notes on methodology

Determination of cholesterol using *o*-phthalaldehyde

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Summary A simple, rapid method for the determination of cholesterol in plasma and tissue using *o*-phthalaldehyde is presented. Comparison of this method with the $FeCl_3$ method gave identical results. However, the *o*-phthalaldehyde determination is three times more sensitive than the $FeCl_3$ determination (molar extinction coefficients of 11,610 and 33,440 for $FeCl_3$ and *o*-phthalaldehyde, respectively), it takes less time to complete, and the color developed is more stable. The *o*-phthalaldehyde method can be used to assay free and esterified cholesterol directly after thin-layer chromatographic separation.

Supplementary key words cholesteryl ester · $FeCl_3$ · thin-layer chromatography

CHOLESTEROL assay methods should be rapid and sensitive to be of maximal value for research use. We have found that the assay of cholesterol as described below using the *o*-phthalaldehyde reagent described by Zlatkis and Zak (1) meets these criteria. When the cholesterol content of plasma of several species was determined, essentially identical results were obtained with the *o*-phthalaldehyde and the $FeCl_3$ assays. However, much time was saved using the former reagent, the sensitivity was greater, and the color developed was more stable.

Chemicals and reagents. All solvents used were reagent grade and were purchased from commercial suppliers. Cholesterol, made to 1 mg/ml in ethanol for use as a standard, was purchased from Applied Science Laboratories, State College, Pa. This cholesterol preparation has been shown chromatographically to be 99+% pure (2). *o*-Phthalaldehyde was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio; a working solution with a concentration of 50 mg/dl in glacial acetic acid was prepared fresh on the day of use. Infrequently, a batch of *o*-phthalaldehyde was received from the supplier which did not give reproducible color development; however, this was true for less than 10% of the shipments obtained. Most of the *o*-phthalaldehyde purchased had a shelf life of at least 6 months. $FeCl_3$ was purchased from Fisher Scientific Co., Ltd., Montreal, Canada. A stock solution was prepared by dissolving 840 mg of $FeCl_3 \cdot 6H_2O$ in 10 ml of glacial acetic acid. A work solution was prepared by making a 1:100 dilution of stock $FeCl_3$ with glacial acetic acid. The work $FeCl_3$ was prepared 1 day prior to use and was stable for several months.

Procedure. In a typical assay, 0.1 ml of plasma or serum, 0.3 ml of 33% (w/v) KOH, and 3 ml of 95% ethanol are placed in a glass-stoppered tube and mixed thoroughly. The tube is then stoppered and placed in a 60°C heating block for 15 min. After the mixture has cooled, 10 ml of hexane is forcefully added to the tube to mix with the lower layer. 3 ml of distilled water is added, and the tube is capped and shaken for 1 min to ensure complete mixing. A blank, a standard, and a sample of pooled plasma are saponified and extracted at the same time. Appropriate aliquots (usually 1 ml) of the hexane layer are pipetted in duplicate into colorimeter tubes, and the solvent is evaporated under nitrogen. 2 ml of the *o*-phthalaldehyde reagent is added to each tube, and the solution is thoroughly mixed to dissolve all of the sample. About 10 min after the addition of the *o*-phthalaldehyde reagent, 1 ml of concentrated sulfuric acid is carefully added by allowing it to run down the inside of the tube; the solutions are immediately mixed on a tube vibrator. Absorbance is read at 550 nm between 10 and 90 min after the addition of the concentrated sulfuric acid.

In the ferric chloride procedure, 1.5 ml of $FeCl_3$ work solution was added to the tube containing the sample; after thorough mixing, the solution was allowed to stand for 10 min. Then 1 ml of concentrated sulfuric acid was added and the solution was mixed and placed in the dark. Absorbance at 560 nm was determined 45 min later. When the *o*-phthalaldehyde procedure was compared with the $FeCl_3$ method, separate portions of the same hexane layer were taken for assay by both color reactions.

In other studies, lymph lipoproteins were isolated from thoracic duct lymph of rabbits by preparative ultracentrifugation. Lipids were extracted with chloroform–methanol 2:1, and portions of the extract were taken for saponification and determination of total cholesterol by the *o*-phthalaldehyde method. Free cholesterol and cholesteryl esters were separated by TLC on prewashed silica gel G (Brinkmann Instruments, Ltd., Rexdale, Ontario) in a developing solvent of hexane–ethyl ether–

Abbreviations: ϵ, molar extinction coefficient; TLC, thin-layer chromatography.

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glacial acetic acid 70:30:1. After separation, free cholesterol and cholesteryl ester bands were visualized with iodine, and the appropriate areas were marked. After the iodine evaporated, the silica gel was scraped into glass-stoppered tubes and extracted with 10 ml of chloroform. The tubes were then centrifuged for 5 min at 1000 *g*. Aliquots of the chloroform layer were taken for colorimetric analysis using *o*-phthalaldehyde.

Aortas from cholesterol-fed rabbits were placed in saponification tubes, saponified for 1 hr, and then extracted as above. Portions of the hexane layers were removed, and cholesterol determination was performed using both the $FeCl_3$ and the *o*-phthalaldehyde procedures.

Results and discussion. A typical standard curve is shown in Fig 1. The absorbance of blank samples was below 0.010. With care, as little as 4–5 μg of cholesterol can be accurately determined. There was a linear relationship between the degree of color development and the amount of cholesterol over the entire range of observation. A similar straight line relationship was described by Zlatkis and Zak (1), although the absorbance per microgram of cholesterol for the present experiments was about two times higher than that reported by these workers. The reason for this difference is not known. In addition, we found that the absorbance did not change from 10 to 90 min after mixing. It was unnecessary to keep samples in the dark during this time.

We then proceeded to determine the applicability of using *o*-phthalaldehyde for the determination of cholesterol present in biological samples. Cholesterol in 25–150-μl aliquots of the same plasma sample was determined, and there was a straight line relationship between absorbance and plasma volume. Thus, the cholesterol concentration determined for individual plasma samples was not affected by the volume of plasma used in the assay. To determine the accuracy of the method, direct comparisons were made with the $FeCl_3$ method. The results are shown in Table 1. A total of 74 plasma samples from four animal species were compared, and the mean values were almost identical using the two methods. The ranges of values and the standard errors of the means

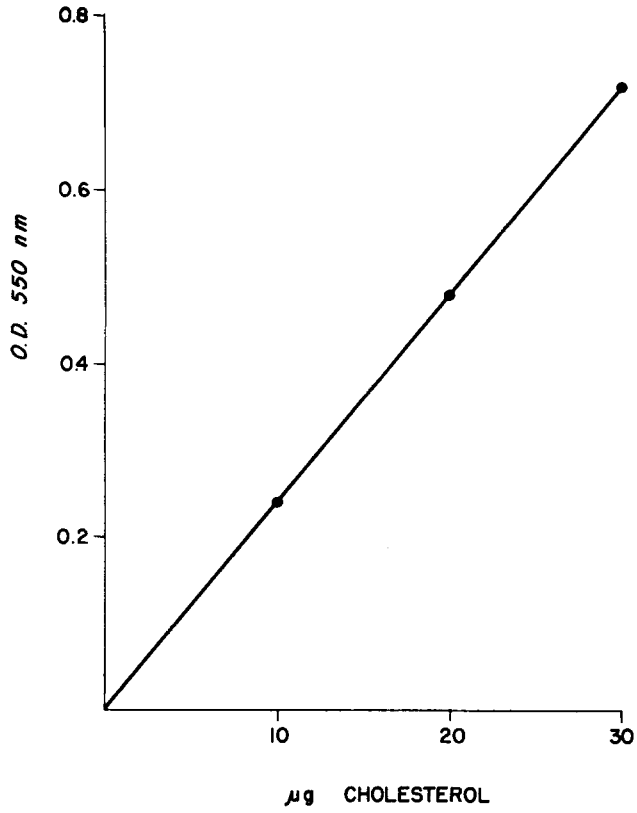

FIG. 1. Standard curve for cholesterol determination using *o*-phthalaldehyde. Absorbance was determined using a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.) in a 1.2-cm light path.

show that the variation within each of the methods is also the same. To determine the applicability of this method for cholesterol analysis in tissue, we analyzed the cholesterol in samples of aorta from five cholesterol-fed rabbits. The results shown in the bottom line of Table 1 demonstrate that the two methods gave identical results.

The reproducibility of the *o*-phthalaldehyde assay method was also studied. A pooled sample of rabbit plasma was divided into small portions, which were frozen; the cholesterol in these samples was then assayed on 20 different days. A mean value of 74.15 ± 1.03

TABLE 1. Comparison of *o*-phthalaldehyde and ferric chloride assay procedures

Species	No. of Samples	*o*-Phthalaldehyde Mean	*o*-Phthalaldehyde Range	*o*-Phthalaldehyde SEM	Ferric Chloride Mean	Ferric Chloride Range	Ferric Chloride SEM
Plasma cholesterol (mg/dl)							
Dog	9	110.3	84–147	6.43	114.7	91–148	6.14
Human	19	217.9	154–260	7.68	213.2	150–275	7.56
Monkey	23	157.9	96–233	7.33	155.7	98–230	7.10
Rabbit	23	117.2	42–209	9.58	113.7	42–214	9.44
Aorta cholesterol (mg/sample)							
Rabbit	5	8.64	1.59–15.50	2.31	8.69	1.61–15.84	2.35

TABLE 2. Recovery of cholesterol by *o*-phthalaldehyde determination

	Free Cholesterol	Ester Cholesterol	Total Cholesterol	% Recovery[a]
	μg/ml	*μg/ml*	*μg/ml*	*μg*
TLC samples	498.65 ±8.14	541.3 ±8.50	1039.9 ±10.50	101.8 ±1.07
Extract direct			1022.0 ±8.97	

Values are means ± SEM.

[a] Eight TLC separations were performed and are compared to direct saponification and determination of cholesterol in the extract, performed in triplicate.

(SEM) (range 64–80) mg/dl was obtained. A mean value of 187.3 ± 0.73 (SEM) (range 183–195) mg/dl was obtained in samples of pooled human plasma analyzed on 20 different days. These data show that this method of cholesterol determination is highly reproducible.

The recovery of free and ester cholesterol in a lipoprotein extract as determined by cholesterol analysis using the *o*-phthalaldehyde method is shown in Table 2. The mean percentage recovery for free + ester cholesterol for the eight TLC separations was 102% of the total cholesterol determined to be present by direct assay. It is important to note that the cholesteryl esters from the TLC plate were assayed intact without prior saponification. It was, however, necessary to saponify the lipoprotein extract before total cholesterol determination. In a few cases, we precipitated free cholesterol with digitonin, and the presence of digitonin did not interfere with the determination of free cholesterol by *o*-phthalaldehyde.

In summary, the use of the *o*-phthalaldehyde reagent for cholesterol assay has been shown to be applicable using biological samples, and it appears to offer several advantages over previously available methods. The most important of these is probably the saving in time. The *o*-phthalaldehyde reagent is easy to prepare, the color development is immediate and complete, and the color is stable and is light insensitive. Another important point is that the assay is relatively specific for cholesterol; no absorption occurs at 550 nm in the presence of cholestanol (1), which is the noncholesterol sterol present in highest concentrations in most mammalian species (3). The sensitivity of the present method is also an important advantage. For the $FeCl_3$ reaction, ϵ is 11,610 compared with 33,440 for the *o*-phthalaldehyde reaction. Quantities of cholesterol in the 4–5-μg range can be accurately determined using the method described. The *o*-phthalaldehyde determination is equally sensitive for esterified as well as free cholesterol. At the same time, the linearity of the concentration curve affords a wide range of sample concentrations to be included.

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