The picomole determination of free and total cholesterol in cells in culture

John G. Heider and R. Lance Boyett

Research and Development Department, Sandoz, Inc., East Hanover, NJ 07936

Summary An enzymatic, fluorometric method for the determination of free and total cholesterol in cells in culture is presented. The method is simple, requiring one reagent that includes all of the enzymes and a second reagent that increases the pH, which enhances the fluorescence of the product. The method is based on the enzymatic hydrolysis of cholesteryl esters to free cholesterol, the oxidation of cholesterol with the liberation of hydrogen peroxide, and the reaction of this peroxide with a fluorogen to form a fluorescent product in the presence of a peroxidase. It is rapid, in that free cholesterol can be read in 5 minutes and total cholesterol after 20 minutes. The precision of the method is greater than that obtained from gas–liquid chromatography.

Supplementary key words fluorescence · enzymatic method

The amount of cholesterol in a cell in culture will vary depending on the cell type and treatment, but it is in the order of femtomoles (10^{-15}). The study of cholesterol metabolism in cells has thus been restricted to large cell populations ($>10^5$ cells) because of the sensitivity limitations of present methods (1–3). Most of these methods, besides using corrosive dehydrating reagents, can only determine total cholesterol and require prior separation of free and esterified cholesterol by chromatography (4). Only the gas–liquid chromatographic methods, such as described by Ishikawa et al. (5), can distinguish free and total cholesterol directly. They, however, have the disadvantage of being tedious and time-consuming.

An enzymatic fluorometric method is described for the determination of either total or free cholesterol using a single aqueous reagent before the addition of base. Cholesteryl esters are hydrolyzed to free cholesterol by cholesteryl ester hydrolase (E.C. 3.1.1.13). The cholesterol oxidase (E.C. 1.11.1.7)-catalyzed oxidation of free cholesterol yields cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide reacts with *p*-hydroxyphenylacetic acid, in the presence of horseradish peroxidase (E.C. 1.11.1.7), to form a fluorescent product with an excitation wavelength at 325 nm and an emission wavelength at 415 nm. The method is reproducible and the reagents are both easy to prepare and relatively stable. The sensitivity has been extended into the picomole range.

The method has been successfully applied to the analysis of free and total cholesterol in arterial smooth muscle cells and fibroblasts from various species.

METHODS

Chemicals

Sodium phosphate, mono and dibasic, reagent grade were obtained from Baker and Adamson, General Chemical Division, Allied Chemical Corporation. Cholesterol oxidase (14.4 IU/mg), horseradish peroxidase (approximately 4000 U/mg) and Carbowax-6000 were from Supelco, Inc. Cholesteryl ester hydrolase (0.27 U/mg) was from P-L Biochemicals, Incorporated. Sodium taurocholate was obtained from Maybridge Chemical Company, Ltd., Cornwall, U.K. The *p*-hydroxyphenylacetic acid (Aldrich) was purified by column chromatography on silica gel 60 (70–230 mesh ASTM) (E. Merck Darmstadt, W. Germany). The column was charged with the *p*-hydroxyphenylacetic acid slurried in methylene chloride and was washed with approximately 2 l of methylene chloride before eluting with 2% methanol in methylene chloride. A narrow cut of the principal fraction was evaporated to dryness and crystallized two times from ether (mp 150–151°C). Isopropyl alcohol, Spectroquality grade, was from Matheson, Coleman, and Bell. Cholesterol U.S.P. was obtained from Sigma Chemical Company, cholesteryl oleate from Eastman Kodak Company, and sodium hydroxide from Fisher Scientific Company. The composition of the reagent solutions is given in **Table 1**.

Cell extraction procedure

The cells (10^4–10^5) were removed from Falcon T25 flasks with the aid of a rubber policeman and transferred to a conical graduated centrifuge tube. The cells were washed three times by suspending them in an isotonic salt solution, centrifuging at 800 *g* for 5

TABLE 1. Composition of reagents for cholesterol determination

A. Free cholesterol reagent	
Sodium phosphate buffer, pH 7.0	0.05 M
Cholesterol oxidase	0.08 U/ml
Horseradish peroxidase	30 U/ml
p-Hydroxyphenylacetic acid	0.15 mg/ml
B. Total cholesterol reagent	
Sodium phosphate buffer, pH 7.0	0.05 M
Cholesteryl ester hydrolase	0.08 U/ml
Cholesterol oxidase	0.08 U/ml
Horseradish peroxidase	30 U/ml
Sodium taurocholate	5 mM
Carbowax-6000	0.17 mM
p-Hydroxyphenylacetic acid	0.15 mg/ml
C. Sodium hydroxide solution	0.5 N

min, and aspirating the supernatant fluid. An appropriate volume of isopropyl alcohol (~0.1–1.0 ml/mg protein) was then added to the cell pellet and the sample was sonicated with a microprobe (140 × 3 mm) for 10 sec with a "LO" setting of 50 on a Bronwell Biosonik IV. Extraction is 95% for total cholesterol. After centrifugation for 15 min at 800 *g* the clear supernatant was decanted and an aliquot was taken for cholesterol analysis.

The residue was dissolved in 0.1 N sodium hydroxide and an aliquot was taken for protein determination by the method of Lowry, et al. (6).

Fluorometric assay

Free cholesterol. The isopropyl alcoholic solutions of standards, samples, and blank (isopropyl alcohol alone) were treated in a similar manner. An aliquot of 0.4 ml of free reagent (Reagent A, Table 1) was added to a 10 × 75 mm disposable glass test tube to which 20 μl of the isopropyl alcoholic solution was added and mixed. After standing at room temperature for approximately 5 min, 0.8 ml of 0.5 N sodium hydroxide (Reagent C, Table 1) was added and mixed. The fluorescence was measured with an Aminco-Bowman spectrophotofluorometer with an excitation wavelength of 325 nm and emission wavelength of 415 nm. A 1-cm light path cuvette was used with a xenon lamp, an IP28 photomultiplier tube, and 2-mm slits.

Total cholesterol. The same procedure described above for free cholesterol was followed for total cholesterol except that the total reagent (Reagent B, Table 1) was used instead of the free reagent and the samples were incubated for 20 min at 37°C before the addition of the 0.5 N sodium hydroxide solution (Reagent C, Table 1).

The gas–liquid chromatographic procedure was as follows. The data obtained from the fluorometric method was compared with that of the GLC method as described by Ishikawa et al. (5) with the following modifications: stigmasterol was used as an internal standard and the cholesterol was extracted into chloroform via the method of Bligh and Dyer (7).

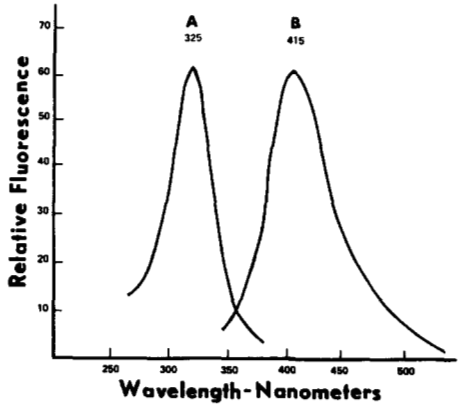

Fig. 1. Excitation and emission spectra of fluorescent product. *A*, Excitation with emission held at 415 nm; *B*, emission with excitation held at 325 nm.

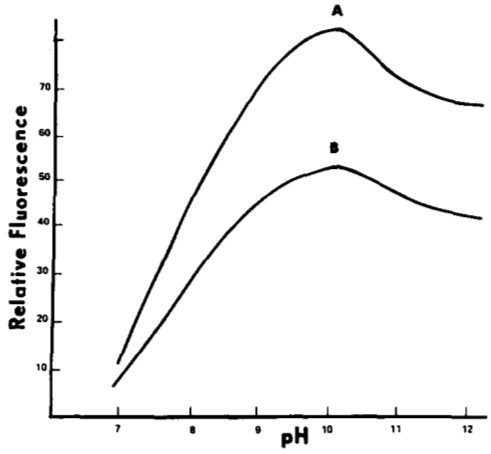

Fig. 2. Effect of pH on fluorescence. *A*, Cholesterol; *B*, blank.

RESULTS

Optimization of conditions

Fluorescence spectra. With the emission wavelength held at 415 nm and the excitation wavelength varied, maximum fluorescence was observed at 325 nm. The emission maximum fluorescence occurred at 415 nm when the excitation wavelength was held at 325 nm (see **Fig. 1**).

Concentration of reagents. The optimal concentrations of reagents are given in Table 1. No significant changes in fluorescent intensity over blank were observed over a relatively broad range of concentrations for all enzymes. Since the method was optimized for the determination of the smallest amounts of cholesterol, the concentration of cholesteryl ester hydrolase was kept to a minimum (0.08 U/ml). Higher concentrations shorten the time for complete hydrolysis only at the expense of greater blanks. A secondary consideration of cost of the enzyme also prompted the use of the lowest concentration possible.

The Carbowax-6000 was used as a solubilizing agent for the cholesteryl esters. Preliminary studies indicate that Triton X-100 may be used as a substitute but further work is required to optimize the concentration.

Varying the concentration of the pH 7 buffer between 0.1 M and 0.01 M had little effect on fluorescence of the sample above the blank. Increased molarity increased both blank and sample fluores-

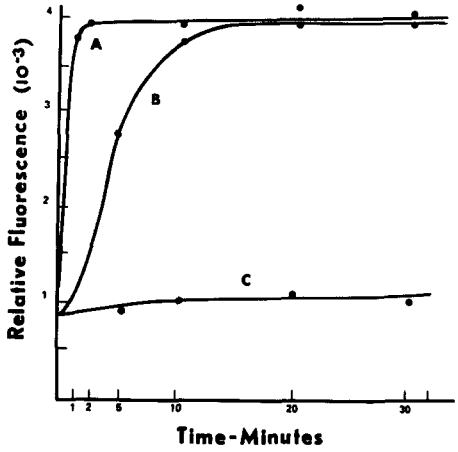

Fig. 3. Time of incubation vs. fluorescence. *A*, Cholesterol (2 nmol); *B*, cholesteryl oleate (2 nmol); *C*, blank.

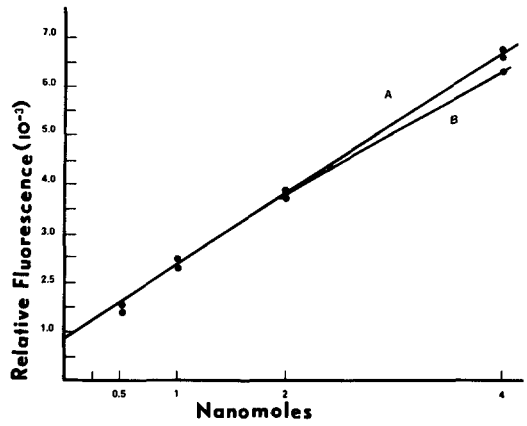

Fig. 4. Concentration of cholesterol free and ester vs. fluorescence (blank not subtracted). *A*, Cholesterol; *B*, cholesteryl oleate.

cence whereas lower molarities had reduced buffering capacity. Therefore, 0.05 M buffer was chosen for the assay procedure.

Effect of pH. Enzymatic activity was not significantly altered within the range of pH 6.5–7.5. Below and above this range activity decreased. However, the effect of pH on the fluorescent product is shown in **Fig. 2.** Although the blank fluorescence increased as well, the difference between sample and blank was greatest at pH 10. For reasons of greater stability of samples and blank, the pH was adjusted to pH 12.5. This was accomplished by adding 0.8 ml of 0.5 N sodium hydroxide solution. A 5-fold increase in fluorescence was obtained at this pH over that found at pH 7.

Incubation. At room temperature, the reaction was almost instantaneous for the determination of free cholesterol (**Fig. 3**); however, reproducibility was greater after 5 min. The determination of total cholesterol, which required cholesteryl ester hydrolase, needed longer incubation at 37°C for completion (see Concentration of reagents, above). Total hydrolysis was accomplished after 20 min (Fig. 3). This was confirmed by thin-layer chromatography semiquantitative analysis. After 20 min no cholesteryl ester spots could be detected. It was observed that both cholesterol oxidase and sodium taurocholate were required for complete hydrolysis.

Stability of reagents and fluorescent product

The stability of the fluorescent product was investigated by measuring the change in fluorescence of blank and standards with time. Although there was a slight increase in fluorescence of both blank and samples, the slopes were parallel for at least 2 hr (e.g., t = 0: blank 190, 250 pmol standard 720; t = 2 hr: blank 210, 250 pmol standard 750).

The free reagent (Reagent A) was found to be stable for more than a month if stored at 4°C in the dark. The total reagent (Reagent B) was prepared daily because of the instability of cholesteryl ester hydrolase.

Linearity of standard curve. In **Fig. 4** fluorescence vs. concentration in nanomoles per sample is plotted for free cholesterol and cholesteryl oleate standards. Above 5 nmol there is a gradual decrease in the slope. Since this was well above the concentrations of our working samples, attempts to increase linearity above this concentration were not pursued. The lower limit of detection is shown in **Fig. 5.** Detection of 25 pmol is possible but reliable quantitative data are only obtained with samples greater than 50 pmol.

Precision of the method

A study of intra- and intertest variability was made with a pooled isopropyl alcoholic extract from sonicated cells. The means and standard deviations for

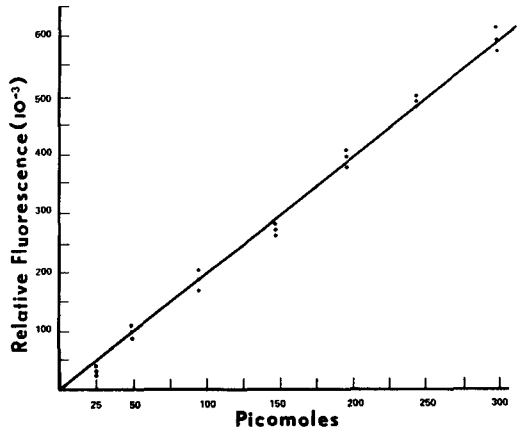

Fig. 5. Concentration of cholesterol vs. fluorescence (blank subtracted).

each day's results are shown in **Table 2.** An intertest mean and standard deviation of 408.4 ± 10.6 and 697.8 ± 9.6 pmol/sample was obtained for free and total cholesterol, respectively.

DISCUSSION

The isolation of cholesterol oxidase from *Nocardia* by Richmond (8) and of cholesteryl ester hydrolase by Hernandez and Chaikoff (9) have led to the development of several enzymatic methods for the determination of cholesterol (10, 11). In these methods, developed primarily for serum samples, a suitable chromogen such as *o*-dianisidine (11) or 4-aminoantipyrene and phenol (10) reacts with hydrogen peroxide to form a colored product. Because of the sensitivity limitations inherent in colorimetric analysis, a search was made for a reagent that would enzymatically react with hydrogen peroxide to yield a more sensitive fluorescent product and that would not interfere with the cholesterol-metabolizing enzymes.

Keston and Brandt (12) reported that dichlorodiacetyl fluorescin was a sensitive fluorometric reagent for the determination of hydrogen peroxide. Applying this to the determination of cholesterol, it was found to be extremely sensitive but was only suitable for free cholesterol standards. Cholesteryl ester hydrolase and cell extracts caused a rapid and complete conversion of the reagent to the fluorescent product.

Guilbault, Brignac, and Summer (13), who initially recommended homovanillic acid, later found that tyramine and *p*-hydroxyphenylacetic acid was equally effective or better for the determination of hydrogen peroxide and had the advantage of being less expensive (14). We found that *p*-hydroxyphenylacetic acid, after purification, was ideally suited for the determination of both free and total cholesterol. It was extremely stable in solution and did not interfere with the enzymatic reactions.

TABLE 2. Reproducibility of the method. Intra- and intertest variability[a]

Exp. No.	Cholesterol	
	Free	Total
1	412.2 ± 12.9[b]	698.3 ± 13.2[b]
2	397.5 ± 3.9	684.8 ± 18.6
3	417.9 ± 4.9	699.3 ± 17.6
4	396.7 ± 7.2	708.1 ± 22.0
5	417.9 ± 8.0	
Intertest mean	408.4 ± 10.6	697.8 ± 9.6
	(416.4 ± 15.0)[c]	(654.0 ± 7.8)[c]

[a] Pmol assayed (n = 4) on a pooled-cell isopropyl alcoholic extract.
[b] Mean ± SD.
[c] Gas–liquid chromatographic analysis on an equal aliquot evaporated to dryness and then assayed by the method of Ishikawa et al. (5) (n = 5).

TABLE 3. Effect of hyperlipemic serum on monkey smooth muscle cells and skin fibroblasts

			Cell Cholesterol (μg/mg Protein)		
			Free	Total	Ester
Exp. 1	SMC[a]	10% FBS[c]	32.5 ± 2.3[e]	31.9 ± 3.1	0
		10% HMS[d]	39.6 ± 0.8	46.2 ± 2.0	6.6
	MSF[b]	10% FBS	49.8 ± 1.8	57.8 ± 2.8	8.0
		10% HMS	53.0 ± 2.1	90.6 ± 3.9	36.9
Exp. 2	SMC	10% FBS	44.4 ± 3.2	44.2 ± 2.9	0
		10% HMS	46.0 ± 3.0	73.4 ± 1.2	27.4
	MSF	10% FBS	49.8 ± 2.0	57.8 ± 3.3	8.0
		10% HMS	53.6 ± 1.4	90.6 ± 4.0	37.0

[a] SMC, smooth muscle cells.
[b] MSF, skin fibroblasts.
[c] FBS, fetal bovine serum.
[d] HMS, hyperlipemic monkey serum.
[e] Mean ± SD (n = 4).
Monkey cells and serum were a gracious gift of K. Fisher-Dzoga, Department of Pathology, University of Chicago.

During the development of the method described here Huang, Kuan, and Guilbault (15) reported on the fluorometric enzymatic determination of total cholesterol in serum using homovanillic acid. Their method measures total cholesterol down to 4 mg/sample with a standard deviation of greater than 160 ng/sample. Furthermore it is a time-consuming, 10-step procedure requiring the sequential addition of reagents. The concentration of cholesterol is determined by rate change. In contrast, the method presented here requires the addition of one reagent that includes all the enzymes and another reagent to increase the pH of the final fluorescent product which results in a 5-fold increase in fluorescence. Concentration is determined by steady state fluorescence. This has the advantage of eliminating carefully timed readings and permits rereading samples up to 2 hr later.

The isopropyl alcohol extraction procedure described above is one of convenience and is not essential to the method. It has the advantage of precipitating the protein and extracting the cholesterol simultaneously. In cases where interference is suspected (e.g., tissue containing oxygen dismustase, which is soluble in alcohol and converts nascent oxygen to hydrogen peroxide) the more conventional (15) or the convenient (7) chloroform–methanol extraction methods have been used.

When sample concentrations of greater than 200

ng are to be assayed the addition of the sodium hydroxide solution can be eliminated. In the determination of free cholesterol this has the advantage of permitting the analysis of several samples in succession with the same reagent. It is useful to have a strip chart recorder attached to the spectrophotofluorometer to be assured the reaction is complete before the next sample is added.

The method has been successfully applied to the analysis of cholesterol of cells in culture with as few as 10^4 cells (0.1 mg of protein) as shown in **Table 3**. Studies are in progress to automate this procedure.

The authors wish to express their thanks to Dr. J. Perrine and R. Hugo for the cell samples used in this study, to Dr. F. Kathawalla and E. Copland for the purification of the *p*-hydroxyphenylacetic acid, and to C. Thummel and L. Macksoud for their technical assistance.

Manuscript received 1 August 1977; accepted 29 November 1977.

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