Effects of Tomatine on the Colorimetric Determination of Cholesterol by the Zak Procedure

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The iron-acetic acid reagent used for cholesterol determinations produces a color with tomatine, which is reported to be a more specific precipitant for cholesterol. To circumvent the interference of tomatine found in a cholesterol-tomatinide complex, a standard solution containing stoichiometric amounts of tomatine and cholesterol was used and proved to be reliable for the determination of free cholesterol in serum.

A new precipitating agent, tomatine, a glycosidal alkaloid, has a greater specificity than digitonin for cholesterol (1). The use of tomatine to precipitate cholesterol-like material and the subsequent direct colorimetric determination by the Zak method (2) resulted in a higher cholesterol content than was actually present. Others (3) reported that tomatine produced a color with the Zak cholesterol reagent; but when the excess tomatine was washed from the cholesterol-tomatinide no interference occurred. Our investigation has resulted in a quantitative Zak cholesterol method which can be carried out in the presence of tomatine.

Materials, Methods, and Results

Purification of Cholesterol and Tomatine

Cholesterol was purified by the dibromide method (4). Crude tomatine was dissolved in hot alcohol and decolorized by the addition of charcoal and by filtration; it was then cooled in carbon dioxide-acetone bath and the precipitated crystalline tomatine filtered. This recrystallization procedure was repeated 3 additional times.

Cholesterol Determination

Determinations of standard cholesterol curves, with and without tomatine, were made by using the Zak colorimetric method (2). Various

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amounts of an alcohol solution containing cholesterol (1 mg./ml.) were pipetted into centrifuge tubes. Nine replicates of each of these concentrations were prepared: 0.00, 0.05, 0.10, 0.20, 0.30, 0.40, 0.50, 0.80, and 1.00 mg. Then to 5 of these solutions was added 0.5 mg. of a tomatine-alcohol solution; the other 5 were used as standards. All of the specimens were evaporated to dryness by placing them in a water bath at 65–85° and using an air stream to increase evaporation. They were further dried in an oven at 90° for 30 min. (This same drying procedure was used throughout.) Ten ml. of Zak’s reagent (764 mg. ferric chloride per liter of glacial acetic acid) was added to each solution. The material in the tubes containing tomatine was dissolved by heating in a water bath at 65–85°. After mixing, a 3-ml. aliquot from each of these solutions was pipetted into separate matched cuvets and to each was added 2 ml. of concentrated sulfuric acid and the mixture stirred immediately. After a 30-min. incubation time at room temperature, the absorbance was determined at 560 mμ on a Bausch and Lomb Spectronic 20 spectrophotometer. A specimen containing only the color reagents was used to set the spectrophotometer at zero absorbance. The absorbance of 5 replicates was averaged for each point on the standard curves (Fig. 1).

![Fig. 1. Effects of 0.5 mg. tomatine on standard cholesterol curve. Solid squares indicate cholesterol; open circles, cholesterol plus 0.5 mg. tomatine.](image)

**Standard Tomatine Curve**

The material for obtaining the standard tomatine curve was prepared by placing various amounts of an alcohol-tomatine solution (1 mg. tomatine/ml.) into a centrifuge tube. The solution was dried,
10 ml. Zak reagent was added, and color developed on a 3-ml. aliquot in the usual way. The absorbance was determined at 560 m\(\mu\). (Fig. 2).

**Tomatine-Cholesterol Color**

The additive properties of tomatine and of cholesterol were determined by preparing 6 experimental groups of 5 specimens each. The first and second groups contained 0.200 mg. and 0.400 mg. cholesterol, respectively; the third and fourth groups, 0.514 mg. tomatine (enough to precipitate 0.200 mg. cholesterol) and 1.028 mg. (enough to precipitate 0.400 mg. cholesterol), respectively; the fifth and the sixth groups contained 0.200 mg. cholesterol plus 0.514 mg. tomatine and 0.400 mg. cholesterol plus 1.028 mg. tomatine, respectively. After drying, each specimen was dissolved in 10 ml. of Zak reagent and color developed on a 3-ml. aliquot in the prescribed manner (Table 1).

**Standard Cholesterol Curves**

A Zak reagent solution composed of 0.10 mg. of cholesterol and 0.2675 mg. of tomatine per milliliter was made up and quantities of 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, and 2.00 ml. were each diluted to 3 ml. with Zak reagent; 2 ml. of concentrated sulfuric acid was then added to each. The solutions were thoroughly mixed and the absorbance determined in the predescribed manner (Fig. 3).
Table 1. Additive Properties of Color Developed by the Zak Reagent on Tomatine and Cholesterol

<table>
<thead>
<tr>
<th>Group*</th>
<th>Cholesterol added (mg.)</th>
<th>Tomatine added (mg.)</th>
<th>Absorbance (± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.200</td>
<td>none</td>
<td>0.293 ± .012</td>
</tr>
<tr>
<td>2</td>
<td>0.400</td>
<td>none</td>
<td>0.606 ± .019</td>
</tr>
<tr>
<td>3</td>
<td>none</td>
<td>0.514</td>
<td>0.077 ± .006</td>
</tr>
<tr>
<td>4</td>
<td>none</td>
<td>1.028</td>
<td>0.174 ± .005</td>
</tr>
<tr>
<td>5</td>
<td>0.200</td>
<td>0.514</td>
<td>0.384 ± .013†</td>
</tr>
<tr>
<td>6</td>
<td>0.400</td>
<td>1.028</td>
<td>0.773 ± .028‡</td>
</tr>
</tbody>
</table>

Addition to Absorbance

<table>
<thead>
<tr>
<th></th>
<th>1 + 3</th>
<th>2 + 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.370 ± .016</td>
<td>0.780 ± .018</td>
</tr>
</tbody>
</table>

* Five determinations each.
† Not significantly different when compared to the addition of Groups 1 and 3.
‡ Not significantly different when compared to the addition of Groups 2 and 4.

Fig. 3. Standard cholesterol-tomatine curves.

Standard Cholesterol-Tomatine Curve

An extracting reagent solution* containing 0.10 mg. of cholesterol per milliliter was made up and quantities of 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, and 2.00 ml. were pipeted into separate centrifuge tubes, then 1 ml. of tomatine solution was added to each. The solution was mixed and the pH adjusted to approximately 7.0–7.5 with methanolic 2.5N KOH,

using phenolphthalein as an indicator. The sides of the tubes were rinsed thoroughly by using 1 ml. of extracting reagent in a syringe and forcibly ejecting this solution through a 26-gauge needle, care being taken to rinse all the cholesterol-tomatinide from the side of the tubes. The mixture was allowed to stand overnight; then 1 ml. of extracting reagent was added to each tube and the mixing and rinsing repeated. The precipitate collected by centrifugation was washed once with 2 ml. of extracting reagent and the mixing and rinsing repeated. The supernatant liquid was discarded. The precipitated cholesterol-tomatinide was collected by centrifugation. After careful decanting of the supernatant liquids, the tubes were inverted for 15 min., then 3 ml. of Zak's reagent was added and color developed in the usual way (Fig. 3).

**Serum Cholesterol Recoveries**

To 60 ml. of extracting reagent* in a 100-ml. volumetric flask was added 4 ml. of a pooled serum and 4 mg. of cholesterol. To another such flask with 60 ml. of extracting reagent was added 4 ml. of serum. Contents of each flask were thoroughly mixed and diluted to 100 ml. with extracting reagent, mixed again, then filtered through Whatman No. 1 filter paper. During this procedure the filtration beakers were covered by larger ones to prevent loss of solution by evaporation. In one series of twelve 15-ml. centrifuge tubes and another series of 4, was placed 2 ml. of cholesterol-serum solution; in another series of 10 and 4, 5 ml. of serum extract was placed. Each volume was concentrated in a 65–85° water bath to approximately 0.5 ml., then 1 ml. of tomatine solution was added, the pH adjusted just to the acid side of phenolphthalein, the solution mixed, and the sides of the tubes rinsed. The specimens were allowed to stand overnight; then 3 ml. of extracting solution was added and again the mixing and rinsing procedure was repeated. The tubes were then centrifuged and the supernatant fluids were discarded. To each precipitate was added 1 ml. of extracting reagent and the mixing and rinsing repeated. The precipitate was collected by centrifugation, dissolved in 3 ml. of Zak reagent, and the color developed in the usual way (Table 2).

**Discussion**

A comparison of a standard cholesterol curve with one in which a constant amount of tomatine had been added revealed that tomatine produced a color with the Zak reagents. The color produced by the Zak reagent on tomatine is linear—as indicated by the standard tomatine curve, which is read at 560 m\(\mu\). (Fig. 1 and 2).

*As described under Standard Cholesterol-Tomatinide Curve.
Table 2. Free Cholesterol Determination and Recovery in Serum

<table>
<thead>
<tr>
<th>No. of determinations</th>
<th>Cholesterol added to serum (mg./ml.)</th>
<th>Av. cholesterol recovered (S.D. (mg./ml.)</th>
<th>Cholesterol recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1.0</td>
<td>1.72 ± 0.12</td>
<td>97</td>
</tr>
<tr>
<td>10</td>
<td>none</td>
<td>0.75 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>1.56 ± 0.06</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>none</td>
<td>0.56 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

The standard cholesterol curves, with and without tomatine, were parallel, and when a blank containing an equivalent amount of tomatine was used to determine the absorbance of the standard cholesterol-tomatine curve, the results were statistically identical to those obtained when no tomatine was added.

Since the colors developed by tomatine and cholesterol (when subjected to the Zak reagent) were additive (Table 1), and cholesterol reacts stoichiometrically with tomatine to form the tomatinide, the amount of cholesterol present in a cholesterol-tomatinide can be determined by adding equivalent molar amounts of tomatine to a standard cholesterol solution. Thus by using various amounts of this solution and developing the color according to the Zak procedure, a standard cholesterol-tomatine curve was obtained (Fig. 3). This was supported by the study in which various amounts of cholesterol were precipitated as the tomatinide and after proper washing and color development, the cholesterol-tomatinide curve was statistically identical to the standard cholesterol-tomatine curve. Other investigators (5) have shown good recoveries of radioactive cholesterol from liver homogenate by using tomatine as the isolating agent. They confirmed the findings of Kabara et al. (1) in showing that tomatine was more selective than digitonin for cholesterol in liver homogenates.

More than 0.05 mg. of cholesterol must be present in the specimen if good recoveries are to be obtained by this method. For this reason 4 or 5 ml. of the usual 1:24 serum extract was used to determine free cholesterol.

The standard cholesterol-tomatine-Zak reagent solution was stable for only 1 week, while the cholesterol-tomatine solution in glacial acetic acid was stable for a longer period.

Summary and Conclusions

Tomatine interferes with cholesterol determination by the Zak method. This difficulty was circumvented by using a standard solution con-
taining molar-equivalent amounts of both cholesterol and tomatine, and developing color using a Zak method for determining cholesterol. This method can be used to determine free cholesterol in serum.

References