We use bilirubin oxidase (EC 1.3.3.5) to remove interference by bilirubin in the assay of cholesterol concentration in bile by standard enzymatic methods. Samples are treated for 10 min with nonlimiting amounts of bilirubin oxidase to form biliverdin from bilirubin before the reagent for cholesterol is added. The relatively small interference by biliverdin is easily eliminated by use of sample blanks. The method is simple, convenient, and not hampered by the "chromogen oxidase" activity (the inherent ability of bilirubin oxidase to oxidize some chromogens) that plagues other assays of this type. Using this assay, we have accurately and precisely determined the concentration of cholesterol in bile. Such elimination of bilirubin will also be useful in assays of other biliary constituents or constituents of urine or icteric plasma.

Additional Keyphrases: analytical error • enzymatic methods • bilirubin oxidase • biliary cholesterol

Bile salts, phospholipids (primarily phosphatidylcholine), cholesterol, and proteins are the major organic solutes in bile (1). The bilirubin also present gives bile its characteristic color. Unfortunately, this color interferes with several spectrophotometric assays used to quantify bile constituents. Moreover, bilirubin can serve as a competitor in the peroxidase reaction by reacting with hydrogen peroxide; as a result, formation of the chromogens is incomplete, resulting in errors in the assay. Although the primary mechanism for disposal of cholesterol from the body is conversion to bile acids, cholesterol in bile also represents an excretory route for excess cholesterol in the body via the feces (2).

The concentration of cholesterol in bile is an important determinant of the proportion of cholesterol dissolved in bile. The tendency of cholesterol gallstones either toward growth or dissolution depends largely on the amount of dissolved cholesterol in bile: as the concentration of cholesterol in bile increases, formation of cholesterol gallstones is more likely if the concentration of other bile components remains unchanged (3).

Biliary cholesterol also has been implicated in a protective mechanism for the gall bladder, preventing damage induced by bile salts to the mucosal lining of the gall bladder, the common, hepatic, and cystic bile ducts, and the small intestine (4).

Analytical techniques currently used for determining concentrations of cholesterol in bile are primarily gas-liquid chromatographic procedures (5), laborious and time consuming. Bleaching with ultraviolet light for 24 h is also commonly used to remove bilirubin from bile or other compounds. This obviously time-consuming method also includes risks of sample contamination, evaporation, or spillage.

We report here an enzymatic assay, similar to that reported from Fromm et al. (6) but more convenient and time saving, for determining biliary cholesterol. Interference by bilirubin in peroxidase-coupled enzymatic procedures involving spectrophotometric measurement of hydrogen peroxide is common (7,8) and can cause erroneous results. Our procedure eliminates interference from bilirubin, and it is potentially applicable to other analyses, such as quantifying compounds other than cholesterol in bile, urine, and icteric plasma.

Materials and Methods

Samples. Hepatic bile was obtained from pigs in which common bile duct and duodenal catheters had been implanted. Samples were stored at −20 °C in screw-top vials and thawed at room temperature just before use.

Chemicals and enzymes. All chemical reagents were analytical grade. Bilirubin oxidase (from Myrothecium species; EC 1.3.3.5), lauryl sulfate (sodium salt, 99% pure), L-ascorbic acid, Diagnostic Kit 352 for total serum cholesterol, cholesterol standard (Accutrol calibrator A2034), and cholesterol calibrators (C0534) were all from Sigma Chemical Co., St. Louis, MO.

Apparatus. A Model 2600 spectrophotometer (Gilford Instrument Laboratory, Inc., Oberlin, OH) was used for all spectrophotometric analyses.

Reagent preparation. Bilirubin oxidase, diluted in distilled, de-ionized water to give a final concentration of 223 kU/L, was stable for at least 10 h in an ice bath. The pH 7.0 reaction buffer used in the assay was 100 mmol/L phos-
phate buffer containing 10 mmol of lauryl sulfate per liter. Stored at 4 °C, this buffer was stable for at least two weeks.

The cholesterol reagent was prepared according to the manufacturer’s directions by adding distilled, de-ionized water. To obtain blank values, we added ascorbic acid to duplicate samples to inhibit cholesterol oxidase in the reagent mix. The ascorbic acid was dissolved in 0.5 mol/L NaCl, and the pH of the solution was adjusted to 7.0.

A standard curve was prepared with Sigma cholesterol calibrators. Adding bilirubin oxidase to these standards produced no deleterious effects; the standard curves were similar to those obtained when bilirubin oxidase was not added. We measured the cholesterol in porcine bile 20 min after pretreatment with bilirubin oxidase.

Procedure. Pipet 30 μL of bile or 10 μL of standard into four 12 × 75 mm test tubes. Add 20 μL of reaction buffer and 30 μL of bilirubin oxidase solution (final concentration, 7 U per tube) to all tubes. Vortex-mix and incubate at 37 °C for 10 min. After incubating, add 50 μL of the ascorbate solution to two of the tubes (these will be the blanks). Then add 1 mL of cholesterol reagent to all tubes, vortex-mix, and incubate for 10 min at 37 °C. Determine the absorbance of the mixture at 505 nm as water as the blank. Determine the net absorbance values of the sample (subtract the blank absorbance values from the sample absorbance values) and read the concentration of cholesterol from the standard curve.

Results and Discussion

This use of bilirubin oxidase to pretreat samples for biliary cholesterol assay is based on the method of Allain et al. (9), with modifications by Sigma Diagnostics, and with use of Sigma Diagnostics’ cholesterol reagent after removal of interference from bilirubin. The cholesterol assay reagent includes cholesterol esterase, cholesterol oxidase, and peroxidase (9). The chromophores 4-aminoantipyrine and 5-hydroxybenzenesulphonate in the presence of hydrogen peroxide and peroxidase yield a quinoneimine dye that has an absorbance maximum at 505 nm. The intensity of the color produced is directly proportional to the total concentration of cholesterol in the sample.

Bilirubin has a peak absorbance at 440 nm but also absorbs some at 505 nm. This so interferes with the assay that use of blank samples is insufficient, not only because of its color but also because bilirubin interferes competitively in peroxidase-coupled reactions. We used bilirubin oxidase to remove this interference by facilitating the conversion of bilirubin to biliverdin, a compound that absorbs at a maximum of 320 nm and thus does not interfere at 505 nm to the extent that bilirubin does. Figure 1 illustrates the spectra of hepatic porcine bile, either treated or not treated with bilirubin oxidase. The sample’s absorbance in the 505-nm range is substantially decreased and is readily corrected with a sample blank.

Bilirubin oxidase has been used to measure bilirubin directly (10, 11) and to remove interference caused by bilirubin when creatinine is measured in serum and urine (12). Artiss et al. (12) noted that pretreatment with bilirubin oxidase resulted in somewhat lower concentrations of apparent creatinine than did the common methods of measurement, because results by commonly used methods are affected by bilirubin interference. Therefore, results obtained by using pretreatment with bilirubin oxidase in spectrophotometric assays may be more nearly accurate. Bilirubin oxidase pretreatment, however, has not been used in assays that measure concentrations of biliary constituents in bile; thus, our procedure is unique.

Table 1 shows data for the linear regression analysis of a standard curve from cholesterol calibrators (four standards in duplicate) and similar curves developed from cholesterol calibrator plus 10 and 20 μL of porcine bile. Curves from calibrator plus bile parallel the standard curve from 0 to 4 g/L and are linear over the same range of cholesterol concentrations. Regression coefficients (r) and coefficients of variation (CV) for the curves also are shown in Table 1. Regression coefficients in the range of 0.998 substantiate linearity, and slopes of 0.015 to 0.016 indicate that the lines are parallel. Thus, the assay has good repeatability. The assay was linear through sample sizes ranging from 0 to 40 μL of bile (concentration of cholesterol in bile from 0 to 3.6 mg/dL) with a slope of 0.009 and a regression coefficient of 0.997.

Our laboratory has measured cholesterol concentration in bile from 66 pigs in two different trials (13, 14). The pigs were fed diets varying in type and amount of fat and in amount of calcium and vitamin D3. The mean concentration of biliary cholesterol was 0.47 g/L, and it did not vary significantly with diet.

The precision of the method was determined by assaying a commercial cholesterol calibrator that contained 1.64 g of cholesterol per liter (Accutrol, Sigma Chemical Co.). We used results from 21 separate runs, performed during seven days, to determine the results shown in Table 2. The mean value for all samples was 1.64 g/L. Means for all runs during a day ranged from 1.77 to 1.53 g/L. Per-run means

**Table 1. Linear Regression Analyses of the Standard Curve and Standards plus Bile for Samples Pretreated with Bilirubin Oxidase**

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Mean ± SEM</th>
<th>r</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std.</td>
<td>15 ± 0.5</td>
<td>0.007 ± 0.10</td>
<td>0.998</td>
</tr>
<tr>
<td>10</td>
<td>16 ± 0.3</td>
<td>0.10 ± 0.006</td>
<td>0.999</td>
</tr>
<tr>
<td>20</td>
<td>15 ± 0.6</td>
<td>0.18 ± 0.013</td>
<td>0.997</td>
</tr>
</tbody>
</table>

*Std.: standard curve (four standards ranging in cholesterol concentration from 0 to 4 g/L); 10: standards plus 10 μL of added bile; 20: standards plus 20 μL of added bile; n = 3 each.
ranged from 1.79 to 1.49 g/L.

Use of bilirubin oxidase to remove interference of bilirubin in certain chromogen systems has been hampered by the inherent ability of bilirubin oxidase to oxidize the chromogen and thus destroy the ability to absorb spectrophotometrically. Chromogens and chromophores affected by bilirubin oxidase were 4-aminophenazone and tribromo-hydroxynbenzoic acid (15). The chromophore used in our procedure, 4-aminonitpyrine, may be less susceptible to oxidation than are other color-producing compounds. The dye complex was stable for at least 30 min, so no further precautions were necessary if absorbance was determined within that time frame. Absorbance values were 0.2955 at 0 min, 0.2958 at 15 min, and 0.2967 at 30 min. Our procedure eliminates the need for ferrocyanide (16), 4-dimethylaminoantipyrine (17), or other compounds that have been incorporated into peroxidase-coupled reagent mixtures to obviate oxidation of chromogen complexes that can occur with bilirubin oxidase.

Bilirubin interferes in spectrophotometric procedures for quantifying several biliary constituents in bile, including biliary cholesterol. In our procedure, bilirubin oxidase removes this interference by changing bilirubin to biliverdin, which absorbs maximally at a shorter wavelength than does bilirubin and therefore interferes less. In addition, pretreatment with bilirubin oxidase removes interference of bilirubin with hydrogen-peroxide-coupled reactions. The small amount of interference caused by biliverdin can easily be eliminated by use of sample blanks. Our procedure is easy, convenient, and time-efficient and may be readily adaptable for colorimetry of other biliary constituents.

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Table 2. Precision of Assay with Bile Samples* Pretreated with Bilirubin Oxidase

<table>
<thead>
<tr>
<th>Category</th>
<th>SD, g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0.09</td>
</tr>
<tr>
<td>Within-run</td>
<td>0.05</td>
</tr>
<tr>
<td>Between-run</td>
<td>0.07</td>
</tr>
<tr>
<td>Within-day</td>
<td>0.02</td>
</tr>
<tr>
<td>Between-day</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Mean = 1.64 g/L for 42 samples in 21 runs over seven days.


References

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