Rapid, Simplified Method for Measuring Total Hepatic Cholesterol

Herbert K. Naito and Lena A. Lewis

Because determinations of hepatic total cholesterol are frequently needed, an automated, continuous-flow method would be useful to have available. Hepatic total cholesterol is extracted with isopropyl alcohol in one step and the extract is then quantitatively analyzed by an accepted AutoAnalyzer procedure for serum total cholesterol. Homogenization of the tissue eliminates the use of proteolytic enzymes or KOH for the digestive process. The one-step extraction of total cholesterol with isopropyl alcohol simultaneously precipitates interfering proteins. Addition of FeCl₃ color reagent to an aliquot of clear extract produces a stable color with a density proportional to the amount of cholesterol present, which can be quantitatively detected by a colorimeter and recorded on a graphic recorder. This automated method eliminates tedious, time-consuming processes, and can greatly increase the reliability and accuracy of hepatic total cholesterol determinations. This method is shown to be rapid, reproducible, and simple. The procedure is adapted for both wet tissue and lyophilized samples, the latter giving slightly more reproducible results.

Additional Keyphrases AutoAnalyzer • tissue extraction with isopropyl alcohol • FeCl₃ color reagent for cholesterol • human and rat hepatic tissue

The increasing number and frequency of hepatic cholesterol analyses in experiments on laboratory animals and on clinical materials emphasize the need for a rapid, precise, and reproducible method. Several methods now used (1-4) require a time-consuming digestion of liver tissue with either proteolytic enzyme or concentrated KOH solution; an extensive and tedious extraction with one or more organic solvents; filtration of the supernatant fluid; evaporation of solvent; and, after the residue is reconstituted in the proper solvent, colorimetric measurement.

A fully automated continuous-flow method for performing colorimetric chemical analyses was first described by Skeggs (5). The “AutoAnalyzer,” in which the principles of this method are used, was introduced later. Since then, a methodology for automated determination of serum total cholesterol has been devised (6) and modified (7), resulting in a procedure that is rapid, simple, and precise (8).

The purpose of our investigation was to develop an equally satisfactory method for assay of hepatic total cholesterol with the AutoAnalyzer.

Materials and Methods

Instrumentation

The instrument system (Technicon Instruments Corp., Tarrytown, N.Y. 10591) consisted of the AutoAnalyzer Sampler II, Proportioning Pump, 95°C Heating Bath, Colorimeter, and Recorder. For greater accuracy, the samples were run at a rate of 20/h rather than at 40/h. Absorbance was read at 550 nm in a 15-mm tubular flowcell. Reagents used were the same as detailed elsewhere (8). In principle, the addition of FeCl₃ color reagent to an aliquot of clear isopropyl alcohol extract produces a stable color with a density proportional to the amount of cholesterol present, which can be quantitatively detected by a colorimeter and recorded on a graphic recorder. All total cholesterol values represent duplicate extractions of hepatic tissue and each extraction was read in duplicate on the AutoAnalyzer.

Male Sprague–Dawley–Rolfsmeyer rats (Dan Rolfsmeyer Co., Route 3, Syene Rd., Madison, Wis.), about 100 days old and weighing 280–320 g, were used. Wayne “Lab Blox” basal diet (Allied Mills, Inc., Libertyville, Ill.) and water were freely provided. The animals were fasted for 12–16 h before killing.

The samples of human liver were from a 69-year-old woman who died of a heart attack, with no evidence of infection or other disease.

Hepatic Tissue Preparation

Each rat’s liver was removed and blotted dry. A 500-mg sample was weighed, placed in a 30-ml tissue grinder (Cat. No. 8-414; Fisher Scientific Co., Cleveland, Ohio 44128) containing 9.5 ml of isopropyl alcohol (2-propanol), and completely homogenized as described by Naito and Griffith (18). The homogenate was decanted into a tube and centrifuged at 2,000 × g for 10 min. The supernatant

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fluid was then poured into a 125 × 16 mm Teflon screw-cap culture tube, capped, and stored at −20°C until analyzed with the AutoAnalyzer as described (8).

The human liver samples were prepared by freeze-drying the material in a VirTis lyophilizer (Model USM-15; Repp Division, VirTis Co., Inc., Gardiner, N. Y. 12525) and homogenizing the lyophilized material in a mortar and pestle to a fine powder; 150 mg of the powder was then placed in the tissue grinder containing 10.0 ml of isopropyl alcohol for complete homogenization. The homogenate was centrifuged and the supernatant fluid was stored as just described.

Results of our method for estimating total cholesterol in rat liver were compared to those obtained by the method of Riccardi and Fahrenbach (4), which is the only procedure that we know of in which the AutoAnalyzer is used to determine hepatic total cholesterol. The procedure involves an extensive digestion with alcoholic KOH solution, extraction of the cholesterol with an aliquot of petroleum ether, evaporation of the extract, and reconstitution with isopropyl alcohol.2 Results of analyses by the two methods were compared, the samples being contiguous portions from the same rat liver.

We estimated the relative completeness of extraction and precision of the cholesterol determination of our method by comparing our values for total cholesterol in rat liver with data obtained by the reference method of Folch et al. (13) for lipid extraction and by the method of Sperry and Webb (1) for cholesterol estimation. We compared the extraction of human hepatic total cholesterol by isopropyl alcohol with the chloroform–methanol (2:1, by vol) extraction method of Folch et al. (13). Extracts of human liver prepared by each of the methods were analyzed on the AutoAnalyzer.

**Evaluation of the Procedure**

**Recovery.** Recovery of added cholesterol was measured by extracting four different samples of rat liver (500 mg) to which a standard amount (237.5 µg in 9.5 ml isopropyl alcohol) of cholesterol standard had been added, which when multiplied by the dilution factor of 20, gives a 50 mg/100 ml standard. Protein precipitates when isopropyl alcohol is added to extract the hepatic total cholesterol, and, on centrifuging, the precipitate forms a pellet at the bottom of the tube. These pellets were washed twice with distilled water, centrifuged, and re-extracted with isopropyl alcohol, which was analyzed for total cholesterol to determine the completeness of recovery.

**Reproducibility and repeatability.** Reproducibility was tested by analyzing five separate aliquots of the same rat liver that were extracted on the same day. This procedure was repeated four times.

Repeatability was tested by preparing one “set” of hepatic total cholesterol determinations each day for 12 days from a single rat liver that was stored frozen. Each “set” contained five samples.

**Results and Discussion**

Results of 24 analyses for total cholesterol content of rat liver averaged 251.0 ± 7.0 (sd) mg/100 g tissue (wt wt); the mean value for 12 runs with the method of Riccardi and Fahrenbach (4) was 245.0 ± 15.0 (sd) mg/100 g tissue (Table 1). The difference between the means for the two methods was insignificant by Student's t-test (14). However, the lower standard deviation (±7.0 mg/100 g) and coefficient of variation (2.01%) of the proposed method as compared to ±15.0 mg/100 g and 6.20%, respectively, for Riccardi and Fahrenbach's method (4) suggests that ours is the more precise method.

When results by our method were compared to those obtained by the reference methods (1, 13) for accuracy and relative completeness of extraction (Table 2), results of our method were lower by an average of about 9.3 mg/100 g tissue (wt wt) than were the corresponding values obtained by the reference method (1), a significant difference (P < .05). Apparently, the extraction with chloroform–methanol (13) and determination of hepatic total cholesterol by the method of Sperry and Webb (1) gives 2.2 to 6.7% higher extraction yields and more reproducible results than does our method.

Recovery values are shown in Table 3. The addition of 237.5 µg of cholesterol in 9.5 ml of isopropyl alcohol (equal to 50 mg/100 ml cholesterol standard) when extracting the rat liver samples resulted in about 50 mg/100 g tissue (wt wt) higher values than the corresponding values for those samples extracted with isopropanol only, in accordance with the Beer–Lambert law. Some cholesterol remained in the pellets even after two washes with distilled water. Actual recovery of total cholesterol

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<th>Table 1. Precision, Reliability, and Relative Degree of Extraction Compared for Two AutoAnalyzer Methods for Determination of Hepatic Total Cholesterol</th>
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<td>Analytical method</td>
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<td>mg/100 g tissue, wt</td>
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* Contiguous samples of liver tissue from the same rat were used for each comparison.
was 86 to 98%. Note that the results of pellet analyses in both methods do not explain the slightly low yields by the proposed method.

Data on reproducibility are given in Table 4. The overall mean for the 20 determinations was 259.8 ± 1.6 (SE) mg/100 g tissue (wet wt). Range (SE) of values for sets one to four was 257.4 ± 1.0 to 262.0 ± 0.9 mg/100 g tissue. The difference between these values is not statistically significant, which indicates that the proposed method is reproducible.

Table 5 shows the results of the repeatability test. The mean was 351.1 ± 1.9 (SE) mg/100 g tissue (range, 348.0 to 353.5 mg/100 g). The difference in values was found to be insignificant at the 95% confidence level.

The above data indicate that the method described is reproducible, repeatable, and precise. It is simpler, faster, and more reliable than the method of Riccardi and Fahrenbach (4). Although the reference procedures (1, 13) gave slightly better yields and possibly better estimation of hepatic total cholesterol as compared to our method, they are time-consuming and laborious.

Freeze-drying hepatic tissues before extracting them with isopropyl alcohol gives more consistent, reliable, and reproducible results. The total cholesterol extracted from human liver by the method we described averaged 400.6 ± 0.3 mg/100 g tissue (dry wt); the amount obtained when the tissue was extracted by the reference method of Polch et al. (15) gave a mean of 399.3 ± 0.3 mg/100 g tissue (dry wt).
We made no attempt to correct for bilirubin content or for possible impurities in the hepatic tissue extracts and possible impurities in glacial acetic acid, which have been shown to affect serum total cholesterol determinations (9–11). However, the similarity, between 520 nm and 650 nm, of absorbance spectra of identical total cholesterol concentrations (300 mg/100 ml, as measured at 550 nm) of standard cholesterol and an extract of rat liver (Figure 1) indicates that readings at 550 nm are essentially free of interference from possible hepatic and reagent impurities. Only readings at lower wavelengths (400–500 nm) seem to reflect the presence of interfering substance(s) in the liver extracts. This does not exclude the possibility that other interfering factors, not present in our particular hepatic extracts and glacial acetic acid, may be encountered. Livers from animals subjected to different physiological conditions or patients with different pathological conditions may (a) introduce new chromogens or inhibitors or (b) increase the concentrations of these chromogens or inhibitors to the point at which they would interfere. Under these conditions the use of zeolite and Lloyd’s reagent in isopropyl alcohol might be warranted.

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References