

Use of a simple enzymatic assay for cholesterol analysis in human bile

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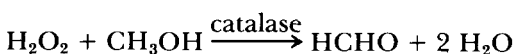
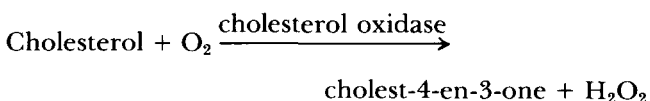
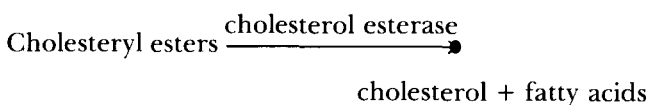
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Summary An enzymatic technique for cholesterol analysis in serum was applied to human bile. The analytical yield was very satisfactory in experiments in which known amounts of cholesterol were added to untreated, as well as Millipore-filtered, samples of human bile. The analytical results of the enzymatic test agreed closely with those of a method utilizing the Liebermann-Burchard reaction. The enzymatic assay of cholesterol in bile proved to be sensitive and precise. In comparison to other methods of biliary cholesterol determination, it has the advantage of being rapid and simple.—**Fromm, H., P. Amin, H. Klein, and I. Kupke.** Use of a simple enzymatic assay for cholesterol analysis in human bile. *J. Lipid Res.* 1980. **21:** 259–261.

Supplementary key words cholesterol esterase · cholesterol oxidase · catalase · Liebermann-Burchard reaction

Biliary cholesterol analysis is an integral part of the assessment of the solubility of cholesterol in bile, which, in turn, gives information as to whether there is a propensity to either growth or dissolution of cholesterol gallstones (1–4). The analytical methods currently used by most investigators are based on the Liebermann-Burchard reaction (2, 5, 6), or gas–liquid chromatographic procedures (7). However, these methods are laborious and time-consuming. We report here our experience with a convenient and relatively simple enzymatic determination of cholesterol in bile. The enzymatic assay represents an application of a method introduced by Röschlau, Bernt and Gruber (8) for measuring cholesterol in serum with the use of cholesterol oxidase.

PRINCIPLE AND METHOD



Formaldehyde reacts with ammonium ions and acetylacetone to form 3,5-diacetyl-1,4-dihydrolutidine. Reagents and procedure of the test are as described in detail for serum analysis, by Röschlau et al. (8), as well as in the manual by Bio-Dynamics, Division of Boehringer-Mannheim, Indianapolis, IN (Cat. Nos. 124079 and 124087). For analysis of biliary cholesterol, we used Millipore-filtered bile (pore size: 0.45 μm , Millipore Corporation, Bedford, MA). If the bile appears concentrated, a dilution, usually 1:5 (v/v), with 0.9% NaCl was prepared. Otherwise, the bile sample was analyzed without prior dilution. The enzymatic test is performed on 100- to 200- μl samples that are added to 10 ml of the reagent mixture (bmc Reagent Set, Bio-Dynamics, Division of Boehringer-Mannheim). The reagent mixture has the following composition: 0.56 M ammonium phosphate buffer (pH 7.0), 19.8 mM acetylacetone, 1.68 M methanol, 0.1% (w/v) hydroxy-polyethoxydodecane, >660 U/ml catalase (25°C), and >26 mU/ml cholesterol esterase (25°C). One half of the assay mixture is mixed with 50 μl of a solution of cholesterol oxidase (>32 mU/ml measured at 25°C). The remaining half serves as a blank. Both sample and sample blank are incubated for 1 hr at 37°C. The blank is set at zero on a Zeiss Spectrophotometer, Model PMQII at 400 to 420 nm, and the absorbance of the sample is recorded. For comparison, biliary cholesterol was also analyzed by a previously published method, which utilizes the Liebermann-Burchard color reaction (2, 5, 6). This procedure requires, as preparative steps, deproteinization in a 95% ethanol solution and extraction into *n*-hexane.

VALIDATION

Analytical yield of enzymatic cholesterol assay

The analytical yield of the enzymatic assay was determined in experiments in which human bile samples were analyzed before and after addition of 50 μg of a cholesterol standard (Bio-Dynamics, Division of Boehringer-Mannheim). The cholesterol used in the standard solution corresponded with the quality of Standard Reference Material 911 of the National Bureau of Standards, Washington, DC.

Analytical yield in unfiltered bile. Recovery experiments were carried out on unfiltered bile samples of nine gallstone patients. Examination by polarizing microscopy showed that these bile samples contained considerable amounts of cholesterol microcrystals. The results of the recovery experiments are presented in **Table 1**. The cholesterol concentrations in these bile samples ranged between 1.02 and 9.82 mM. The recovery of the cholesterol standard, which was

TABLE 1. Analytical yield of enzymatic assay in unfiltered samples of human bile

Patient	Cholesterol Concentration	Cholesterol Standard ^a Recovered
	mM	%
1	3.50	90
2	1.80	114
3	1.83	118
4	9.82	100
5	1.55	106
6	2.66	86
7	3.16	100
8	4.60	92
9	1.54	92

^a 50 µg of cholesterol standard was used.

99.8 ± 3.48% (mean ± SEM), did not differ between the bile samples with high and those with low cholesterol concentrations (Table 1).

Analytical yield in millipore filtered bile. The analytical yield of the enzymatic assay was also assessed in studies of Millipore-filtered bile samples of 19 gallstone patients. The recovery data are shown in **Table 2**. The cholesterol concentrations in these samples ranged between 0.64 and 5.88 mM. The recovery of the cholesterol standard was 100.6 ± 4.36%. Again there was no noticeable difference in the recovery between the samples with higher and those with lower cholesterol concentrations.

TABLE 2. Analytical yield of enzymatic assay in Millipore-filtered samples of human bile^a

Patient ^b	Cholesterol Concentration	Cholesterol Standard Recovered
	mM	%
1	2.38	96
2	1.02	92
3	1.16	106
4	5.60	90
5	1.09	104
6	1.38	96
7	1.46	118
8	2.88	114
9	1.34	104
10	2.18	96
11	1.11	102
12	0.75	98
13	1.51	94
14	5.88	90
15	1.14	104
16	1.60	112
17	1.63	86
18	0.64	108
19	1.79	102

^a Pore size of filter was 0.45 µm.

^b Samples of patients 1–9 in this table represent those of Table 1 after Millipore filtration.

^c 50 µg of cholesterol standard was used.

TABLE 3. Comparison between enzymatic assay and Liebermann-Burchard reaction in the cholesterol analysis of human bile samples

Sample No.	Measured Cholesterol Concentration (mM)	
	Liebermann-Burchard Reaction	Enzymatic Assay
1	0.61 ± 0.043	0.76 ± 0.047
2	0.26 ± 0.006	0.24 ± 0.018
3	0.14 ± 0.008	0.07 ± 0.005
4	0.25 ± 0.015	0.22 ± 0.013
5	0.07 ± 0.006	0.05 ± 0.013
6	0.55 ± 0.008	0.57 ± 0.017
7	0.28 ± 0.008	0.32 ± 0.012
8	0.14 ± 0.015	0.07 ± 0.005
9	0.24 ± 0.013	0.25 ± 0.022
10	0.57 ± 0.012	0.58 ± 0.009
11	0.26 ± 0.008	0.24 ± 0.007
12	0.20 ± 0.010	0.21 ± 0.013
13	0.21 ± 0.008	0.24 ± 0.008
14	1.95 ± 0.231	2.03 ± 0.029
15	2.02 ± 0.150	2.03 ± 0.030
16	1.85 ± 0.121	2.02 ± 0.044
17	1.29 ± 0.087	1.09 ± 0.046
18	1.25 ± 0.108	1.35 ± 0.038
19	1.16 ± 0.079	1.22 ± 0.071
20	1.13 ± 0.100	1.13 ± 0.059
21	1.07 ± 0.110	1.22 ± 0.032
22	1.03 ± 0.125	1.11 ± 0.096
23	1.39 ± 0.220	1.43 ± 0.035
24	2.12 ± 0.110	2.07 ± 0.095
25	0.98 ± 0.100	1.09 ± 0.053
26	3.37 ± 0.039	3.54 ± 0.086
27	3.58 ± 0.090	3.64 ± 0.023
28	3.84 ± 0.033	3.89 ± 0.035
29	3.80 ± 0.267	4.17 ± 0.100
30	5.90 ± 0.080	5.91 ± 0.098

Comparison between enzymatic assay and Liebermann-Burchard reaction

Sextuplicate analyses by both methods were carried out in human bile samples. The results of both tests are listed in **Table 3** and plotted on **Fig. 1**. The least square regression coefficient associated with the fitted line is $r = 0.955$. A 95% confidence region for the slope and intercept of the best fit line includes 0 and 1 as intercept and slope, respectively, i.e., $y = x$ can be used to describe the data. The coefficient of variation of the sextuplicate analyses is 7.04% for the Liebermann-Burchard reaction and 4.20% for the enzymatic assay.

DISCUSSION

Our studies show, as also reported by Roda et al. (9), that the enzymatic cholesterol assay, which was introduced for analysis of serum, is also applicable to bile. In comparison to other methods of cholesterol analysis, the enzymatic assay has the advantage of being simpler

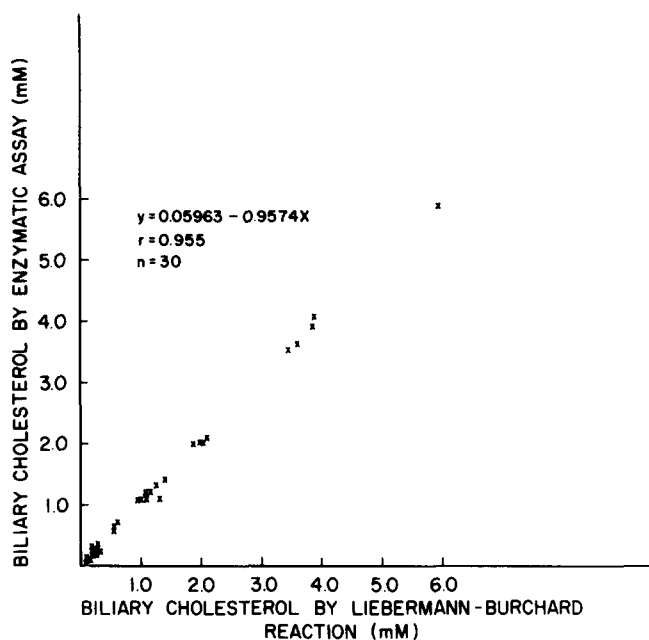


Fig. 1. The biliary cholesterol concentrations obtained by the enzymatic assay are plotted against those measured by a method using the Liebermann-Burchard reaction.

and less time-consuming, because it involves no extraction steps. The analytical yield of the assay is very satisfactory in unfiltered bile, which contains a considerable amount of cholesterol microcrystals, as well as in bile which is processed through a Millipore filter. Millipore filtration, which removes the majority of microcrystals of cholesterol, results in a marked decrease in the enzymatically measured cholesterol concentrations. These observations indicate that the enzymatic test allows the reliable measurement of the micellar as well as the microcrystalline cholesterol in bile.

The analytical results of the enzymatic test agree closely with those of the Liebermann-Burchard reaction (Fig. 1, Table 3). The correlation of increasing values of cholesterol concentrations is linear and through the origin. The enzymatic assay has no tendency to measure either higher or lower values than the Liebermann-Burchard reaction. The reproducibility of the enzymatic assay is good and similar to that of the Liebermann-Burchard reaction.

In summary, the enzymatic technique proved to be sensitive and precise. In comparison to other methods of biliary cholesterol determination, it has, in particular, the advantage of being rapid and simple, as no extractions need to be carried out.

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