Determination of $^{14}$CO$_2$ in Breath and $^{14}$C in Stool after Oral Administration of Cholyl-1-$[^{14}$C]glycine: Clinical Application

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Twenty patients with intestinal bacterial overgrowth and 20 control subjects were investigated for bile acid deconjugation, by measuring $^{14}$CO$_2$ in the breath after cholyl-1-$[^{14}$C]glycine administration. $^{14}$CO$_2$ output/24 h was 11.0 ± 5.2% (mean ± SD) in controls and 54.2 ± 14.0% (mean ± SD) in bacterial-overgrowth patients ($P < .001$). $^{14}$CO$_2$ excretion rate in 12 h, when normalized to 100% of the dose at the 12th hour, gave an even finer discrimination between the two groups (no false responses). $^{14}$C in stool, analyzed in 20 malabsorption patients and 20 controls by two different techniques, was 6.6 ± 4% and 31.38 ± 21.7% (mean ± SD), respectively. Results by the two different techniques described here correlated well ($r = .99$). Bile acid malabsorption was in reasonable agreement ($r = .67$) with percentage of "chenoid" (chenodeoxycholic acid plus orsodeoxycholic acid) in the stool by gas–liquid chromatography; a poorer correlation was observed when "chenoid" plus "choclid" (cholic acid plus its epimers) were plotted vs. $^{14}$C in stool ($r = .57$, $n = 15$).

Additional Keyphrases: bile acid deconjugation in vivo • normal values • values for patients with intestinal bacterial overgrowth • bile acid malabsorption • gas–liquid chromatography

Recently the $^{14}$CO$_2$ breath test (1) has been shown to be clinically useful in the diagnosis of various gastrointestinal and metabolic disorders, thanks to the use of various radiolabeled compounds that are involved in various metabolic pathways (2–6).

The cholyl-1-$[^{14}$C]glycine breath test is based on the assumption that the linkage between cholic acid and glycine may be broken by intestinal bacteria. 1-$[^{14}$C]glycine thus formed is converted into H$_2$O and $^{14}$CO$_2$, which promptly diffuses into the intestinal wall, is carried to the lungs in the blood, and is exhaled. If there is bile acid malabsorption, most of the labeled bile acid administered is excreted in the stool, without chemical transformation. In this study, we describe and validate a method for $^{14}$CO$_2$ detection in the breath. In addition, two different techniques for $^{14}$C recovery in the stool are described and compared.

Materials and Methods

Apparatus

Analytic Isocap 300 (Searle Analytic Inc., Des Plaines, Ill. 60018) was used as beta counter; a Model 3920 instrument (Perkin-Elmer Corp., Norwalk, Conn. 06856) was used for gas chromatography.

Reagents

The reagents, all of analytical grade, were as follows: Hyamine hydroxide 10-X (methylbenzethonium hydroxide, Rohm & Haas, 1 mol/liter, in methanol); "Soluene-350" (Packard Instrument Co., Downers Grove, Ill. 60515); phenylethylamine (Sigma Chemical Co., St. Louis, Mo. 63178), "Spectafluor PPO-PPOP" scintillation cocktail (Amersham/Searle, Arlington Heights, Ill. 60005); carmine (Merck, Darmstadt, G.F.R.); chenodeoxycholic, cholic, deoxycholic, and lithocholic acids, free and conjugated with glycine or tauroine (Calbiochem, La Jolla, Calif. 92037); [carboxyl-$^{14}$C]-cholic acid 50 kCi/mol (The Radiochemical Centre, Amersham, England); N-nitrosomethylurea (ICN, Isotope and Nuclear Division, Irvine, Calif. 92664); 3% AN 600 Anakrom Q, 110–120 mesh (Analabs, Inc., New
Haven, Conn. 06473); and nordeoxycholic acid methyl-ester, kindly provided by Dr. A. F. Hofmann (Mayo Clinic, Rochester, Minn. 55901).

Subjects

Bacterial overgrowth: A full study of intestinal bacteria was done of 20 control subjects and 20 patients with blind loop syndrome. In addition, all underwent complete radiological examination of the digestive tract.

Bile acid malabsorption: We investigated 20 controls and 20 patients with bile acid malabsorption. Controls and patients were discriminated by previous quantitative gas-liquid chromatography of 24-h stool collection. (Bile-acid fecal loss mass was 300–600 mg/day in controls and >900 mg/day in malabsorption patients.) Only patients with a normal breath test and no ileal dysfunction revealed by roentgenogram were admitted to the latter group. All subjects gave their informed consent for the study. The small dose of 14C-labeled substrate that was administered and the short biological half-life of the bile acid led to a comparatively low radiation burden.

Procedures

Breath analysis. The fasting patient was given 5 μCi of cholyril-1-[14C]glycine (100 g/liter ethanolic solution), 5 ml, orally. Fifteen minutes later, this was followed by a liquid meal (40 g of glucose, 15 g of corn oil, and 20 g of milk in 250 ml of water), to which 1 g of carmine was added as nonabsorbable stool marker. The breath was analyzed 1, 2, 4, 6, 8, and 12 h after isotope administration, by means of a disposable 14CO2 breath trap (Figure 1). Exhaled CO2 was collected in a combination of 2 ml of a 1 mol/liter solution of Hyamine hydroxide in methanol and 1 ml of methanol. Two drops of thymolphthalein indicator were added to indicate complete neutralization. After addition of one or two drops of glacial acetic acid and 15 ml of liquid scintillation cocktail (PPO 6 g/liter, POPOP 75 mg/liter, “Spectro-fluor”; Amersham/Searle), radioactivity of 14C in the vials was measured in a beta counter giving a counting efficiency of 77.8 ± 3.2%.

Stool analysis. Red-colored feces were collected quantitatively in plastic bags, weighed, and made homogeneous inside the bag by external kneading of the bag. When the stool was nondiarrheic, a known amount of water was added. The samples were lyophilized.

Radiochemical Analysis

Digestion method. “Soluene-350” solution, 2 ml, was added to the dried samples (20 to 40 mg, as weighed in a glass scintillation vial). The samples were incubated for 2 h at 60 °C. After cooling at room temperature, 0.5 ml of isopropanol and 0.5 ml of hydrogen peroxide (300 g/liter solution) were added. After 10 min at room temperature, the samples were incubated at 60 °C for 2 h, allowed to stand for 15 min at room temperature, and then diluted with 4 ml of water and 16 ml of “Unisolve.” After 24 h of dark equilibration at 4 °C, the radioactivity of the samples was measured in a beta counter.

Combustion method. Duplicate 0.1-g aliquots of dried samples, rolled into filter paper, were placed in a quartz basket and 1 or 2 ml of amyl alcohol was added. The basket was put in a combustion flask (Figure 2) and the flask was filled with pure oxygen. Combustion was
triggered electrically and was usually complete within 1 min (7). After the flask was cooled in an ice bath for 15 min, the $^{14}$CO$_2$ formed was trapped in 8 ml of phenylethylamine/ethanol/water (30/40/1, by vol) mixture.

The radioactivity of 2 ml of trapping solution was measured in a glass counting vial after adding 15 ml of scintillation-cocktail mixture.

Gas–liquid chromatography. We used the method of Grundy et al. (8). Twenty milliliters of a 90/10 by vol mixture of ethanol/NaOH (1 mol/liter) and some boiling chips were added to 0.5 g of dried fecal powder. Nordeoxycholic acid, 5 mg/g, was added as internal standard. The mixture was refluxed for 2 h, cooled to room temperature, and 10 ml of water and 50 ml of petroleum ether (bp 60–80 °C) were added. The flask was shaken vigorously and allowed to stand for 15 min. The upper phase was removed, and the extraction was repeated twice with the same volume of petroleum ether. The aqueous (lower) phase, containing conjugated and free bile acids, was concentrated and hydrolyzed with 10 ml of an equivalent mixture of NaOH (2 mol/liter) and methanol at 120 °C for 4 h in a nickel bomb at 14 kPa. The mixture was then acidified with 5 mol/liter HCl and extracted once with 50 ml of chloroform/methanol (2/1 by vol) and then twice more with 50-ml portions of chloroform.

The lower phase was evaporated, and the residue, dissolved in 2 ml of chloroform/methanol (2/1), was then methylated with diazomethane, freshly produced by the reaction between $N,N$-nitrosomethylurea in ethereal solution and a 20 g/liter ethanolic solution of KOH. The sample was dried under a stream of nitrogen, and the residue was dissolved in 0.2 ml of chloroform/methanol (2/1) solution and purified by thin-layer chromatography (silica gel plates, 250-μm thickness; Merck, Darmstadt, G.F.R.), with benzene as the developing solvent. The area of the plate containing fatty acid methyl esters was identified by exposure to iodine vapors; then the plate was developed again in isoctane/isopropanol/acetic acid (2/40/1, by vol), again exposed to iodine vapors to make methylcholate visible, and corresponding areas were then scraped from the plate and bile acid was desorbed with 10 ml of methanol. The dried sample was acetylated at 4 °C by treatment with 4 ml of acetic anhydride/glacial acetic acid/perchloric acid (10/14/0.1, by vol) for 2 h.

Two milliliters of NaCl solution (200 g/liter) was added to the sample; then the bile acids were extracted with three 10-ml portions of ethyl acetate. Finally, the solution was evaporated to about 0.1 ml, and 0.2 to 1 μl was injected onto the column.

Analysis of the Sample

A 2 m × 0.3 cm column packed with 3% AN-600 Anakrom Q (110–120 mesh) was used as follows: column temperature: 260 °C, injector temperature: 260 °C (all-glass system), carrier gas: N$_2$, 20–30 ml/min, detector: flame ionization.

Qualitative analysis was based on the retention time for deoxycholic acid. Nordeoxycholic acid was used as the internal standard for the quantitative analysis, correcting the areas for the detector response.

Expression of the Data

Excretion of $^{14}$CO$_2$ in the breath was expressed in three ways (9):

(a) percentage of administrated $^{14}$C excreted in 24 h (output/24 h)

(b) excretion rate of $^{14}$CO$_2$ at various time intervals (this measurement converts a specific activity determination obtained on a single breath collection to the amount of $^{14}$C calculated to be present during 1 h of collection by the formula: % of dose excreted per mmol CO$_2$ × endogenous CO$_2$ output; each measurement obtained is added to the preceding ones), and

(c) excretion rate of $^{14}$CO$_2$ at various intervals of time, during 12 h, assuming that all of the dose is excreted in the breath by hour 12. This determination was done because of those negative breath analyses with highly positive $^{14}$C in stool, to avoid any false response due to the isotope loss. $^{14}$C in stool was expressed as per cent of the dose of radioactivity administered.

Results

Bacterial Overgrowth

Patients with bacterial overgrowth presented a higher $^{14}$CO$_2$ output/24 h than did controls, in spite of a wide range of variation ($P < .001$) (Figure 3).

When the values were expressed as excretion rate of $^{14}$CO$_2$, the bacterial-overgrowth patients appeared to be better discriminated from controls: $^{14}$CO$_2$ in the breath peaked sooner and higher in the former than in the latter group (Figure 4). Typical kinetics of $^{14}$CO$_2$ excretion could be observed in this way. When the percentage radioactivity excreted in 12 h was taken to be 100%, discrimination between controls and bacterial overgrowth patients was best (Figure 5), no overlap
Fig. 4. $^{14}$CO$_2$ excretion rate in controls (N) and stagnant loop syndrome (SLS) patients

Fig. 5. $^{14}$CO$_2$ excretion rate, assuming 100% dose excreted in 12 h in controls (N) and stagnant-loop-syndrome (SLS) patients

being observed, and the two negative breath analyses became positive.

**Bile Acid Malabsorption**

Figure 6 shows $^{14}$C in stool of controls and of bile acid malabsorption patients. In spite of the rather wide variation, the two groups are well discriminated ($P < .01$). The fecal bile acid pattern of a control subject (Figure 7) is quite different from that for a bile acid malabsorption patient.

Mean values ± SD of fecal bile acid composition in controls and bile acid malabsorption subjects are shown in Figure 8. A good correlation was found between percentage "chenoid" in stool and per cent of $^{14}$C recovered ($r = .67$) (Figure 9); a poorer correlation was found when cholic plus chenodeoxycholic acid epimers were plotted vs. per cent $^{14}$C recovered ($r = .57$). The precision and accuracy of the digestion is illustrated in Table 1. The intra-assay precision, evaluated from five determinations on the same sample, was satisfactory. When a fixed amount of choly-1-$^{[14]}$Cglycine was added to a known sample, the analytical recovery was 94–98% and was not dependent on the percentage of $^{14}$C recovered previously in stool.

Figure 10 shows the good correlation between the amount of $^{14}$C recovered in stool with the two methods.
Discussion

We have shown the cholyl-1-[^14]C]glycine breath test to be satisfactory in identifying ileal dysfunction, providing there is correct expression and interpretation of the data. Good agreement has been found between 24-h ^14CO2 output and the presence of intraluminal deconjugating bacteria, although two false-negative results were found, which could be theoretically ascribed either to the presence of non-deconjugating bacteria, to bile acid malabsorption, or to rapid intestinal transit with insufficient time for contact between substrate and bacterial enzymes. On the other hand, a false-positive breath test might be found in the presence of diarrhea patients because of rapid passage of bile acids into the colon, where much of the deconjugation occurs (9). In such cases, bacterial overgrowth can be better discriminated from other conditions by expressing the data as rate of ^14CO2 excretion in breath: in case of bacterial overgrowth ^14CO2 recovery in breath typically peaks earlier and higher than in other conditions. Because fecal ^14C is reciprocally related to ^14CO2 excretion in breath, in the presence of bile acid malabsorption a falsely negative breath test may be properly expressed as rate of ^14CO2 excretion when 12-h recovery in breath is taken to be 100%. In this way the results are unaffected by fecal losses of ^14C, and any further false-negative results are eliminated.

Bile acid malabsorption. Analytical recovery of ^14C in stool well discriminates bile acid malabsorption. Values of <10% of the dose cannot be considered pathological and samples with a <10% loss do not show a fecal bile acid pattern typical for malabsorption.

We found good correlation between fecal ^14C and percentage chenoid in stool, but a poorer correlation when cholic and chinic acid epimers were plotted vs. ^14C,

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<th>Table 1. Precision and Accuracy of the Digestion Method (Six Samples)</th>
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thus suggesting a critical role of chenodeoxycholic acid in determining diarrhea.

The two proposed techniques appear to give results that correlate well. The combustion procedure requires more work and apparatus; with the digestion of stool by Soluene it is simpler and easier.

Many analyses can be done at the same time, therefore it appears to be the technique of choice when stool analysis is to be done routinely.

References