 100 mL of whole milk, 50 g of flour, and 25 g of beaten egg. Cholesteryl-$[^{14}$C]octanoate, 5 μCi (18.5 × 10$^5$ Bq), dissolved in 0.2 mL of n-hexane, was carefully mixed into the pancake batter together with 1.5 g of cholesteryl octanoate. The pancake was fried in 10 g of butter and spread with jam. Finally, 1000 mg of NBT-PABA and 500 mg of PAS were sprinkled onto the pancake. The meal contained 1527 kJ (365 kcal), and its calculated composition was 50% carbohydrates, 37% fat, and 13% proteins. Subjects ate their complete pancakes. During ingestion of the pancake, they also drank 75 mL of water.

For the first 3 h after the test meal, subjects were not allowed additional fluids (which could have diluted gastric contents). After 3–6 h, they were encouraged to drink at least 500 mL of water or tea. They also received a standardized light lunch 4 h after the pancake meal.

**Analyses**

Breath $^{14}$CO$_2$ was measured by liquid scintillation counting. The specific activity of expired $^{14}$CO$_2$ was divided by the dose of radioactivity to give a normalized specific activity, % dose per millimole CO$_2$. This figure was then multiplied by body weight (kg) to correct for the influence of endogenous CO$_2$ production on $^{14}$CO$_2$ specific activity.

Concentrations of PABA in plasma (mg/L) were determined by HPLC, as were the concentrations of PABA and PAS in urine, and the cumulative 6-h urinary PABA and PAS recoveries were expressed as the percentage of the amount of substrate recovered in the 6-h urine collection compared with the total amount administered orally. The HPLC assays were performed essentially as described previously with some modifications (7). In brief, to determine PABA in plasma, we mixed 0.3 mL of plasma with 0.9 mL of water, 0.3 mL of 0.35 mol/L ZnSO$_4$, and 0.3 mL of 0.75 mol/L NaOH containing 16 mg/L m-hydroxybenzoic acid as internal standard. The protein precipitate was pelleted by 10 min of centrifugation at 1000g and discarded. The supernate remaining was lyophilized.
and then redissolved in 0.2 mL of 0.75 mol/L NaOH. After heating the solution for 50 min at 120°C in a capped vial, we neutralized it with glacial acetic acid, filtered it through a nylon 0.22-mm-pore filter and injected 10 μL into a 100 × 3.0 mm Inersil C18 column (Chrompack, Middelburg, The Netherlands). To determine PABA and PAS concentrations in urine, we mixed 650 μL of urine with 650 μL of 10 mol/L NaOH containing the internal standard. After 50 min of hydrolysis at 120°C we added 5 mL of acetic acid (300 mML/L), then diluted the mixture to 50 mL with water before injection of 10 μL into the chromatograph. Elution was performed with a gradient of 0.01 mol/L sodium acetate (pH 4.0) and methanol (0 to 300 mLL/L), at a flow rate of 0.7 mL/min. All reagents and solvents used were of analytical grade. All analyses were performed in triplicate, including analyses of PABA calibrator solutions and blanks.

Statistics

Results are expressed as mean ± SD. Data were compared by using the Mann–Whitney test (Wilcoxon rank sum test). To calculate the area under the curve (AUC) for both ^14CO2 outputs and plasma PABA concentrations, we connected measurements at consecutive time points by straight-line interpolation. The lower limits for “normal” values were obtained according to recommendations of the International Federation of Clinical Chemistry, by calculating the α = 0.025 fractile of the values for the healthy control subjects (21). We also calculated the 90% confidence limits of the α = 0.025 fractiles (21).

Results

Cholesteryl octanoate breath test: ^14CO2 output. The time course of the mean ^14CO2 output in breath ([% dose/mmol CO2] × kg) in patients with EPI and healthy volunteers is shown in Fig. 1. In patients with EPI, ^14CO2 output in breath increased slowly, reaching a maximum at 180–210 min, the mean ± SD output at 210 min being 0.15 ± 0.11 (% dose/mmol CO2) × kg. In contrast, in healthy volunteers ^14CO2 output in breath rose quickly, reaching a maximum at 90–120 min [the output at 120 min was 0.37 ± 0.05(% dose/mmol CO2) × kg].

The ^14CO2 output in breath from 0 to 360 min, as determined by the AUC, was much lower in patients with EPI [39.1 ± 25.0(% dose/mmol CO2) × kg × min] than in healthy volunteers [90.5 ± 9.7(% dose/mmol CO2) × kg × min] (P = 0.0007).

According to the recommended fractile for lower limits of normal (21), ^14CO2 output gave the best separation between patients with EPI and healthy volunteers at 120 min after the test meal was ingested (Fig. 2.). The calculated lower limit for normal values gave a cutoff value of 0.26(% dose/mmol CO2) × kg [90% confidence interval, 0.21–0.31(% dose/mmol CO2) × kg]. Using this cutoff, we diagnosed eight of the nine patients correctly, with no false positives in the healthy volunteers.

Plasma PABA concentration. The time course of the mean plasma PABA concentration (mg/L) in patients with EPI and in healthy volunteers is shown in Fig. 3. In patients with EPI, plasma PABA concentrations rose slowly, reaching a maximum at 240 min (1.47 ± 1.08 mg/L). In healthy volunteers, plasma PABA concentrations rose more quickly, peaking at 120 min (3.47 ± 1.07 mg/L).

The AUC for plasma PABA concentration from 0 to 360 min was considerably lower in the patients with EPI (346 ± 231 mg/L per minute) than in healthy volunteers (782 ± 197; P = 0.003).

Again using the recommended lower limit of normal (21), we found that plasma PABA concentrations gave the best separation between patients with EPI and healthy volunteers in the 90-min sample (Fig. 2). The calculated lower limit for normal values (α = 0.025 fractile) was 1.14 mg/L (90% confidence interval, 0.38–1.90 mg/L). This lower limit allowed us to diagnose seven of the nine patients correctly, with no false positives in the healthy volunteers.

PABA and PAS recovery in urine. Fig. 4 shows the cumulative 6-h urinary PABA recovery (%) and the

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Fig. 1. Time course of the mean ^14CO2 output (% CO2 dose) during the cholesteryl-[^14C]octanoate breath test in patients with EPI (——) and healthy volunteers (●●●). Vertical bars represent the 95% confidence interval of the mean.

Fig. 2. ^14CO2 output in breath ([% CO2 dose] at 120 min [left] and plasma PABA concentration (mg/L) at 90 min [right] in patients with EPI and healthy volunteers (HV).

Lower limits of normal values (α = 0.025 fractile) (——) and 90% confidence intervals (——) were 0.23(% dose/mmol CO2) × kg (0.21–0.31) for ^14CO2 output in breath and 1.14 mg/L (0.38–1.90 mg/L) for plasma PABA concentration.
cumulative 6-h urinary PABA/PAS ratio. The mean cumulative PABA recovery in 6-h urine from patients with EPI was 21.8% ± 16.2%, much less than that for the healthy volunteers (63.7% ± 10.0%; P = 0.0003). The calculated lower limit for normal values was 44% (90% confidence interval, 34–54%). This cutoff gave a correct diagnosis for eight of the nine patients, with no false positives in the healthy volunteers.

The cumulative 6-h urinary PABA/PAS ratio in patients with chronic pancreatitis was 0.36 ± 0.20, also much less than that in healthy volunteers (0.92 ± 0.16; P = 0.0002). The calculated lower limit for normal values, 0.59 (90% confidence interval, 0.43–0.75), allowed a correct diagnosis for eight of the nine patients, with no false positives in the healthy volunteers.

Discussion

This study, in which we performed the cholesteryl-[14C]octanoate breath test and the bentiromide test (with plasma PABA measurements) in the same subjects at the same times, shows that both tests gave similar results with respect to sensitivity (the number of false-negative results for the patients with EPI) and specificity (the number of false-positive results for the healthy control subjects). The optimal time point for collection of breath (for the cholesteryl octanoate breath test) was 120 min; for plasma (for the bentiromide test), 90 min. However, the broad variability in the outcomes of the single-sample plasma PABA test in healthy volunteers led to a wide 90% confidence interval (0.38–1.90 mg/L) for the lower limit for normal values (α = 0.025 fractile), such that many of the measurements obtained in patients were included in this interval. This is in contrast to the results for the single-sample cholesteryl-[14C]octanoate breath test, the cumulative 6-h urinary PABA recovery, and the cumulative 6-h urinary PABA/PAS ratio.

The use of plasma PABA measurements obviates the problem of obtaining a complete urine collection. The latter is often difficult in children, severely ill patients, and outpatients. Even among our inpatients, one patient failed to provide a complete collection. Measurements of the cumulative 6-h urinary PABA recovery, the cumulative 6-h urinary PABA/PAS ratio, or the AUC of plasma PABA concentrations and 14CO2 output in breath provided no additional diagnostic information over to the single-sample test results.

One difference between the cholesteryl-[14C]octanoate breath test and the plasma PABA test is the labor required for analysis of the samples. In our hands, it was easier to determine 14CO2 radioactivity in breath (by liquid scintillation spectrometry) than to measure PABA in plasma by HPLC. PABA in plasma can also be determined with a more simple colorimetric procedure, e.g., the Bratton and Marshall reaction for aromatic amines (22). HPLC, however, is less sensitive to interferences than colorimetric procedures and thus is more specific. Indeed, the plasma PABA concentrations we determined by HPLC were lower than those obtained by colorimetric methods (9–13, 15).

The cholesteryl-[14C]octanoate breath test is a simple test to assess EPI based on the activity of pancreatic cholesterol esterase in the small intestine. Although a radioactive label is involved, radiation exposure when performed as described is very low, the total whole-body radiation being 100 mrem in the 5 μCi in the pancake batter (200 Sv/Ci, or 54 nSv/Bq) (23). The determination of radioactivity of the breath samples in a scintillation counter is quick. Moreover, it should be possible to replace the 14C label in the octanoate moiety by a 13C label, and determine 13CO2 enrichment in breath by mass spectrometry (24); the analytical procedure, although more complex, is available in referral laboratories, and would eliminate exposure to radioactivity. Because cholesterol esterase activity requires the presence of bile acids (25), a decrease of 14CO2 measured in breath may be due either to true EPI or to a decreased concentration of bile acids (e.g., in cholestatic liver disease or ileal dysfunction resulting in bile acid malabsorption). Additional studies are required to determine whether the addition of exogenous bile acids should become part of the test procedure.

The time course of 14CO2 output in breath and of the plasma PABA concentration was similar in patients.
with EPI and in the healthy control subjects. In the patients, however, both markers increased much more slowly than in the healthy volunteers. Perhaps the presence of a large oil phase of unabsorbed lipid or a solid phase of unabsorbed protein trapped the cholesteryl octanoate and NBT-PABA, slowing their rates of hydrolysis. The slower increase in breath $^{14}$CO$_2$ and plasma PABA in patients was unlikely to be caused by delayed gastric emptying: Results of gastric emptying studies in five of the patients with EPI (who ingested a $^{99m}$Tc-labeled pancake of otherwise the same composition as that used in this study) were all normal. Hydrolysis of NBT-PABA by chymotrypsin-like activity present in the brush border of the enterocyte (26, 27) or in enteric bacteria (28) may have also contributed to the concentrations of plasma PABA measured. Microbial hydrolysis of cholesteryl octanoate in the distal intestine may have contributed to $^{14}$CO$_2$ generation (29, 30).

One patient showed values within the normal range for both the cholesteryl-$^{14}$C octanoate breath test and the PABA test (plasma and urine samples). A recently performed endoscopic retrograde cholangiopancreatography in this patient, however, showed extensive destruction of the pancreas from chronic pancreatitis. This patient also had steatorrhea with diarrhea and abdominal cramps. On medication with pancreatic enzymes, these complaints disappeared.

The number of patients studied was too small to indicate whether performing both tests provides more information than performing either of the tests alone. Some patients had low $^{14}$CO$_2$ output in breath and a relatively high plasma PABA concentration, while others had the opposite. Discrepancies in results from the two tests might indicate that pancreatic enzyme activities (e.g., cholesterol esterase and chymotrypsin) do not decrease in parallel in patients with EPI.

If reliable information regarding EPI can be obtained, the assay of either a single blood sample (PABA concentration) or a single breath sample ($^{14}$CO$_2$ output) may appear to be considerably simpler than measuring the cumulative 6-h urinary PABA recovery. Our finding that plasma PABA measurements in blood sampled 90 min after dosing best discriminated between patients and healthy volunteers agrees with several previous studies, and supports the recommendation of a 90- or 120-min sampling time for plasma PABA (8, 9, 11, 13, 15).

We conclude that, in future studies using these tests, breath (cholesteryl-$^{14}$C octanoate breath test) or plasma collection (PABA test) can be limited to one sample. However, for safety reasons (e.g., loss of sample) we recommend sampling at both 90 and 120 min. Previous studies of the single-sample plasma PABA test reported a sensitivity ranging from 62.5% to 100% and a specificity ranging from 90% to 100% (9-14). Future studies with more EPI patients are desirable to determine whether the cholesteryl octanoate breath test can detect patients with moderate loss of pancreatic exocrine function, i.e., to establish the true sensitivity of the test; in addition, patients with non-pancreatic gastrointestinal disease (e.g., celiac disease, lactase deficiency, Crohn disease) should be included, to determine the true specificity of the test.

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References