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A Rapid and Sensitive Method for HPLC Cholesterol Determination in Bile

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A relatively little time consuming simple method based on the treatment of bile with cholesterol oxidase and subsequent high performance liquid chromatography measurement of the 3-ketocholesterol produced in order to determine the level of the cholesterol concentration is described. The method avoids bilirubin interferences, has high reproducibility and recovery assays give 100 % values. It is highly sensitive and suitable for use in the determination of cholesterol concentrations in bile and other bilirubin containing biological fluids.

Key words: Cholesterol measurement, Bile, HPLC, Bilirubin interference.

The secretion into the bile of cholesterol and its degradation products, bile acids, represents the major route for cholesterol elimination from the liver, and ultimately, from the body (7). In addition of basic research on cholesterol metabolism, there are several physiological and pathological conditions in which the amount of cholesterol in the bile should be measured. However, the high bilirubin content of bile is a major interference factor in the measurement of cholesterol in these samples. On one hand, bilirubin competes with the hydrogen peroxide reaction (8), and on the other, it absorbs light at 505-510 nm, which is also the wavelength for absorption by most cholesterol-color products (5, 7), and thus, bilirubin gives artifactual cholesterol values. Several methods have been developed to obviate these problems, but they are not yet free of difficulties. The FROMM *et al.* (2) method uses catalase to oxidize ethanol to acetaldehyde by the H_2O_2 produced in the cholesterol-oxygen reaction in the presence of cholesterol oxidase; however a light absorption of bilirubin is not avoided. Another method uses a high resolution

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gas liquid chromatographic technique (1) but it is very slow due to the prolonged retention time of cholesterol, which is about 19 min. An enzymatic method with cholesterol oxidase and peroxidase after incubation with bilirubin oxidase to prevent bilirubin interferences (5) is acceptable, but requires four tubes per sample, which makes it tedious and sample-consuming. In order to avoid these inconveniences, GOH *et al.* (3), have recently described a method based on the oxidative conversion of cholesterol and other related compounds with cholesterol oxidase, followed by high performance liquid chromatography (HPLC) elution and sterol quantification by measuring UV absorption at 240 nm. An adaptation of this method is here described, which substantially enhances its sensitivity and reduces column retention time for oxidized cholesterol.

Materials and Methods

Reagents. — Cholesterol reagent from the kit for total Cholesterol N.° 791440 for Automated Analysis by the BM/Hitachi System 737 from Boehringer Manheim GmbH (Germany) which, in the final reaction mixture, is composed of Tris buffer, 100 mmol/l, pH 7.7; Mg^{2+}, 50 mmol/l; sodium cholate, 10 mmol/l; phenol, 6 mmol/l; 3,4-dichlorophenol, 4 mmol/l; fatty-alcohol-polyglycol ether, 0.3 %; cholesterol esterase ≥ 0.4 U/ml; cholesterol oxidase ≥ 0.25 U/ml; peroxidase ≥ U/ml. Sodium cholate 98 %, ox or sheep bile (Sigma), cholesterol P.A. (IGODA-Merck, Barcelona, Spain) and isopropyl alcohol and ethyl alcohol for HPLC and acetic acid A.G. (FEROSA, Barcelona, Spain).

Apparatus. — Chromatograph for HPLC composed of a 440 ultraviolet and visible light detector, a model 510 pump, a U6K injector, a Data Module 730 integrator and a P/N 080040 precolumn Module with a RCSS C_{18} cartridge from Millipore-Waters (Milford, MA, USA) and a Nucleosil 5 C_{18} column (120 mm × 3 mm I.D.) from Knauer, FIOSA (Barcelona, Spain).

Animals. — Male Sprague-Dawley rats weighing 180-200 g fed *ad libitum* with purina chow diet (Panlab, Barcelona, Spain) were anaesthetized i.p. with 0.33 ml of a mixture containing 25 mg Ketolar, 5 mg Valium and 0.0005 mg atropine per ml. The bile duct was cannulated and bile was collected during the following 15 min.

Cholesterol measurement. — Standard: A cholesterol stock solution in isopropanol containing 1 mg cholesterol per ml was prepared. From this solution, fresh working standards were prepared by diluting 1/10 different amounts of the stock solution in 0.5 % sodium cholate.

Procedure: This is a modification of the GOH *et al.* method (3) which consists in reducing both the amount of isopropanol used to prepare the samples (2 µl instead of 50 µl), and the incubation time (30 min instead of 60), and uses a methanol/acetic acid eluting mixture instead of the methanol/acetonitrile. To 1.5 ml Eppendorf tubes, 10 µl bile and 20 µl 0.5 % sodium cholate/isopropanol (10/1) were added. In the case of the standards, 10 µl of saline and 20 µl of the working standard were used. After thoroughly mixing, 200 µl of cholesterol reagent diluted 1/10 in deionized water were added, mixed and incubated at 37 °C for 30 min. At this time the reaction was stopped by adding 600 µl ethanol. After mixing, and letting them stand at 4 °C for 30 min, the tubes were centrifuged at 3,000 *g* for 10 min.

Chromatography was carried out by eluting 50 µl of each supernatant with methanol/acetic acid 99/1 as eluant at a flow of 1.2 ml/min and the detector was set at 254 nm.

Results and Discussion

Standard curve and validation of the method. — The validity of the method was always verified in duplicate by processing a standard curve of 0, 0.5, 1.0, 1.5 and 2.0 μg cholesterol; by testing the reproducibility of the assay by measuring the cholesterol concentration in six aliquots of a bile sample; by processing a curve of increasing amounts of the same bile; and by determining the recovery of 5 μl bile aliquots to which 0, 0.5, 1.0 or 1.5 μg cholesterol were added.

As shown in figure 1, when the amount of cholesterol (μg) was plotted on the X axis against the peak areas (arbitrary units) on the Y axis, a linear regression with a high correlation coefficient appeared. The reproducibility study gave a mean value of 9.43 mg/100 ml with a variation coefficient of 2.48. The regression equation of the curve made by processing increasing amounts of bile was Y = 40.75 + 26.63 X (r = 0.99926) when μl of bile was plotted on the X axis against the peak areas on the Y axis. Figure 2 shows the results corresponding to the recovery assay with the expected values on the abscissae and the obtained ones on the ordinate. The different amounts of cholesterol used gave a linear and significant regression between these two parameters and a mean recovery of 100.02 ± 1.96 (mean ± SE) with a variation coefficient of 4.8 %.

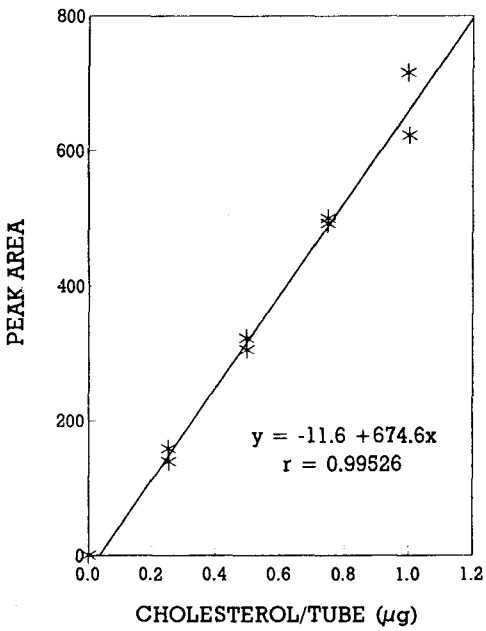

Fig. 1. Peak area values obtained in the processing of different amounts of standard cholesterol.

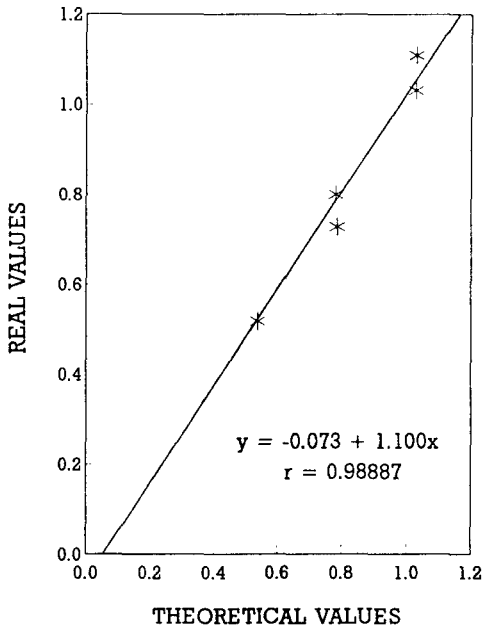

Fig. 2. Plot between the actual (real) and theoretical values of different amounts of cholesterol standard processed in the presence of 5 μl aliquots of the same rat bile samples.

The application of the present method in measuring the cholesterol concentration of six rats gave an average value of 10.13 mg/100 ml with a standard error of 1.39, which is a value in the range of that found by others in rat bile (4).

The method described herein has revealed several beneficial aspects which de-

serve emphasis: a) The method directly measures the 3-ketocholesterol produced by the reaction of cholesterol with O_2 in the presence of cholesterol oxidase and posterior separation by HPLC. Thus, it avoids any interference due to the bilirubin in the sample. b) This method is very sensitive (0.5 μg of cholesterol can be quantified) and, therefore, requires very little sample. Moreover, sensitivity can be increased by detecting the peak of 3-ketocholesterol at 240 nm —peak absorption of this compound (6)—, instead of 254 nm, which is the nearest available wavelength with the detector used here. c) The chromatographic retention time of 3-ketocholesterol found here was about 3 min which is less than half of the retention time (7.5 min) found for this same compound when following another HPLC method (3) and much shorter (19 min) than with cholesterol (1).

In conclusion, based on the simplicity of this method and the obtained results, we think it is a reliable and relatively simple method which can be applied in both clinical and basic studies.

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Resumen

Se describe un método sencillo y rápido para determinar las concentraciones de colesterol en bilis, basado en la formación de 3-cetocolesterol, mediante el tratamiento con colesterol oxidasa, y su medida por cromatografía de alta resolución. El método evita las interferencias con la bilirrubina y presenta una alta reproducibilidad. Los ensayos de recuperación dan valores del 100 %. El método es de alta sensibilidad y apropiado para cuantificar el colesterol en bilis y en otros fluidos biológicos que contengan bilirrubina.

Palabras clave: Determinación de colesterol, Bilis, HPLC, Interferencias con la bilirrubina.

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