Determination of cholesterol as the tomatinide using the iron reagent*

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»Tomatine has been reported to be a more specific precipitating agent for cholesterol than is digitonin (1, 2). The subsequent solubilization of the tomatinide and the colorimetric determination of the cholesterol by the Liebermann-Burchardt reaction (3) have demonstrated that tomatine does not interfere with the color reaction (2). The purpose of this investigation was to determine the feasibility of using the iron reagent with tomatine for the analysis of cholesterol. The colorimetric method used was that suggested by Zlatkis, Zak, and Boyle (4) as modified by Rosenthal et al. (5). Digitonin was prepared as a 0.5% solution in ethanol–

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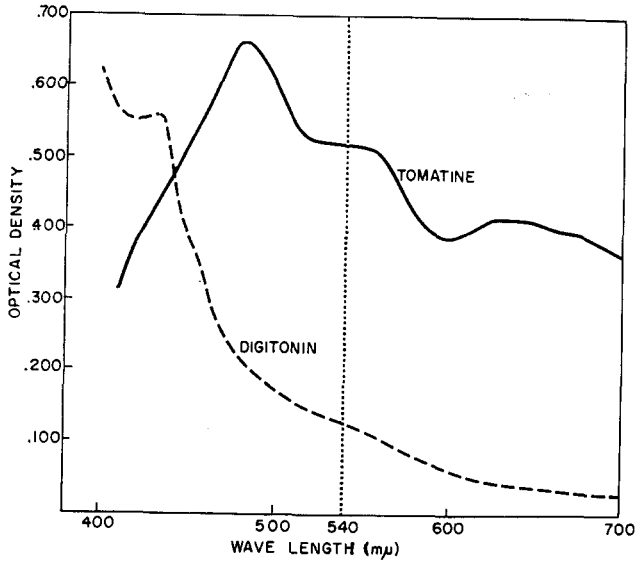

FIG. 1. Absorption spectra of digitonin and tomatine in glacial acetic acid with reagents of Zlatkis, Zak, and Boyle.

water 1:1 and tomatine[1] as a 0.5% solution in ethanol–water–glacial acetic acid 55:44:1. The standard cholesterol solution was 0.1% in ethanol–acetone 1:1.

To determine the interference that tomatine might contribute to the reaction, reagents were added to 1 mg of tomatine and a like amount of digitonin, and the absorption spectra were plotted and compared (Fig. 1). It is apparent that the free tomatine reacting with the $FeCl_3$-H_2SO_4 color reagent produces a great deal more color than does digitonin. This is especially true in the region of 540–560 mμ where the color reaction is measured. Excess tomatine must be completely removed and the tomatinide thoroughly washed to prevent falsely high values.

The digitonide and tomatinide of cholesterol were prepared and their absorption spectra were compared (Fig. 2). The maximum absorption with both glycosides prepared with 100 μg of cholesterol is 560 mμ, and their spectra over the visible range are similar. In the preparation of the digitonide and tomatinide for the color reaction, the precipitates were subjected to three (0.5 ml) washings with ethanol–acetone 1:1, ethanol–ether 1:3, and ether. That the tomatinide curve exhibits no significant peak at 480 mμ (the maximum optical density for tomatine) attests to the effectiveness of the washing procedure.

In handling and washing the precipitates, a marked contrast is noted between digitonin and tomatine. The tomatinide precipitates are more easily suspended

[1] Chemical Concentrates, Fort Washington, Pennsylvania.

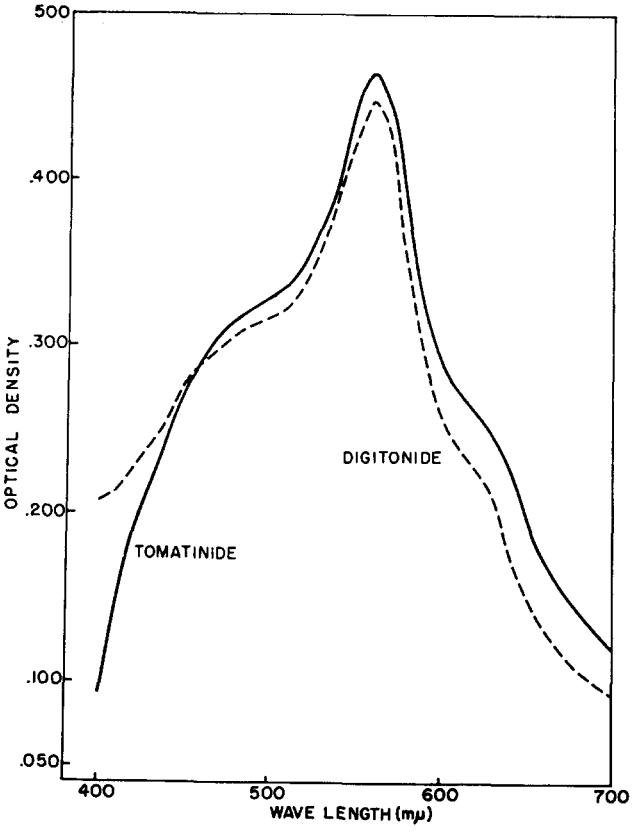

FIG. 2. Absorption spectra of the digitonide and tomatinide of cholesterol obtained with the reagents of Zlatkis, Zak, and Boyle.

and are, therefore, easier to wash. One of the disadvantages of tomatine, however, lies in the difficulty encountered in effecting complete sedimentation of the precipitates upon centrifugation. Care must be exercised in the removal of the supernatant fluid from the tomatinide or unsedimented material may be lost. By contrast, the digitonide sediments readily, removal of supernatant fluid is simple, but resuspension during the washing procedure is more difficult.

Tomatine forms a visible precipitate with cholesterol more slowly than does digitonin. The time required to develop visible turbidity increases rapidly from only a few seconds at higher concentrations (ca. 1 mg) to approximately $3\frac{1}{2}$ hr when the cholesterol concentration is less than 100 μg/ml. By contrast, this latter concentration of cholesterol forms a visible precipitate almost instantaneously with digitonin. That the tomatinides in these experiments form more slowly than found by Kabara (2) may possibly be attributed to the fact that in his experiments the cholesterol was precipitated from an acetone–alcohol–ether 4:4:1 extract rather than an alcohol–acetone 1:1 system as reported here. In all of these experiments precipitation was permitted to occur overnight at room

TABLE 1. COMPARISON OF DIGITONIN AND TOMATINE AS PRECIPITATING AGENTS FOR CHOLESTEROL FROM ALCOHOL–ACETONE 1:1 EXTRACTS OF RAT SERUM. VALUES EXPRESSED IN MG/100 ML

Sample Number	Direct Method	Digitonin Precipitation	Tomatine Precipitation
1	125	100	130
2	111	98	97
3	118	131	130
4	125	100	106
5	125	102	103
6	118	97	105
Avg.	120	105	112
% Deviation from direct method		−12.5	−6.6

temperature to insure complete precipitation and colorimetric determinations were carried out the following day. No attempt was made to determine the minimum time required to effect complete precipitation.

Digitonin and tomatine were next compared in precipitating cholesterol from alcohol–acetone 1:1 extracts of rat serum and liver. The serums were extracted in duplicate. Twenty-five milliliters of alcohol–acetone was placed in a centrifuge tube. One milliliter of the serum was taken up in a syringe fitted with a 24-gauge needle. The serum was then forcefully ejected into the solvent, with the tip of the needle under the surface of the solvent. The mixture was allowed to stand 1 hr, then was shaken for 1 min, and centrifuged; the extract was removed, reduced in volume to 5 ml and 1 ml of 0.5% tomatine or digitonin was added to each sample. The samples were allowed to precipitate overnight and analyzed the following morning. Two-gram samples of rat liver were finely ground with a motor-driven glass homogenizer in the presence of 50 ml of alcohol–acetone. The liver suspensions were centrifuged; the extracts were divided into two equal parts, each of which was reduced to 5 or 6 ml; and 3 ml of either digitonin or tomatine was added to each portion. These samples likewise were permitted to precipitate overnight. The results of these experiments are shown in Tables 1 and 2. In Table 1, the results with rat serum are compared with those obtained by the direct Zlatkis method (4). Both the digitonin- and tomatine-precipitated samples gave results slightly lower than those obtained by the direct method. These lower results with the glycosides may be the result of incomplete precipitation of the cholesterol or of losses incurred during the washing procedure. However, it might also be pointed out that slightly higher values might be expected from the direct method due to the presence of chromogenic material in the serum. In Table 2, using rat liver extracts, the values for tomatine-precipitated cholesterol compare favorably with those obtained with digitonin.

TABLE 2. COMPARISON OF DIGITONIN AND TOMATINE AS PRECIPITATING AGENTS FOR CHOLESTEROL FROM ALCOHOL–ACETONE 1:1 EXTRACTS OF RAT LIVER*

Sample Number	Digitonin Precipitation	Tomatine Precipitation
1	2.48	2.69
2	2.63	2.57
3	2.20	2.38
4	2.24	2.30
5	2.60	2.46
6	2.42	2.50
Avg.	2.43	2.48

* Values expressed in mg/g liver free cholesterol.

In summary, the iron reagent may be satisfactorily utilized for the determination of cholesterol in alcohol-acetone extracts of serum and tissue when tomatine is substituted for digitonin as the precipitating agent, provided the following precautions are taken: (1) more time must be allowed for precipitation of samples containing small amounts of cholesterol, and (2) more care must be exercised in handling and washing the precipitates.

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