Enzymatic measurement of choline-containing phospholipids in bile

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Summary An enzymatic method, previously used to determine choline-containing phospholipids in serum, was applied to the estimation of phospholipid concentration in bile. The method combines three enzymatic reactions: *a*) release of choline by phospholipase D; *b*) choline oxidation by choline oxidase, a reaction which generates hydrogen peroxide; and *c*) formation of a red quinone dye by peroxidase. The method is an endpoint, spectrophotometric determination. It is simple, sensitive, and reproducible. Accuracy was demonstrated both by linear recoveries of the choline-containing phospholipids in bile and a good correlation with a chemical determination. No interferences by bile acids and/or cholesterol were observed; bile pigment interference was removed by bleaching with white light. This method which determines lipid choline is simpler than the widely used chemical determination of lipid phosphorus. —**Gurantz, D., M. F. Laker, and A. F. Hofmann.** Enzymatic measurement of choline-containing phospholipids in bile. *J. Lipid Res.* 1981. **22:** 373–376.

Supplementary key words lecithin · lysolecithin · sphingomyelin · phospholipase D · choline oxidase

Measurement of the phospholipid content of bile is required for calculating the degree of saturation with cholesterol (1, 2). Traditionally, the biliary phospholipid level has been estimated by the chemical determinations of lipid phosphorus. These methods require extraction into a lipid solvent such as chloroform (3), wet ashing to liberate inorganic phosphate, and chemical determination of phosphate as a molybdate complex (4–6). This technique is not only lengthy but, in addition, is not ideal for samples such as duodenal contents obtained during digestion, which may have a high concentration of lysolecithin (7). The latter is difficult to extract completely into chloroform (8).

It would be preferable to have a method that required neither extraction nor ashing and would determine both lecithin and lysolecithin. Takayama et al. (9) have recently described an enzymatic method for the measurement of serum choline-containing phospholipids, and we reasoned that it could be applied to the estimation of biliary lipid choline, i.e., lecithin and lysolecithin. This study reports the validation and application of this enzymatic method for biliary phospholipids. While our work was in progress, Igimi et al. (10) and Seki and Horiguchi (11) also reported the use of this method for the measurement of biliary phospholipids.

PRINCIPLE OF THE METHOD

Phospholipase D from a *Streptomyces* species liberates choline from lecithin, lysolecithin, or sphingomyelin. Choline, in turn, reacts with choline oxidase to yield hydrogen peroxide and betaine. Hydrogen peroxide in the presence of peroxidase induces formation of a red quinone dye with a peak absorption of 500 nm which is determined spectrophotometrically (9) (**Fig. 1**).

MATERIALS AND METHODS

The reagent kit for determination of phospholipids in serum was obtained from Nippon Shoji Kaisha Ltd., Osaka, Japan. It is a single vial reagent. The assay reaction mixture contained 45 units of phospholipase D (from a *Streptomyces* species), 100 units of choline oxidase (from an *Arthrobacter* species), 220 units of horseradish peroxidase, 12 mg of 4-aminoantipyrine, 20 mg of phenol, and 2 mg of Triton X-100 in 100 ml of 50 mM Tris/HCl buffer, pH = 7.8. Phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, and sphingomyelin were obtained from Sigma, St. Louis, MO. Cholesterol was obtained from Supelco, Bellefonte, PA. Four bile acid conjugates (taurocholate, taurochenodeoxycholate, glycocholate, and glycochenodeoxycholate) were synthesized using a modification of the method of Tserng, Hachey, and Klein (12).

Assay procedure

Bile was diluted with isopropanol to a phospholipid concentration of 0.5 to 3 mM, and the protein removed by centrifugation. The degree of dilution differed with the source of bile (human gallbladder (1:15), human intestinal aspirate (1:5), dog hepatic bile (1:15), or hamster bile (1:3)). Twenty-five μl of diluted bile was added to 1.5 ml of reaction mixture and incubated at 37°C. After 20 min of incubation, the color absorbance was determined at 500 nm. For each sample, we ran a sample blank which contained 1.5 ml reaction mixture in which the enzyme had been inactivated at 60°C for 30 min. In addition, each set of determinations included two reagent

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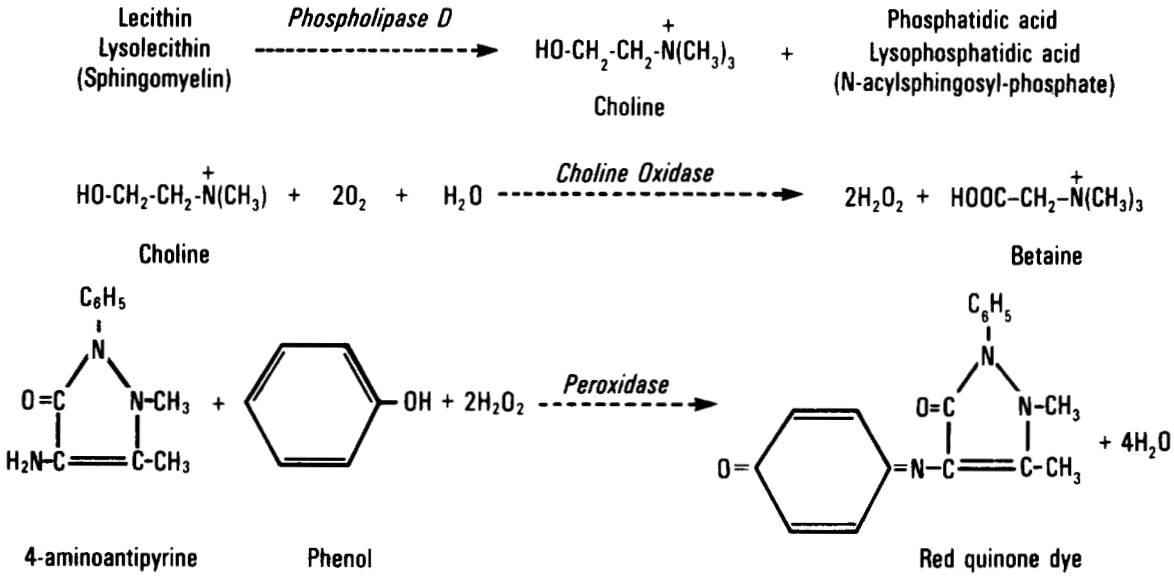

Fig. 1. Reaction sequence of the enzymatic determination. The figure has been redrawn from that published by Takayama et al. (9); a similar figure is shown in the descriptive brochure obtained from the manufacturer, Nippon Shoji Kaisha, Ltd.

blanks, one with inactivated and one with active enzymes. For a standard curve, synthetic dipalmitoyl phosphatidylcholine was dissolved in isopropanol, with a concentration range of 0.5–5.0 mM (**Fig. 2**). These standard solutions must be kept at room temperature since dipalmitoyl phosphatidylcholine is insoluble at 4°C at concentrations higher than 3.0 mM. Bile samples with high pigment concentration were bleached by overnight exposure to white light (20V plant light GRO-LUX, Sylvania) (13, 14). The photodegradation of the biliary pigments was faster and more complete when samples were diluted and exposed to light immediately after being obtained.

For validation of the enzymatic method, lipid phosphorus was determined chemically on a chloroform extract of bile (3). Digestion of the phospholipids was carried out at 245°C in the presence of 1:9 (v/v) perchloric acid–10N H_2SO_4; color development was according to Rouser, Fleischer, and Yamamota (5).

RESULTS

Linearity and recovery

The linearity of the assay was examined by adding known amounts of lecithin or lysolecithin to a bile sample. The results shown in **Fig. 3** indicate that the linearity was maintained in the working range of the assay (0.25–5.0 mM). Extrapolation to the 'y' axis gave a value in good agreement with the amount of phospholipid originally present in that bile sample. Overall recovery of six different lecithin concentrations ranged from 92–111%, with a mean value of 101%.

Reproducibility

Reproducibility of the assay was determined *a*) within batch in low concentrations (0.49 ± 0.02 mM, C.V. = 3.5%, n = 10) and in high concentrations (2.55 ± 0.11 mM, C.V. = 4.2%, n = 10) and *b*) between batch, day to day variations, (1.79 ± 0.1 mM, C.V. = 5.5%).

Interferences

Possible interferences of other biliary constituents were examined by assaying lecithin in their presence. Other non-choline containing phospholipids (phosphatidylethanolamine, phosphatidylserine, and phos-

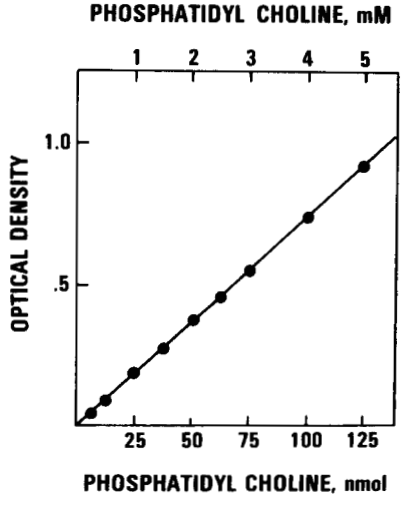

Fig. 2. Standard curve for phosphatidylcholine dipalmitoyl (synthetic, pure).

phatidylinositol) neither generated color nor interfered with the determination.

Bile acids: The biliary phospholipid:bile acid ratio varied over a wide range in samples from different species. Therefore, we chose to test bile acid interference for a single high concentration of lecithin (2.5 mM) with one concentration of bile acid (75 mM). No interference was observed with any of the four representative bile acid conjugates: cholyltaurine, chenodeoxycholyltaurine, cholylglycine, and chenodeoxycholylglycine.

Cholesterol: The highest cholesterol:phospholipid ratio that has been observed in bile is 1:2 (15). No interference of cholesterol was found over the entire range of phospholipid determination with that ratio.

Bile pigments: Interference with the determination was observed in bile abnormally rich in pigments. The interference could easily be removed by overnight photodegradation (13, 14). The linearity and recovery experiments (Fig. 2) were carried out on prebleached bile.

Agreement with chemical method

Twenty-three bile samples were analyzed enzymatically and chemically. The results, expressed as mM in the original bile sample, are shown in **Fig. 4.** The correlation coefficient was 0.95, the slope, 0.99, and the intercept, 1.33. The middle range of concentrations (15–25 mM) is characteristic of human gallbladder bile and dog hepatic bile; the lower range (5–6 mM) represents bile obtained from postoperative T-tube drainage.

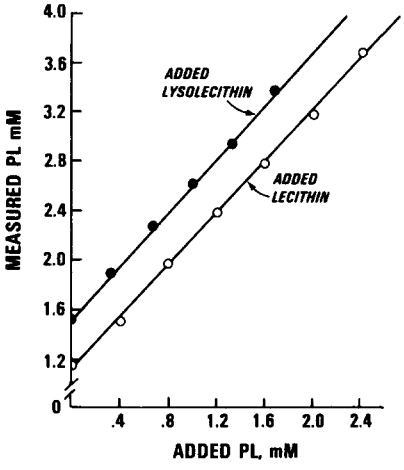

Fig. 3. Linearity and recovery: a) Various amounts of lecithin were added to bile samples rich in pigments. Bile was bleached by white light and total phospholipids were determined. A linear regression line through the points has an intercept which is in good agreement with the level of phospholipid originally present in the bile sample ($r = 0.998$, $y = 1.115 + 1.054x$). b) Various amounts of lysolecithin were added to a low pigment bile sample and total phospholipid was determined ($r = 0.999$, $y = 1.517 + 1.099x$). PL, phospholipid.

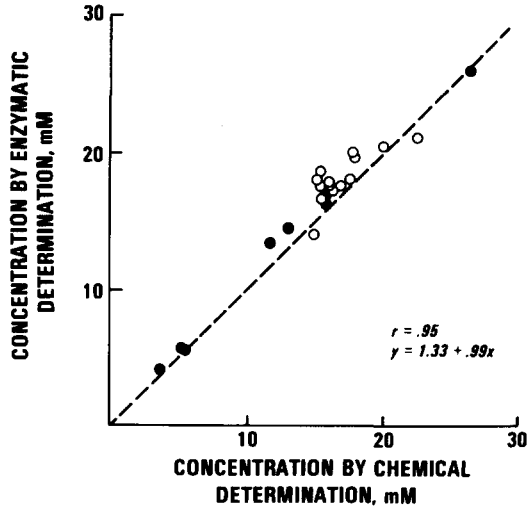

Fig. 4. Agreement between values for lipid phosphorus determined chemically (x axis) and lipid choline determined enzymatically (y axis). Bile samples (n = 23) were obtained by gallbladder aspiration during surgery or by collection of hepatic bile from T-tube drainage in post-cholecystectomy patients (●), and by collection of hepatic bile from dogs through an external bile fistula (○) ($r = 0.95$, $y = 1.33 + 0.99x$).

DISCUSSION

Our results indicate that this single-step enzymatic procedure is a satisfactory alternative to the chemical determination of biliary phospholipids. It is sensitive and specific and thus allows one to use minute amounts of unextracted bile. It is also accurate and reproducible. Since biliary phospholipids in most mammals are composed predominantly of lecithin (8, 16), a method that measures lipid choline gives results identical to those obtained by methods which measure lipid phosphorus.

The linearity of the assay was demonstrated for both lecithin and lysolecithin by complete recovery when these substances were added to bile (Fig. 3). The slopes of the regression lines calculated were the same and the intercepts with 'y' axis gave a value in good agreement with the amount of phospholipids originally present in each bile sample. This indicates that lysolecithin, if present in bile or duodenal aspirates containing bile, can be determined with the same efficiency as lecithin.

We found no interference with the method other than by bile pigments. No attempt was made to define the chemical identity at the interference pigments, since reference substances are not available. The pigment molecules that most likely interfere are bilirubin, biliverdin, and their conjugates. Bile pigments presumably consume hydrogen peroxide, one of the intermediates in the reaction sequence. Interference by bile pigments is to be suspected when the sample blank has an absorbance greater than 10% of the re-

action sample. Bleaching with light eliminated such interference completely without further complicating the determination.

Determination of choline-containing phospholipid could give erroneous results for duodenal content samples if they were contaminated with choline. We did not detect such contamination in our samples, but such could be tested for by running a phospholipase D blank.

In the course of this work, we also explored enzymatic determination of biliary phospholipid using phospholipase C to generate diglyceride, which is then determined using an enzymatic method for lipid glycerol. This method, which has previously been described by Sugiura, Oikawa, and Hirano. (17), is unsatisfactory because it does not measure lysolecithin accurately; the phospholipase C hydrolyzes it incompletely. In addition, separate determinations of tri- and diglycerides in the sample are required to avoid overestimation of the phospholipids.

Development of an enzymatic method for determination of biliary phospholipids supplements already existing enzymatic methods for determination of biliary bile acids (18) and cholesterol (19, 20). Thus, it should now be possible to develop automated systems or convenient kits for the rapid and accurate determination of the cholesterol saturation of any bile sample.

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Note added in proof: After our work had been completed and submitted for publication, we learned of studies by Qureshi, Murphy, and Dowling which also have validated this method for determination of phospholipids in bile. (Qureshi, M. Y., G. M. Murphy, and R. H. Dowling. 1980. *Clin. Chim. Acta.* **105:** 407–410.)

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