Code No. 435-35801

Free Cholesterol E

(COD-DAOS Method)

For Research Use Only. Not for use in diagnostic procedures.

Intended use

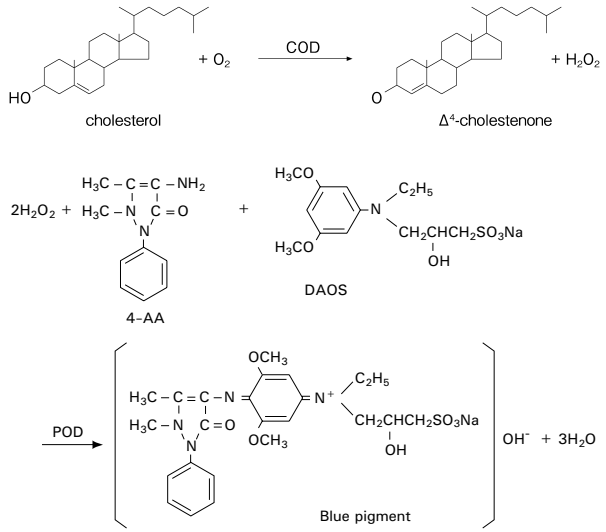
The Free Cholesterol E is an *in vitro* assay for the quantitative determination of free cholesterol in serum.

Summary and explanation of the test

Cholesterol concentration in serum is closely related to the formation, absorption and catabolism of cholesterol in liver and in intestine and to the metabolism of lipoproteins in blood.
Early methods for determining cholesterol levels were based on chemical colorimetric methods. Since W.Richmond reported an enzymatic method employing cholesterol oxidase (COD) in 1972, highly specific enzymatic methods, which are very simple procedures under ordinary conditions, have been used extensively with growing popularity.
The Free Cholesterol E is reagent based on the enzymatic method, employing 3,5-dimethoxy-N-ethyl-N-(2-hydroxy-3-sulfopropyl)･aniline sodium (DAOS) that produces blue pigment.

Principle of the method

Free cholesterol in the serum is oxidized by cholesterol oxidase to Δ^4-cholestenone and produces simultaneously hydrogen peroxide. The hydrogen peroxide formed causes DAOS and 4-aminoantipyrine to undergo quantitatively an oxidative condensation in the presence of peroxidase, to produce a blue color.
The amount of free cholesterol in the test sample is determined by measurement of the absorbance of the blue color.



Reagents

(1) Buffer Solution 2 × 75 mL
50 mmol/L Good's buffer (pH6.1)
Surfactant
Store at 2-10℃.

(2) Color Reagent 2 × for 75 mL
23.75 U/vial Cholesterol oxidase (COD)
400 U/vial Peroxidase (POD)
2.99 mg/vial 4-Aminoantipyrine (4-AA)
25.1 mg/vial DAOS
Ascorbate oxidase (AOD)
Store at 2-10℃.

(3) Standard Solution 1 × 10 mL
100 mg/dL Cholesterol
Store at 2-10℃.

Warnings and precautions

(1) For Research Use Only. Not for use in diagnostic procedures.
(2) Do not use the reagents described above in any procedures other than those described herein. Performance cannot be guaranteed if the reagents are used in other procedures or for other purposes.
(3) Operate the instruments according to operator's manuals under appropriate conditions. Consult the instrument manufacturer for details.
(4) Store the reagents under the specified conditions. Do not use reagents past the expiration date stated on each reagent container label.
(5) Do not use reagents which were frozen in error. Such reagents may give false results.
(6) After opening the reagents, it is recommended to use them immediately. When the opened reagents are stored, cap the bottles and keep them under the specified conditions.
(7) Do not use the containers and other materials in the package for any purpose other than those described herein.
(8) The vial is stoppered at reduced pressure. Slowly remove the stopper in order not to release the powder in the vial.
(9) Do not use the reagents at any reaction temperatures or reaction periods other than described herein.
(10) When discarding the reagents, dispose of them according to local or national regulations.
(11) All the devices including reagents and reagent bottles that come in contact with specimens should be considered potentially infectious.
(12) If the reagents come in contact with the mouth, eyes or skin, wash off immediately with a large amount of water. Consult a physician if necessary.
(13) Do not mouth pipette directly. Use a safety device for pipetting.
(14) Be careful not to cut yourself with the aluminum cap when removing it from the vial.

Physical or chemical indications of instability

The presence of precipitates in the reagents or values of control sera outside the manufacturer's acceptable range may be an indication of reagent instability.

Specimen collection and preparation

(1) Samples
(a) Sample can be stored for 3 days at 2-10℃ or 2 months in frozen state without significant effect on the measured value.
(2) Interfering substances
(a) Anticoagulants such as heparin, citrate, oxalate, EDTA do not have significant influence on the assay when they are used in their usual amounts.
(b) Ascorbic acid gives slightly positive influence on the assay.
(c) Hemolysis gives slightly positive influence on the assay.
(d) Bilirubin does not have significant influence up to 20 mg/dL on the assay.

Materials required but not supplied

Pipettes (0.05 mL and 3.0 mL), Water bath or heating block capable of maintaining 37℃, Spectrophotometer or colorimeter, capable of measuring absorbance at 600 nm.

Reagent preparation

Color reagent solution : Dissolve the contents of one bottle of Color Reagent in one bottle (75 mL) of Buffer Solution. This solution is stable for 2 weeks at 2-10℃. Do not freeze.
Since the bottle of Color Reagent is under negative pressure, the rubber stopper must be removed slowly to avoid loss of contents.

Test procedure

Temperature : 37℃

(1) Free cholesterol assay

	Sample(serum)	Standard	Reagent Blank
Specimen	0.05 mL	0.05 mL	0.05 mL
Color Reagent Solution	3.0 mL	3.0 mL	3.0 mL
Mix well and after 5 min, measure the absorbance of S (A_S), Std (A_{Std}) reagent Blank (A_{Bl}) at 600 nm within 1 hour. If double wavelength photometry is used, main wavelength 600 nm and sub waverength 700 nm are applied.			

(2) Calibration

(a) Prepare a diluted standard solution by adding the distilled or deionized water to the Standard Solution as directed below in Table 1.

Table 1

Standard Solution (100 mg/dL)	Distilled or deionized water	Amount of cholesterol
1.0 mL	1.0 mL	50 mg/dL

(b) Take the dilute standard solution prepared as above (1) and the Standard Solution (undiluted) in the quantities shown in Table 2 into 3 test tubes.

Table 2

Test tube No.	Diluted standard solution	Stnd Soltn. (undiluted)	Amount cholesterol (mg/dL)
1	0.05 mL	–	50
2	–	0.05 mL	100
3	–	0.10 mL	196.8 (note)

(Note) Though the amount of sample to be taken as a rule is 0.05 mL, 0.10 mL was taken in this case and hence the total volume is increased slightly, the value shown in Table 2 is the corrected value.

(c) Proceed as directed in Procedures of Measurement for the Standard Solution and then plot absorbances of each tube against the amount of free cholesterol; a calibration curve is obtained.

Results

(1) Reading from the calibration curve:
The amount of free cholesterol corresponding to the absorbance of a test sample is read from the calibration curve prepared in advance.

(2) Determine from the calculation formula:
Determine the absorbance of a sample (A_S) and that of Standard Solution (A_{Std}) against Blank and calculate the amount of free cholesterol from the following formula.

Amount of free cholesterol in the sample (mg/dL)

$$= \frac{A_S}{A_{Std}} \times 100$$

Amount of cholesterol ester (mg/dL) =
Total cholesterol amount (mg/dL)-Free cholesterol amount

Ester ratio (%) =

$$\frac{\text{Cholesterol ester amount (mg/dL)}}{\text{Total cholesterol amount (mg/dL)}} \times 100$$

Quality control

A quality control program is recommended for all laboratories. The analysis of control material in both the low and high ranges with each assay is recommended for monitoring the performance of the procedure. The values obtained for controls should fall within the manufacturer's acceptable ranges. If values are to be established for unassayed control material, the laboratory should assay each level of control material a sufficient number of times to generate a valid mean and acceptable range.

Limitations of the procedure

The calibration curve is linear up to 300 mg/dL on the Shimadzu UV-240. If the sample free cholesterol exceeds this level, it should be diluted appropriately with distilled or deionized water and reassayed.

Performance characteristics

Accuracy

When a control serum of known concentration is assayed, the assay value falls within the range of ± 18% of the known concentration.

Precision

Within-run precision
When a sample is assayed 5 times or more in a run, CV of the absorbance is not more than 2 %.

Sample #	Replicates	Mean (μg/dL)	SD	CV %
1	10	26.48	0.15	0.57
2	10	37.76	0.33	0.87
3	10	80.02	0.55	0.69

Sensitivity

(1) When purified water is assayed, the absorbance is not more than 0.08.
(2) When a calibrator of given concentration (Cholesterol 100 mg/dL) is assayed, the absorbance is 0.17-0.44.

Correlation

A comparison of the Free Cholesterol E and a similar Free Cholesterol C (A Product of Wako Pure Chemical Ind., Ltd) was performed using the spectrophotometer. The test results provided the following data.

Specimen	Serum
Correlation coefficient	r = 0.987 (n = 40)
Regression equation	y = 1.03x + 0.3
x	Free Cholesterol C (mg/dL)
y	Free Cholesterol E (mg/dL)

Specificity

Additive study

Hemoglobin (mg/dL)	None	50	100	200	300
Free cholesterol (mg/dL)	49.5	50.2	52.9	57.3	57.3

Bilirubin (mg/dL)	None	5	10	20	40
Free cholesterol (mg/dL)	36.3	35.0	34.1	34.7	33.8

Ascorbic acid (mg/dL)	None	5	10	20	50
Free cholesterol (mg/dL)	49.7	46.3	45.0	44.0	41.6

Uric acid (mg/dL)	None	5	10	20	30
Free cholesterol (mg/dL)	47.7	47.7	47.7	47.0	46.4

References

(1) Richmond, W. Clin. Chem., **19**, 1350 (1973).
(2) Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W. and Fu, P. C. : Clin. Chem., **20**, 470 (1974).

Ordering information

Code No.	Product	Package
435-35801	Free Cholesterol E	50 Tests

435-35801F

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