A Simple Enzymatic Quantitative Analysis of Triglycerides in Tissues

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Summary We determined a method to measure the triglyceride levels in tissues by using a modified enzymatic kit. This enzymatic kit was originally designed to be used to measure the triglyceride levels in plasma. Our method of triglyceride level determination includes dissolving the tissue lipid extracts in an alcohol. Before using the enzymatic kit directly, the lipids were dissolved in tert-butyl alcohol, then a Triton X-100/methyl alcohol mixture was added (1/1 by volume). The presence of organic surfactants such as tert-butyl alcohol and methyl alcohol, and of a surfactant such as Triton X-100, did not interfere with the enzymatic activity. This method enabled us to determine triglyceride levels between 10 and 90 nmol, by using a spectrophotometer to measure the absorbances.

Key Words tissue triglycerides, tert-butyl alcohol, methyl alcohol, Triton X-100, enzymatic analysis

Triglyceride levels have been determined by various methods. Commonly used methods are: the chromotropic acid method (1-4), the acetylacetone method (5, 6), the Randrup method (7) and the Mendelsohn method (8). In these procedures, triglyceride levels are measured by assaying glycerol, after removing phospholipids by using absorbefacients. Although reliable, these techniques require either time-consuming steps or careful treatments, because the absorbance capabilities of absorbefacients change with temperature and elapsed time after the activation. Alternatively, we can eliminate the need to use absorbefacients by using lipoprotein lipase. However, the utility of this method is restricted to measuring only serum triglyceride levels, and it is comparatively difficult to determine these levels from the lipid extracts. Organic solvents used as dissolvents interfere with enzymatic reactions. By making use of an enzymatic reagent kit for measuring serum triglycerides, we tried to study the triglyceride levels in lipid extracts. These were the requirements for the organic solvents to be used in the method: they must dissolve lipids rapidly, they must be water-soluble, they must not interfere with
enzymatic reactions and they must contain no peroxides. Since a series of enzymatic reactions transform triglycerides into peroxides in the enzymatic triglyceride reagent kit, we decided that the organic solvents must contain no peroxides, and we skipped over the process of deoxidization. The cho. B. H. S. method (9) determines tissue cholesterol levels with the use of an enzymatic reagent kit. The organic solvent used in that method is a dioxane/isopropyl alcohol mixture (1/1 by volume). We found that a muddiness was induced by using that solvent to measure triglyceride levels in tissues, and that it was impossible to measure absorbances. We abandoned a plan to use dioxane because it easily produced peroxides. After testing numerous organic solvents in the determination of triglyceride levels in lipid extracts, we decided to use 30 µl of tert-butyl alcohol, 20 µl of a Triton X-100/methyl alcohol (1/1 by volume) mixture and 1 ml of the enzymatic kit reagent to determine triglyceride levels of between 10 and 90 nmol. The tubes were then incubated at 37°C for 18 min. Triton X-100 was used to remove the muddiness. Methyl alcohol was added in order for it to be possible to pipette Triton X-100 accurately. When using isopropyl alcohol as an organic solvent, comparatively, it took a long time to dissolve the lipids thoroughly. Isobutyl alcohol and n-butyl alcohol interfered with taking sufficient stable absorbance measurements.

Reagents
1. Chloroform/methyl alcohol mixture: Two parts chloroform in one part methyl alcohol.
2. Triglyceride solution: Triolein (99+%; Sigma Chemical Co.)
3. tert-butyl alcohol.
4. Triton X-100/methyl alcohol mixture: One part Triton X-100 (Wako Pure Chemical Industries; Osaka, Japan) in one part methyl alcohol.
5. Enzymatic triglyceride reagent kit: Triglyceride G-test wako (Wako Pure Chemical Industries; Osaka, Japan), (50 mM of Tris-HCl buffer at pH 7.5, 5.4 mM of p-chlorophenol, 36 U/ml of lipoprotein lipase from Chromobacterium, 1.8 mM of adenosine-5'-triphosphate disodium salt trihydrate, 2.2 U/ml of glycerol kinase from Streptomyces, 4.4 U/ml of glycerol-3-phosphate oxidase from Aerococcus, 2.1 U/ml of peroxidase from horseradish, 0.69 mM of 4-aminooantipyrine).

To prepare lipid extracts from liver tissues, homogenize 0.5 g (wet weight) each of hamster liver tissues with 15 ml of the chloroform/methyl alcohol mixture (2/1 by volume), and extract according to Folch et al. (10). Evaporate the extraction on a centrifugal concentrator instead of on a rotary evaporator, then redissolve the residue in a small amount of benzene. Transfer this new mixture to a 25 ml volumetric flask and dilute to the mark with more benzene.

Transfer aliquots of the known working standards and of the liver lipid extracts in benzene into test tubes. Evaporate the solvents with a centrifugal concentrator, and redissolve the standards and liver samples in 30 µl of tert-butyl alcohol and 20 µl of the Triton X-100/methyl alcohol mixture. These redissolved materials should be mixed. To each test tube, add 1.0 ml of enzymatic reagent and
Fig. 1. Results obtained with the older method and the present method on ten different samples from liver tissue. Each sample is stemmed from liver lipid of rats.

Fig. 2. Triglyceride concentration and absorbance at 5050 nm. Curve was obtained with triolein standards alone and without tissue influences or extraction losses. The reaction mixture is composed of 30 µl of tert-butyl alcohol, 20 µl of equi-volume Triton X-100/methyl alcohol mixture and 0.995 ml of the "enzymatic kit".

Table 1. Recovery studies of triglycerides added to lipid from liver.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Present (µg)</th>
<th>Added (µg)</th>
<th>Recovered (µg)</th>
<th>% recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.0^a</td>
<td>30.0</td>
<td>49.5^a</td>
<td>105.0</td>
</tr>
<tr>
<td>2</td>
<td>22.6</td>
<td>30.0</td>
<td>53.6</td>
<td>103.3</td>
</tr>
<tr>
<td>3</td>
<td>28.2</td>
<td>30.0</td>
<td>57.5</td>
<td>97.7</td>
</tr>
<tr>
<td>4</td>
<td>30.6</td>
<td>30.0</td>
<td>61.0</td>
<td>101.3</td>
</tr>
<tr>
<td>5</td>
<td>35.7</td>
<td>30.0</td>
<td>65.9</td>
<td>100.7</td>
</tr>
<tr>
<td>6</td>
<td>39.0</td>
<td>30.0</td>
<td>69.2</td>
<td>100.7</td>
</tr>
<tr>
<td>7</td>
<td>44.1</td>
<td>30.0</td>
<td>73.4</td>
<td>97.7</td>
</tr>
</tbody>
</table>

*Each value is the average of three independent experiments.

mix carefully. Incubate the standards, samples, and appropriate blanks for 18 min at 37°C, and then measure the absorbances at 505 nm vs. a reagent blank.

The line in Fig. 1 represents the function of the new method equals that of the older method (4). Each sample was designed by diluting the liver lipid extracts from rats with chloroform, and was determined by the new method or by chromotropic acid method. Chromotropic acid method is one of the confirmed methods to measure the triglyceride levels. The relationship between the concentration of
Fig. 3. Time course of color development of triglyceride using three kinds of butyl alcohol as a solubilizing reagent. Triolein was solubilized in 30 μl of a butyl alcohol and 20 μl of equi-volume Triton X-100/methyl alcohol mixture and incubated at 37°C for 18 min. The absorbance was measured at 505 nm.

Fig. 4. Glycerol concentration and absorbance at 505 nm. The reaction mixture is composed of 1.0 ml of the "enzymatic kit".

triolein and the absorbance at 505 nm is shown in Fig. 2. A good linearity is established from 0 to 84.7 nmol. The extent of triglyceride recovery was studied by using various concentrations of triglycerides. The results of recovery experiments are given in Table 1. Mixing known amounts of triglycerides from liver lipids with 30 μg of triolein and carrying out this series of steps, gave recoveries which ranged from 97.7% to 105.0%. After the next stage of experimentation, tert-butyl alcohol was chosen as a solvent to dissolve the lipid extracts. Figure 3 shows the time course of color development for the three kinds of butyl alcohol after incubating at 37°C for 18 min. The color developed within 2 min and was kept up to 90 min in tert-butyl alcohol; it was kept from 14 to 90 min in isobutyl alcohol and from 22 to
90 min in n-butyl alcohol. Figure 4 shows that a good linearity is established for the glycerol standards. Therefore, it is possible to utilize glycerol instead of triglycerides to produce a standard curve.

REFERENCES


