Enzymatic Assay of CHOLESTEROL ESTERASE (EC 3.1.1.13)

PRINCIPLE:

Cholesterol Oleate $\text{Cholesterol Esterase} \rightarrow$ Cholesterol + Oleic Acid

Cholesterol + O$_2$ + H$_2$O $\text{Cholesterol Oxidase} \rightarrow$ H$_2$O$_2$ + Cholestenone

2H$_2$O$_2$ + 4-AAP + Phenol $\text{Peroxidase} \rightarrow$ 4H$_2$O + Quinoneimine Dye

Abbreviations:
4-AAP = 4-Aminoantipyrine

CONDITIONS: T = 37°C, pH = 7.0, A$_{500\text{nm}}$, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 400 mM Potassium Phosphate Buffer, pH 7.0 at 37°C
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Potassium Phosphate Dibasic, Trihydrate. Adjust to pH 7.0 at 37°C with 1 M NaOH.)

B. 0.9% (w/v) Sodium Chloride Solution
(Prepare 25 ml in deionized water using Sodium Chloride.)

C. 8.6 mM Cholesteryl Oleate Solution (Chol-Oleate)
(Prepare by first dissolving 56.0 mg of Cholesteryl Oleate, in 1 ml of Polyoxyethylene Ether, 9 Lauryl Ether. While stirring, add 9 ml of hot Reagent B. Store at room temperature.)

D. 15% (w/v) Taurocholic Acid Solution (Tauro)
(Prepare 10 ml in deionized water using Taurocholic Acid, Sodium Salt.)
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REAGENTS: (continued)

E. 1.75% (w/v) 4-Aminoantipyrine Solution (4-AAP)  
(Prepare 1 ml in deionized water using 4-Aminoantipyrine, Free Base.)

F. 6% (w/v) Phenol Solution  
(Prepare 10 ml in deionized water using Phenol.)

G. Cholesterol Oxidase Enzyme Solution (Chol Oxid)  
(Immediately before use, prepare a solution containing 20 – 30 units/ml of Cholesterol Oxidase, in cold Reagent A.)

H. Peroxidase Enzyme Solution (POD)  
(Immediately before use, prepare a solution containing 5 mg/ml of Peroxidase Type II from Horseradish, in cold deionized water.)

I. Cholesterol Esterase Enzyme Solution  
(Immediately before use, prepare a solution containing 0.25 – 0.50 unit/ml of Cholesterol Esterase in cold Reagent A.)

PROCEDURE:

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into a suitable container:

<table>
<thead>
<tr>
<th>Test</th>
<th>Reagent A (Buffer)</th>
<th>31.80</th>
<th>Reagent C (Chol-Oleate)</th>
<th>7.50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reagent D (Tauro)</td>
<td>1.50</td>
<td>Reagent E (4-AAP)</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Reagent F (Phenol)</td>
<td>1.50</td>
<td>Reagent G (Chol Oxid)</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>Reagent H (POD)</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 37°C.
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PROCEDURE: (continued)

Pipette (in milliliters) the following reagents into a suitable container:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Cocktail</td>
<td>2.90</td>
<td>2.90</td>
</tr>
<tr>
<td>Reagent I (Cholesterol Esterase)</td>
<td>0.10</td>
<td>----</td>
</tr>
<tr>
<td>Reagent A (Buffer)</td>
<td>----</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Immediately mix by inversion and record the increase in A$_{500nm}$ for approximately 5 minutes. Obtain the $\Delta$A$_{500nm}$/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/mg enzyme} = \frac{\Delta A_{500nm}/\text{min Test} - \Delta A_{500nm}/\text{min Blank}}{(0.5) (13.78) (\text{mg enzyme/ml RM})}$$

13.78 = Millimolar extinction coefficient of Quinoneimine Dye at 500 nm under the assay conditions

RM = Reaction Mix

0.5 = Conversion factor based on one mole of H$_2$O$_2$ produces half a mole of Quinoneimine Dye.

UNIT DEFINITION:

One unit will hydrolyze 1.0 µmole of cholesteryl oleate to cholesterol and oleic acid per minute at pH 7.0 at 37°C in the presence of taurocholate.

FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 299 mM potassium phosphate, 0.50% (w/v) taurocholic acid, 0.05 mg peroxidase, 1.4 mM cholesteryl oleate, 1.7% (w/v) polyoxyethylene 9 lauryl ether, 0.14% (w/v) sodium chloride, 0.2% (w/v) phenol, 0.4-0.6 unit cholesterol oxidase and 0.025 - 0.05 unit cholesterol esterase.
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This procedure is for informational purposes.