Porcine Pancreas: A Superior Source of Cholesterol Esterase for Total Serum Cholesterol Assay by the Enzymatic Kinetic Method

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Background: Accurate determination of cholesterol requires complete hydrolysis of cholesteryl esters and must be very fast for the kinetic cholesterol assay. We investigated the properties of cholesterol esterase derived from *Pseudomonas fluorescens*, *Candida cylindracea*, bovine pancreas, and porcine pancreas for cholesterol determination in human serum. **Methods:** Optimization of four enzymes and effect of sodium cholate concentration were performed. We evaluated and compared their performances in enzymatic kinetic cholesterol determination. **Results:** The optimal sodium cholate concentration was 3, 5, 15, and 12 mmol/l with the enzyme activities at 200, 100, 100, and 100 U/l for *P. fluorescens*, *C. cylindracea*, bovine pancreas, and porcine pancreas, respectively.

Key words: serum cholesterol; cholesterol esterase; kinetic property; atherosclerosis; coronary heart disease; *Pseudomonas fluorescens*; *Candida cylindracea*; bovine pancreas; porcine pancreas

Linearity obtained from all enzymes was up to 16.3 mmol/l. All assays were compared favorably with standardized endpoint method. Only the cholesterol esterase derived from porcine pancreas demonstrated acceptable precision within the acceptable criteria (%CV < 3.0). Also, this esterase was least affected by interfering substances and showed longer stability than that of *C. cylindracea* and bovine pancreas. **Conclusion:** Porcine pancreas cholesterol esterase is superior to that obtained from *P. fluorescens*, *C. cylindracea*, and bovine pancreas for total serum cholesterol determination by the kinetic method because of its lower cost, better accuracy and precision, less interference, and longer stability. J. Clin. Lab. Anal. 26:420–430, 2012. © 2012 Wiley Periodicals, Inc.

INTRODUCTION

The determination of cholesterol is widely used for the assessment of atherosclerosis, cardiovascular disease, and its equivalents, which are a worldwide health problem in men and women and through a range of ages (1). Cholesterol circulates in blood in lipoproteins, in free form and partially as a cholesteryl ester. In human serum, an approximately 70% of total cholesterol is esterified with long-chain fatty acids (2). Cholesterol oxidase cannot act on cholesteryl esters, but hydrolysis must completely occur in order to obtain accurate measure of cholesterol.

The linked enzymatic reactions are as follows:

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Received 28 October 2011; Accepted 15 June 2012

DOI 10.1002/jcla.21538

Published online in Wiley Online Library (wileyonlinelibrary.com).

The assay is generally performed as an endpoint (3,4) or kinetic method (5,6). Advantages of the kinetic method over the endpoint method are shorter analysis time, reduced effects of interfering substances, and elimination of sample blank measurement (7). Using kinetic cholesterol determination, the cholesterol oxidase reaction was the rate-limiting step of whole reaction and directly proportional to cholesterol concentration. According to previous studies (5), we found that *Streptomyces* cholesterol oxidase was the best source of enzyme for the kinetic cholesterol method. Analytical performance of the assay was precise and accurate and did not show interference from hemoglobin as high as 7.5 g/l.

An accurate first-order kinetic assay of total cholesterol determination depends on the properties of the cholesterol esterase reaction. The reaction rate of cholesterol esterase must be very fast. Moreover, the hydrolysis of cholesterol esters must be complete before time measurement begins. Cholesterol esterase (EC 3.1.1.13) is derived from the several bacterium sources, such as *Pseudomonas fluorescens*, *P. aeruginosa*, and *Candida cylindracea*, and the pancreatic juice of bovine and porcine (8–12). Wiebe and Bernert (13) showed that the lipolytic efficiency-cleavage of cholesteryl ester was related to the sources of enzyme and the reaction matrix. In addition, bile salts and its conjugates are required to stabilize the enzyme activity. According to Siedel et al., they demonstrated that cholesterol esterase isolated from *Pseudomonas* strain was suitable for complete cleavage of cholesterol ester within minimum incubation period (14).

This study evaluated and compared the performance characteristics of cholesterol esterase isolated from *P. fluorescens*, *C. cylindracea*, bovine pancreas, and porcine pancreas for the measurement of total serum cholesterol by the kinetic method. The eligible sources were also selected for the enzymatic kinetic assay.

MATERIALS AND METHODS

The study was approved by the ethics committee of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand.

Equipment

We used the Vitalab Selectra (E. Merck, Dramstadt, Germany) to determine the reaction rate of total cholesterol in human serum by the kinetic method.

Reagents

For the experimental reagents, all enzymes and chemicals were obtained from the Sigma Chemical company (St.

Louis, MO), except the *C. cylindracea* cholesterol esterase from Roche Diagnostics GmbH (D-68305, Mannheim, Germany), 4-aminophenazone from BDH (Dorset, England), and 3,4 dichlorophenol from Aldrich Chemical Inc. (Milwaukee, WI).

To study the optimization of cholesterol esterase activity, we prepared the reagent base A without cholesterol esterase by dissolving *Streptomyces* cholesterol oxidase 1,000 U/l, peroxidase 10,000 U/l, sodium cholate, 3 mmol/l, 4-aminophenazone, 0.5 mmol/l, phenol 20 mmol/l, 3,4 dichlorophenol 5 mmol/l, and Brij 35, 4.5 g/l in phosphate buffer, 0.1 mol/l, pH 7.0. We added 0, 100, 200, 300, and 400 U/l of each cholesterol esterase in the reagent base A.

To study the optimization of sodium cholate concentration, we prepared the reagent base B by dissolving the suitable activity of *P. fluorescens* (B1), *C. cylindracea* (B2), bovine pancreas (B3) or porcine pancreas (B4) cholesterol esterase, *Streptomyces* cholesterol oxidase 1,000 U/l, peroxidase 10,000 U/l, 4-aminophenazone, 0.5 mmol/l, phenol 20 mmol/l, 3,4 dichlorophenol 5 mmol/l, and Brij 35, 4.5 g/l in phosphate buffer, 0.1 mol/l, pH 7.0. Four sets of working cholesterol reagents containing each cholesterol esterase were prepared by adding sodium cholate 0, 1, 3, 5, 10, and 12 mmol/l in reagent base B1–B4. For bovine pancreas cholesterol esterase, we extended the sodium cholate concentration to 15 mmol/l.

Procedures

Optimization study

The user-defined parameter of the Vitalab Selectra analyzer was set as following: Reaction mode: kinetic, Delay: 148 sec, Wavelength: 505 nm, Reagent volume: 300 μ l, and Sample volume: 3 μ l. Optimizing each cholesterol esterase and sodium cholate concentration was performed by using normal appearance human sera containing cholesterol concentrations of 2.4, 5.3, 10.8, 16.3, and 20.9 mmol/l. The absorbance change per minute was determined and plotted versus each cholesterol concentration. The linearity was evaluated from the guideline of Clinical and Laboratory Standards Institute (CLSI) EP06-A (15). We selected the reagent containing the minimal enzyme activity and sodium cholate concentration necessary to produce the optimal cholesterol linearity.

Analytical performances of cholesterol esterase

We used the Siemens Dimension RxL's endpoint cholesterol method (16) as the reference comparison method. Our laboratory was standardized through the Centers for Disease Control and Prevention (CDC) National Heart Lung and Blood Institute Lipid Standardization

Program. The accuracy and precision of the measurements during the study were within the acceptable criteria ($\%CV \leq 3.0$) of the National Cholesterol Education Program (NCEP) (17).

Linearity and reportable range

To assess the linearity of each method, we used a set of sera with cholesterol ranging from 2.6 to 23.3 mmol/l, plotting the absorbance change per minute versus cholesterol concentration. Linearity was evaluated using the CLSI EP06-A guideline and the linear region reported on the reportable range (15).

Imprecision

We selected sera with low (~ 2.8 mmol/l), middle (~ 5.0 mmol/l), and high (~ 10.0 mmol/l) cholesterol. The within-run (20 replicates in the same run) and between-run (20 consecutive days) imprecisions were determined in each serum sample. The means, standard deviations (SD), and coefficients of variation ($CV = SD/\text{mean} \times 100\%$) were calculated and tabulated.

Recovery

We performed the recovery study by mixing sera contained low, middle, and high cholesterol interchanging by the ratio of 1:1. We calculated the recovery of cholesterol from the test method compared to the Siemens Dimension RxL's method in percentage.

Comparison

We compared cholesterol in 50 hospitalized sera obtained from the test kinetic method to the reference end-point method. The regression equations, correlation coefficients, bias, and the SD of the residuals ($S_{y/x}$) values obtained between the methods were calculated.

Interference study

We prepared the sets of hemolyzed, icteric, and turbid sera samples. The hemolyzed samples were prepared from lysis of clotted red cells to obtain hemoglobin concentration ranging from 0 to 15.0 g/l. The icteric samples were prepared from purified bilirubin (Sigma Chemical) to obtain bilirubin concentration ranging from 0 to 1368 $\mu\text{mol/l}$. The turbid samples were prepared from pooled milky sera and diluted the pool with NaCl, 0.9%, to obtain the various degree of turbidity (absorbance at 670 nm of 0–2.334). A 1:2 dilution of each interference sample with pooled serum of low, middle, and high cholesterol concentrations was made to obtain three sets of various degrees of interfering substances. Cholesterol in each sample's set was determined by using the experimental cholesterol reagents. Interfering substances are considered significant when their effects disturb the results by $\pm 9\%$ or

more according to total analytical goal for cholesterol determination (17).

Stability

The aliquots of two sera with normal (4.7 mmol/l) and high (8.8 mmol/l) cholesterol concentrations kept at -80°C were determined using the assessment cholesterol reagent for a studied period of 2 months. The reagents were stored in a refrigerator at $2-8^\circ\text{C}$.

Statistic Methods

Mean, SD, coefficients of variation ($\%CV$), and the correlation and regression analyses were performed with Microsoft EXCEL. Bias error was calculated from the regression analysis at cholesterol decision cut-point of 5.2 and 6.2 mmol/l. Random error was obtained from $\%CV$ base on the between-run imprecision study.

RESULTS

Optimizing of Cholesterol Esterase

Figure 1 shows the optimal activities of *P. fluorescens* (Fig. 1A), *C. cylindracea* (Fig. 1B), bovine pancreas (Fig. 1C), and porcine pancreas (Fig. 1D) used in the kinetic cholesterol assay. All reaction patterns demonstrated hyperbolic curves. Increasing the enzyme activities from 100 to 400 U/l slightly increased the change in absorbance for the cholesterol reactions. The upper end of the cholesterol linearity for the reagent using *P. fluorescens*, *C. cylindracea*, bovine pancreas and porcine pancreas isolated enzymes was 16.3 mmol/l at the enzyme activities of 200, 100, 100, and 100 U/l, respectively.

Optimizing of Sodium Cholate

The rate of hydrolysis for various sources of cholesterol esterase may be modulated using bile salts such as cholate. Figure 2 illustrates the various absorbances of enzyme activity for increasing sodium cholate concentrations for each source of enzyme. The optimal absorbance patterns of *P. fluorescens* (Fig. 2A) were similar to that of *C. cylindracea* (Fig. 2B). Addition of sodium cholate slightly increased the reaction rates of the enzymes. However, increasing sodium cholate concentration greater than 5 mmol/l suppressed the reaction rate of *C. cylindracea* enzyme. The absorbance patterns of bovine pancreas (Fig. 2C) and porcine pancreas cholesterol esterase (Fig. 2D) differed from those of the *P. fluorescens* and *C. cylindracea* enzymes. Increasing the sodium cholate concentration from 0 to 12 mmol/l increased the reaction rate for the bovine pancreas enzyme. A sodium cholate concentration of 12 mmol/l yielded the greatest

Fig. 1. Optimization curves of cholesterol reagents containing *Pseudomonas fluorescens* (A), *Candida cylindracea* (B), bovine pancreas (C) or porcine pancreas cholesterol esterase (D).

sensitivity for the cholesterol reaction, surpassing that at 10 mmol/l. To maximize the reaction rate, we extended the sodium cholate concentration to 15 mmol/l. The optimal concentrations of sodium cholate were 3, 5, 15, and 12 mmol/l for *P. fluorescens*, *C. cylindracea*, bovine pancreas and porcine pancreas enzymes, respectively.

Analytical Performances of Cholesterol Esterase in Sera

To use the *P. fluorescens* source of enzyme requires a higher enzyme activity (200 U/l), thus making it the most costly of all the enzyme sources. Because of its higher cost, we decided to discontinue its evaluation.

Linearity

Figure 3 displays the linearity of serum cholesterol obtained from the kinetic assay using the reagents containing

the optimal activities for each cholesterol esterase. The upper end of the analytical range for the cholesterol reagents containing *C. cylindracea*, bovine pancreas and porcine pancreas enzymes was 16.3 mmol/l, as demonstrated by the polynomial method (CLSI EP06-A) (15).

Reproducibility

Table 1 shows the within-run and between-run precision obtained from the quality control sera with low (2.20 mmol/l), middle (5.66 mmol/l), and high (9.40 mmol/l) cholesterol concentrations. The average CVs for within-run imprecisions were 1.28, 1.22, and 1.28% and between-day imprecisions were 3.24, 4.46, and 2.87% for *C. cylindracea*, bovine pancreas and porcine pancreas enzymes, respectively.

Fig. 2. Optimization patterns of cholesterol reagents containing *Pseudomonas fluorescens* (A), *Candida cylindracea* (B), bovine pancreas (C) or porcine pancreas cholesterol esterase (D) with various sodium cholate concentrations.

Accuracy

The analytical recovery of serum cholesterol obtained from the kinetic method using the reagents containing *C. cylindracea*, bovine pancreas and porcine pancreas enzymes are tabulated in Table 2. An average recovery was 100.8, 99.8, and 99.7%, respectively.

We compared cholesterol measured between the test kinetic methods with the reference endpoint method (12) by least-square linear regression analysis (Fig. 4). The regression lines according to the least-squares technique and the correlation coefficient (r) were $y = 1.00x + 0.044$ (95% confidential interval (CI), 0.985–1.007 for the slope and -0.038 – 0.126 mmol/l for the y -intercept), $r^2 = 0.998$, for the *C. cylindracea* enzyme; $y = 0.962x + 0.054$, (95% CI, 0.937–0.987 for the slope and -0.116 – 0.226 mmol/l for the y -intercept), $r^2 = 0.985$ for the

bovine pancreas enzyme; and $y = 0.992x + 0.035$ (95% CI, 0.979–1.005 for the slope and -0.057 – 0.127 mmol/l for the y -intercept), $r^2 = 0.998$ for the porcine pancreas enzymes. The biases (test enzyme mean minus Siemens mean) were 0.018, -0.175 , and -0.017 mmol/l and the SD of the residuals ($S_{y/x}$) was 0.117, 0.261, and 0.132 mmol/l, respectively. In addition, the paired t -test shows no significant mean difference ($P > 0.29$) for *C. cylindracea* and porcine pancreas enzymes but shows significant mean difference ($P < 0.001$) for bovine pancreas enzyme.

Analytical error of the implemented methods

We estimated the systematic error as a percent of each cholesterol assay at the clinical decision cut-point of serum cholesterol (5.2 and 6.2 mmol/l) from the regression

Fig. 3. Linearity of serum cholesterol obtained from cholesterol reagents containing *Candida cylindracea* (A), bovine pancreas (B) or porcine pancreas cholesterol esterase (C).

equations as shown in Table 3. The systematic errors of all kinetic cholesterol methods compared with the Siemens Dimension RxL's endpoint cholesterol method ranged from -2.9 to 0.4% , which were within the allowable bias recommended by the NCEP guidelines for cholesterol, $<3\%$ at decision levels (16).

Interference

The effects of hemoglobin, bilirubin, and turbidity on the total cholesterol assays are shown in Table 4. Hemoglobin concentrations up to 7.5 g/l did not interfere with results for cholesterol obtained from porcine pancreas enzyme. However, hemoglobin interfered with cholesterol results at concentrations of 3.5 and 6.5 g/l for *C. cylindracea* and bovine pancreas enzymes, respectively. The effect of turbidity varied for the different kinetic assays. A four-plus turbidity (equivalent to an absorbance

of more than 1.2 at 670 nm) did not interfere with results for the porcine pancreas enzyme. A one-plus turbidity (equivalent to an absorbance of more than 0.40 at 670 nm) decreased the results for the *C. cylindracea* enzyme, and a three-plus turbidity (equivalent to an absorbance of more than 1.0 at 670 nm) increased results for the bovine pancreas enzyme. Bilirubin concentrations greater than 171.0 $\mu\text{mol/l}$, imparted a negative bias for all cholesterol determinations.

Reagent stability

The stability of reagents for the kinetic cholesterol determinations depended on the source of enzyme (Fig. 5). The reagent containing porcine pancreas gave the longest stability (5 weeks), followed by *C. cylindracea* (4 weeks) and bovine pancreas enzyme (3 weeks).

TABLE 1. The Within-Run and Between-Day Imprecision of Total Serum Cholesterol Obtained From the Cholesterol Reagent Containing *Candida*, Bovine Pancreas or Porcine Pancreas Cholesterol Esterase

Imprecision	Coefficient of variation (CV,%)		
	E1 ^a	E2 ^b	E3 ^c
Within-run (<i>n</i> = 20)			
Low	1.25	1.45	1.80
Middle	1.39	1.57	1.12
High	1.19	0.65	0.92
Average	1.28	1.22	1.28
Between-day run (<i>n</i> = 20)			
Low	3.95	4.63	3.08
Middle	3.31	4.21	2.95
High	2.47	4.55	2.63
Average	3.24	4.46	2.87

^aE1: *Candida* cholesterol esterase.^bE2: Bovine pancreas cholesterol esterase.^cE3: Porcine pancreas cholesterol esterase.

DISCUSSION

Measurement of cholesterol is important for risk assessment of coronary heart disease (1). The absorbance change, resistance to interference, and storage stability plays an important role in reagent robustness for conventional and biosensors. More robust reagents mean greater dynamic range and lower cost for conventional assays.

Cholesterol biosensors are very rapidly developing for various applications including clinical diagnostics, pharmaceuticals, and food industries (18–21). Cholesterol biosensors are based on the immobilization of cholesterol esterase and cholesterol oxidase on a desired biosensor surface (18). These biosensing devices must be easy to use, self-sufficient, and quick in response time. The enzymatic kinetic cholesterol biosensor is of increased interest in the development of biosensor devices because the assay offers the advantages such as short turnaround time and low sensitivity to interfering substances (7).

According to previous studies (3, 5), *Streptomyces* cholesterol oxidase is an excellent source of oxidase in the kinetic cholesterol assay. It appears to work well with serum samples not only with esterified cholesterol, but also with lipoproteins. Since cholesterol in human blood is present in the form of ester, the first step in the estimation of cholesterol is complete hydrolysis of cholesterol esters to free cholesterol. In our previous study of different sources for cholesterol oxidase for the kinetic cholesterol method, the only source used for cholesterol esterase was that isolated from *Psuedomonas fluorescens* (5, 6). Other sources for cholesterol esterase are available, but not much is known about the analytical properties of enzymes isolated from *C. cylindracea*, bovine pancreas, and porcine pancreas.

The maximal cholesterol linearity was observed for reagents containing *P. fluorescens* enzyme of 400 U/l, which is similar to report of Deeg and Ziegenhorn (6). However, we selected the enzyme activity at 200 U/l, which gave the suitable linearity up to 16.3 mmol/l. A similar linearity was obtained for the reagents containing minimum enzyme activities at 100, 100, and 100 U/l for *C. cylindracea*, bovine pancreas, and porcine pancreas, respectively.

Sodium cholate acts as an emulsifier, which may provide a more favorable environment for the cholesterol esterase to completely disrupt the cholesterol-containing lipoproteins, dispersing the cholesterol and cholesterol esters into micelles (13). The enzymes then react with these micelles. According to our studies, we observed that the cholesterol reagent with the addition of sodium cholate less than 3 mmol/l appeared turbid. Therefore, a suitable surfactant is necessary to achieve the optimal esterolytic activity and to stabilize the enzyme.

Regarding to the source of cholesterol esterase, we can classify the cholesterol esterase sources into two groups; microbial (*P. fluorescens* and *C. cylindracea*) and animal (bovine pancreas and porcine pancreas). The characteristics of enzyme between the microbial and the

TABLE 2. The Recovery of Total Serum Cholesterol Was Obtained From the Reagent Containing *Candida*, Bovine Pancreas or Porcine Pancreas Cholesterol Esterase

Level	Expected value (mmol/L)	Observed value (mmol/L)			Recovery %		
		E1 ^a	E2 ^b	E3 ^c	E1	E2	E3
	Siemens method						
L + M (1 + 1) ^d	3.93	3.99	3.95	3.92	101.5	100.5	99.7
L + H (1 + 1)	5.80	5.88	5.77	5.77	101.3	99.5	99.5
M + H (1 + 1)	7.53	7.52	7.48	7.52	99.8	99.3	99.8
Average					100.8	99.8	99.7

^aE1: *Candida* cholesterol esterase.^bE2: Bovine pancreas cholesterol esterase.^cE3: Porcine pancreas cholesterol esterase.^dCholesterol concentration: Low [L] = 2.2 mmol/l; Middle [M] = 5.66 mmol/l; High [H] = 9.4 mmol/l.

Fig. 4. Correlation of total serum cholesterol obtained between the kinetic cholesterol assay using *Candida cylindracea* (A), bovine pancreas (B) or porcine pancreas cholesterol esterase (C) and the Siemens Dimension RxL's endpoint method.

TABLE 3. Error Analysis of Cholesterol Assays as Calculated at Clinical Decision Cut-Points (5.2 and 6.2 mmol/l)

Cholesterol (mmol/l)	Analytical error (%)		
	E1 ^a	E2 ^b	E3 ^c
5.2	5.244 (0.4%)*	5.056 (-2.7%)	5.193 (-0.1%)
6.2	6.244 (0.3%)	6.018 (-2.9%)	6.185 (-0.2%)

^aE1: *Candida* cholesterol esterase.

^bE2: Bovine pancreas cholesterol esterase.

^cE3: Porcine pancreas cholesterol esterase.

*The percentage errors are shown in parentheses. They were calculated from (Test value - critical value) divided with critical value and multiplied by 100.

animal sources differed greatly with the addition of sodium cholate. Using microbial sources, sodium cholate concentration has an insignificant effect on their activities. In contrast, the enzymes derived from the animal source are activated by increasing sodium cholate concentration. The difference in the effect of sodium cholate may be due to the mass of enzyme. Hyun et al. (22,23) has purported that sodium cholate aids in the polymerization of the enzyme, thereby increasing its hydrolytic activity toward the steroid ester and protecting the enzyme from proteolytic agents. The molecular weights of *P. fluorescens* (129 kD) and *C. cylindracea* (129 kD) were larger than porcine pancreas and bovine pancreas enzymes (15–64 kD) (8–11). Therefore, the polymerization of cholesterol

TABLE 4. Effect of Hemoglobin, Turbidity, and Bilirubin on Serum Cholesterol Determined by the Cholesterol Reagent Containing *Candida*, Porcine Pancreas or Bovine Pancreas Cholesterol Esterase

Degree	Hemoglobin (g/l)	Cholesterol (mmol/l)			Turbidity absorbance at 670 nm	Cholesterol (mmol/l)			Bilirubin (μmol/l)	Cholesterol (mmol/l)		
		E1 ^a	E2 ^b	E3 ^c		E1	E2	E3		E1	E2	E3
Baseline	0	2.62	2.55	2.51	0	2.62	2.55	2.51	0	2.78	2.68	2.60
±	0.1	2.68	2.51	2.54	0.2	2.48	2.49	2.48	42.8	2.62	2.54	2.55
1+	0.9	2.77	2.55	2.59	0.4	2.10^e	2.55	2.40	85.5	2.47	2.53	2.42
2+	1.8	2.89	2.60	2.68	0.8	1.65	2.64	2.45	171.0	2.12^e	2.23^e	2.19^e
3+	3.5	3.12^d	2.78	2.77	1.0	0.56	2.80^d	2.48	342.0	1.37	1.17	1.52
4+	6.7	3.39	2.96^d	2.91	1.2	-0.64	2.97	2.38	513.0	0.87	1.01	1.07
Baseline	0	4.67	4.55	4.64	0	4.67	4.55	4.64	0	4.67	4.82	4.79
±	0.1	4.65	4.43	4.60	0.2	4.57	4.67	4.78	42.8	4.73	4.67	4.77
1+	0.9	4.82	4.68	4.73	0.4	4.26^e	4.66	4.73	85.5	4.39	4.42	4.49
2+	1.8	4.90	4.65	4.78	0.8	3.67	4.73	4.65	171.0	4.02^e	4.22^e	4.30^e
3+	3.5	5.21^d	4.83	4.91	1.0	2.87	5.27^d	4.87	342.0	3.25	3.74	3.83
4+	6.7	5.49	5.09^d	5.03	1.2	1.25	5.42	4.82	513.0	2.32	3.23	3.09
Baseline	0	7.64	7.99	7.73	0	7.64	7.99	7.73	0	8.09	7.90	8.12
±	0.1	7.69	7.98	7.74	0.2	7.60	8.07	7.79	42.8	8.05	7.89	8.03
1+	0.9	7.91	8.11	7.86	0.4	7.50^e	8.31	7.80	85.5	7.68	7.79	7.78
2+	1.8	7.96	8.19	7.93	0.8	6.62	8.47	7.77	171.0	7.39^e	7.54^e	7.73^e
3+	3.5	8.32^d	8.40	8.14	1.0	5.39	8.89^d	7.88	342.0	6.07	7.58	6.92
4+	6.7	8.67	8.62^d	8.36	1.2	3.98	9.11	8.01	513.0	5.42	7.11	6.83

^aE1: *Candida* cholesterol esterase.^bE2: Bovine pancreas cholesterol esterase.^cE3: Porcine pancreas cholesterol esterase.^dStarting point of positive interference (>9% increasing from baseline cholesterol concentration) (17).^eStarting point of negative interference (>9% decreasing from baseline cholesterol concentration).

Note: Bold indicates cholesterol concentrations that significantly differ from the original cholesterol concentrations.

esterase isolated from the microbial sources is unnecessary while it is required in the animal sources.

Pseudomonas fluorescens cholesterol esterase does not appear to be the best source for kinetic cholesterol assay because its requirement for twofold higher activity made it more costly compared with the other enzymes. In comparison, the cost of the other enzymes was 45.7, 58.6, and 30.2% for *C. cylindracea*, bovine pancreas, and porcine pancreas, respectively. Cholesterol reagents containing *C. cylindracea*, bovine pancreas or porcine pancreas enzyme possessed sufficient activities to provide linear responses up to 16.3 mmol/l. The average analytical recoveries of serum cholesterol obtained from three cholesterol reagents were excellent.

Although the results of linear regression showed the good association with the reference commercial endpoint method, a significant mean difference ($P < 0.001$) was obtained from the method using bovine pancreas enzyme. We also calculated the percentage error at the decision cut-points of 5.2 and 6.2 mmol/l. The assays using *C. cylindracea* and porcine pancreas appeared accurate near the clinical decision cut-points. However, the differences obtained from the bovine pancreas method (−2.7 and −2.9%) were absolutely higher than *C. cylindracea* (0.4 and 0.3%) and porcine pancreas (−0.1 and −0.2%).

A difference in the reproducibility was seen between each other reagent. Although the average value of within-run obtained from all experimental kinetic method showed good reproducibility, the between-run obtained from *C. cylindracea* and bovine pancreas was not. The analytical imprecision of porcine pancreas enzyme only appeared within the acceptable criteria (%CV < 3.0) recommended by the US NCEP (17).

Interfering substances varied in their effect depending on enzymatic origin. We demonstrated that porcine pancreas cholesterol esterase was less sensitive to interfering substances than *C. cylindracea* and bovine pancreas. Bilirubin consistently demonstrated a negative interference for all three enzymes, but to a greater degree for the *C. cylindracea* source of cholesterol esterase. The difference may relate to variations in solubility of the reagent mix. It might be possible that the addition of potassium ferrocyanide into cholesterol reagent may successfully eliminate bilirubin interference with the enzymatic kinetic method. Hemoglobin demonstrated a positive interference with the esterase from *C. cylindracea* and bovine pancreas, but the enzyme from *C. cylindracea* was more markedly affected, which may result from small changes in the kinetics of the reaction or spatial variations. The interference caused by turbidity is noteworthy, in the sense

Fig. 5. Stability of cholesterol reagent, containing *Candida cylindracea* (A), bovine pancreas (B) or porcine pancreas cholesterol esterase (C), kept at 2–8°C.

that it demonstrated a marked negative interference with the *C. cylindracea* esterase, but a small positive interference with the bovine esterase and no interference with the porcine esterase. Because we used a kinetic method, the negative interference with the *C. cylindracea* esterase may have resulted from a slow decrease in the turbidity of the sample–reagent mix, diminishing the effect of chromophore production. If the esterases from the animal sources show a more rapid clearing of the sample or fail to clear the sample, they are less likely to produce a negative interference.

We found the shelf life of porcine pancreas was of the longest stability (5 weeks), followed by *C. cylindracea*

(4 weeks) and bovine pancreas enzyme (3 weeks). In the stability study, we only performed with an aqueous solution of reagent. To adapt these cholesterol reagents for clinical use, preparing reagent in the lyophilized state with vacuum-dried or freeze-dried may improve the stability.

In this study, we have emphasized the source of the esterases, based on their microbial or animal origin. Clearly, the source of the esterase provides most of the difference one might observe for reaction characteristics. We did not investigate different manufacturing processes in the production of any of the particular enzymes. While it is true that variations in manufacturing processes may introduce variation in the performance of a particular enzyme activity, this is less of a problem when one is using purified reagents, and even though we did not investigate in this particular study, it should become part of the quality control of the reagent manufacturer when the reagents are commercialized. Using cholesterol esterase from the different manufacturers may affect the hydrolysis rate of cholesterol esters in biological samples, but one would expect the effect to be much less than the differences based on the source of enzyme itself. Because the lipolytic activity of cholesterol esterase must be very fast for the kinetic cholesterol determination, extending our results to other commercially available enzymes would require additional performance studies, but as commonly practiced today, these studies would be part of the quality control of reagent manufacture.

In conclusion, the analytical performances of *C. cylindracea*, bovine pancreas and porcine pancreas enzyme indicated their potential utility for the determination of total serum cholesterol by the kinetic method. We propose that the porcine pancreas is a superior source of cholesterol esterase because of its lower cost, better accuracy and precision, less interference, and longer stability. Its performance characteristic might prove useful in the development of new reagents for the measurement of cholesterol and of biosensors for the measurement of lipid and lipoproteins.

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