Simple Enzymatic Determination of Total Cholesterol in Gallstones

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In this simple method for rapid enzymatic determination of total cholesterol in human gallstones, gallstone powder is dissolved in an 80/20 by vol mixture of N,N-dimethylformamide and dimethyl sulfoxide and reacted directly with the aqueous enzymatic reagent, without further treatment. The organic solvents do not interfere with enzymatic activities of cholesterol oxidase, esterase, and peroxidase in the assay mixture. The method is reproducible (CVs 3.7% to 6.6%), analytical recoveries ranged from 98.6% to 102%, and linearity is good. Furthermore, results correlated well with those obtained by infrared spectroscopic measurements.

Human gallstones generally fit into two categories, pigment stone and cholesterol stone (1–3), classified according to their cholesterol content. Gallstones containing more than 70–75% of their dry weight as cholesterol are considered cholesterol stones, and those having less than 15–20% of their dry weight as cholesterol are pigment stones; the remainder are considered to be of mixed type. This differentiation between pigment stone and cholesterol stone is becoming increasingly important, because at least some cholesterol stones can now be dissolved in situ with oral medication (4–6).

Methods used for determining total cholesterol in gallstones include infrared spectrometry (2), gas-liquid chromatography (5), and differential scanning calorimetry (7).

Enzymatic determination of total cholesterol in various biological tissues has been reported (8–11). However, analysis for cholesterol in biological specimens usually requires time-consuming organic solvent extraction and evaporation. Also, some of the organic solvents previously used for cholesterol extraction (e.g., chloroform, dioxane, and methanol) are not harmless. Therefore, efforts have been made to use amphiphilic solvents such as N,N-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) to dissolve cholesterol in gallstones for analysis.

Materials and Methods

Materials and chemicals. Gallstones obtained at cholecystectomy were washed with water, dried, pulverized, and desiccated. DMF was obtained from Union Chemical Works Ltd., Hain Chu, Taiwan, R.O.C., and DMSO from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Cholesterol standard (cat. no. C0284) and cholesterol assay reagent (cat. no. 351-50) were from Sigma Chemical Co., St. Louis, MO.

Cholesterol determination. The enzymatic assay was typically performed as follows: Dissolve 5 mg of gallstone powder in 5 mL of DMF/DMSO (80/20, by vol). Mix 10 lL of the resulting solution with 1 mL of the reagent mixture, which contains phosphate buffer, 0.1 mol/L 4-aminopyrine, 0.8 mmol/L p-hydroxybenzenesulfonate, 20 mmol/L cholesterol oxidase (microbial, EC 1.1.3.6), 200 U/L; esterase (microbial, EC 3.1.1.1), 150 U/L; peroxidase (horse-radish, EC 1.11.1.7), 25 kU/L; and stabilizers and fillers. Incubate at 37 °C for 10 min, then measure the absorbance of the reaction mixture at 500 nm vs a blank containing sample and distilled water (we used an UVIDEC-610 spectrophotometer, Jasco, Tokyo, Japan).

FTIR measurement. We also measured the cholesterol content of gallstone samples by infrared spectrometry, using the KBr tablet method and a Model 5DXB FT-IR spectrometer (Nicolet Instrument Corp., Madison, WI).

Results and Discussion

A unique characteristic of DMF and DMSO—ther solubility in both water and other organic solvents—prompted our attempts to dissolve gallstones for enzymatic determination of cholesterol.

The accuracy of the determination of cholesterol content in a gallstone depends on the amount of dissolved cholesterol in the assay mixture. Figure 1 represents three different stones, assayed after they were dissolved in various volume ratios of DMF and DMSO. Neither DMF nor

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Received May 22, 1989; accepted July 28, 1989.

3 Nonstandard abbreviations: DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; and FTIR, Fourier transform infrared spectrometry.
DMSO alone can extract cholesterol from gallstone samples with 100% efficiency. The 80/20 volume ratio of DMF and DMSO represents the best combination for cholesterol dissolution, allowing the measurement of more than 99% of the cholesterol content of certain samples (e.g., the top curve in Figure 1). The cholesterol standard and the cholesterol in gallstone powder dissolved instantly in this solvent system, without turbidity. Colored insoluble particles in certain gallstone samples presumably belong to an inorganic compound/pigment complex; in any case, this has no effect on assay results. Incubation of the gallstone sample with this solvent system at 50 °C for 3 min showed no significant difference from sample treatment at room temperature (data not shown).

Addition of the DMF and DMSO in the assay mixture did not interfere with the catalytic activities of cholesterol esterase, cholesterol oxidase, or peroxidase, as demonstrated by the fact that the gallstone samples (with DMF/DMSO added) and the cholesterol control showed virtually the same kinetic patterns.

To validate the linearity and specificity of this enzymatic assay, we compared absorbance values (500 nm) vs sample volume (10, 20, 30, 40, and 50 μL) by linear regression. Corresponding equations expressing the linear regressions (figures not shown) for three different gallstones were

\[ y = 0.024x + 0.0054 \quad (r = 0.998) \] for a gallstone with 98% cholesterol by weight; \[ y = 0.013x + 0.0014 \quad (r = 0.999) \] for a gallstone with 56% by weight; and \[ y = 0.007x + 0.0022 \quad (r = 0.997) \] for a gallstone with 31% by weight.

Table 1 demonstrates that cholesterol standard added to four gallstone samples was recoverable in the enzymatic method. Percent recovery was defined and calculated based on the relationship between added and measured cholesterol standard, not on the relationship between the predicted and obtained assay values. Two cholesterol stones with 98% and 75% cholesterol by weight showed recoveries of 99.7% and 99.2%, whereas two mixed-type stones with 45% and 31% cholesterol by weight showed recoveries of 98.6% and 102%, respectively.

We determined within-run precision of this method by analyzing two gallstones. Based on calculations from 15 runs, the coefficient of variation (CV) was 3.7% for a mean cholesterol content of 86% by weight for one gallstone, whereas the CV was 6.6% (n = 16) at a mean cholesterol content of 27% by weight for the other.

We compared the results for total cholesterol content of gallstones assayed by this enzymatic method and FTIR (Figure 2). The regression line (x axis, data for the FTIR method; y axis, the enzymatic) gave a correlation coefficient for the total cholesterol comparison of 0.956, with a slope of 0.98 and intercept of −0.6. The data in Figure 2 suggest that the enzymatic cholesterol assay might have a sensitivity problem if the proportion of cholesterol is below 30% to 35%. Indeed, when we examined a pigment stone containing 14% cholesterol by weight as measured by the FTIR method, the value for the linear-regression analysis of the enzymatic concentration plot was as low as 0.783.

In the present technique for analysis of cholesterol in gallstones by dissolving the stones in a specific organic solvent, the cholesterol can be measured by any routine method involving a Trinder-type cholesterol assay. This could be useful to many laboratories, in view of the widespread use of enzymatic methods for measuring cholesterol in serum. Compared with other methods of gallstone cholesterol determination, our method is faster and simpler.

This investigation was supported by grant CMRP 190 from Chang Gung Memorial Hospital, Taipei, Taiwan, R.O.C. We thank...
Drs. Delon Wu and Chau-Hsiung Chang for their interest and encouragement during this study. We also thank Dr. Miin-Fu Chen for supplying gallstone samples.

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