SIGMA QUALITY CONTROL TEST PROCEDURE
Enzymatic Assay of CHOLESTEROL ESTERASE
(EC 3.1.1.13)

PRINCIPLE:

\[
\begin{align*}
\text{Cholesterol Esterase} & : 
\text{Cholesterol Oleate} + \text{H}_2\text{O} \rightarrow \text{Cholesterol} + \text{Oleic Acid} \\
\text{Cholesterol Oxidase} & : 
\text{Cholesterol} + \text{O}_2 \rightarrow \text{H}_2\text{O}_2 + \text{Cholestenone} \\
\text{Peroxidase} & : 
2\text{H}_2\text{O}_2 + \text{4-AAP} + \text{Phenol} \rightarrow 4\text{H}_2\text{O} + \text{Quinoneimine Dye}
\end{align*}
\]

Abbreviation:

\[4\text{-AAP} = 4\text{-Aminoantipyrine}\]

CONDITIONS: \(T = 37^\circ\text{C}, \text{pH} = 7.0, A_{500\text{nm}}, \text{Light path} = 1\ \text{cm}\)

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, Sigma Prod. No. P-5379. Adjust to pH 7.0 at 37°C with 1 M KOH.)

B. 0.9% (w/v) Sodium Chloride Solution
(Prepare 25 ml in deionized water using Sodium Chloride, Sigma Prod. No. S-9625.)

C. 8.6 mM Cholesteryl Oleate Solution (Chol-Oleate)
(Prepare 10 ml by first dissolving the Cholesteryl Oleate, Sigma Prod. No. C-9253, in 1 ml of Polyoxyethylene, 9 Lauryl Ether, Sigma Prod. No. P-9641. Stir gently, with heat, until the solution is clear and colorless. Then add 9 ml of hot Reagent B and continue for 5 minutes. Allow the solution to return to ambient temperature prior to use. The solution clears upon cooling.)
Reagents: (continued)

D. 15% (w/v) Taurocholic Acid Solution (Tauro)
(Prepare 10 ml in deionized water using Taurocholic Acid, Sodium Salt, Sigma Prod. No. T-4009.)

E. 15% (w/v) Cholic Acid Solution (Chol)
(Prepare 10 ml in deionized water using Cholic Acid, Sodium Salt, Sigma Prod. No. C-1254.)

F. 1.76% (w/v) 4-Aminoantipyrine Solution (4-AAP)
(Prepare 1 ml in deionized water using 4-Aminoantipyrine, Free Base, Sigma Prod. No. A-4382.)

G. 5% (w/v) Phenol Solution (Phenol)
(Prepare 10 ml in deionized water using Phenol, Sigma Prod. No. P-4161.)

H. Cholesterol Oxidase Enzyme Solution (Chol Oxid)
(Immediately before use, prepare a solution containing 20 - 30 units/ml of Cholesterol Oxidase, Sigma Prod. No. C-1512, in cold Reagent A.)

I. Peroxidase Enzyme Solution (POD)
(Immediately before use, prepare a solution containing 40 - 60 units/ml of Peroxidase Type II from Horseradish, Sigma Prod. No. P-8250 in cold deionized water.)

J. Cholesterol Esterase Enzyme Solution
(Immediately before use, prepare a solution containing 0.25 - 0.85 unit/ml of Cholesterol Esterase in cold Reagent A.)

Procedure:

Pipe (in milliliters) the following reagents into suitable cuvettes:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A</td>
<td>2.10</td>
<td>2.10</td>
</tr>
<tr>
<td>(Buffer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent D</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>(Tauro)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent E</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>(Chol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent I</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>(POD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent C</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>(Chol-Oleate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagent G</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>(Phenol)</td>
<td></td>
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</tbody>
</table>
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PROCEDURES: (continued)

Mix by inversion and equilibrate to 37°C. Then add:

| Reagent F (4-AAP) | 0.05 | 0.05 |
| Reagent H (Chol Oxid) | 0.05 | 0.05 |

Mix by inversion and obtain the baseline at 500 nm. After approximately 5 minutes add:

<table>
<thead>
<tr>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent J (Cholesterol Esterase)</td>
<td>0.05</td>
</tr>
<tr>
<td>Reagent A (Buffer)</td>
<td>-----</td>
</tr>
</tbody>
</table>

Immediately mix by inversion and record the increase in $A_{500\text{nm}}$ for approximately 10 minutes. Obtain the $\Delta A_{500\text{nm}}$/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

\[
\text{Units/ml enzyme} = \frac{(\Delta A_{500\text{nm}}/\text{min Test} - \Delta A_{500\text{nm}}/\text{min Blank})(3)(df)}{(0.5)(13.78)(0.05)} >
\]

3 = Total volume (in milliliters) of assay  
df = Dilution factor  
0.5 = Conversion factor based on one mole of H$_2$O$_2$ produces half a mole of Quinoneimine Dye  
0.05 = Volume (in milliliters) of enzyme used  
13.78 = Millimolar extinction coefficient of Quinoneimine Dye at 500 nm under the assay conditions

\[
\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}
\]

\[
\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}
\]

UNIT DEFINITION:

One unit will hydrolyze 1.0 $\mu$ mole of cholesteryl oleate to cholesterol and oleic acid per minute at pH 7.0 at 37°C in the presence of taurocholate.
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FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mix, the final concentrations are 287 mM potassium phosphate, 0.25% (w/v) taurocholic acid, 0.25% (w/v) cholic acid, 4 - 6 units peroxidase, 1.4 mM cholesteryl oleate, 1.7% (v/v) polyoxyethylene 9 lauryl ether, 0.14% (w/v) sodium chloride, 0.083% (w/v) phenol, 0.03% (w/v) 4-aminoantipyrine, 1 - 1.5 units cholesterol oxidase and 0.013 - 0.043 unit cholesterol esterase.

REFERENCE:


NOTES:

1. Add the reagents in the order written.
2. The fastest rate is usually between 4-8 minutes after addition of the Cholesterol Esterase.
3. Cholesterol Oxidase Unit Definition: One unit will convert 1.0 μmole of cholesterol to 4-cholesten-3-one per minute at pH 7.5 at 25°C.
4. Peroxidase Unit Definition: One unit will form 1.0 mg purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20°C.
5. This assay is based on the cited reference.
6. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

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