Estimation of Total Cholesterol in Serum by a Micro Method

K. J. Carpenter, A. Gotsis, and D. M. Hegsted

Albers and Lowry (1) have published a method for the estimation of cholesterol on a micro scale in animal tissue by fluorometry involving the reaction of extracted cholesterol dissolved in trichloroethane with acetic anhydride and sulfuric acid. The present paper describes a simple and tested use of this reaction for the estimation of total cholesterol in 0.02 ml. of serum after extraction by a standard procedure (2) and without use of special apparatus.

EXPERIMENTAL

APPARATUS
Farrand fluorometer, set to aperture size 6, with Corning filters 4010 and 5120 in the primary path (a thin holder to take the extra width being used) and filter 2424 in the secondary path (1).

REAGENTS
1. Alcoholic KOH, freshly prepared from 6 ml. of 33% KOH and 94 ml. of redistilled absolute alcohol.
2. Petroleum ether, fraction boiling at 68°, redistilled.
3. "Trichloroethane-acetic anhydride," 5 parts of 1, 1, 2-trichloroethane (Eastman Kodak) used without redistillation and 1 part acetic anhydride. This reagent should be used within one hour after preparation.

From the Department of Nutrition, Harvard School of Public Health, Boston, Mass.
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4. Sulfuric acid, reagent grade.
5. Cholesterol standard, 400 µg. cholesterol (recrystallized four times) per ml. of absolute alcohol.
6. Rhodamine B standard, 0.032 µg. Rhodamine B (Eastman Kodak) per ml. of H₂O.

PROCEDURE

One milliliter of alcoholic KOH is measured into a 15 ml. glass-stoppered vial, and 0.02 ml. of serum is added with a hemoglobin pipet (with rinsing). The vial is stoppered and placed in a water bath at 37-40° for 55 minutes. After the vial has been cooled to room temperature, 4 ml. of petroleum ether and 1 ml. of water are added. The vial is shaken for 2 minutes, and the layers are allowed to settle. Two ml. of the supernatant (ether) layer are then transferred to a 125 x 15 mm. tube.

The solvent is evaporated from the tube by placing it in a water bath at approximately 60° and injecting an air stream from a nozzle placed in the top of the tube. The tube is allowed to cool to room temperature; 2 ml. of trichloroethane-acetic anhydride are added and the tube is shaken gently. After 15 minutes, 0.08 ml. of sulfuric acid is added with a 0.2 ml. pipet graduated in hundredths and the tube is shaken immediately.

After 15 to 60 minutes, approximately one-quarter of the contents is used to rinse out a 9 x 75 mm. fluorometer tube; the remainder is then tipped in, and the fluorescence is measured with the sensitivity control of the instrument set to give 50 per cent of “full deflection” with the Rhodamine B solution which is kept in a second tube. (This adjustment is checked before each “unknown” reading).

Two levels of cholesterol and a blank are run simultaneously through the entire analytical procedure including the extraction. Usually 0.10 and 0.20 ml. of standard cholesterol solution, each in triplicate, have been used. These amounts are measured into the alcoholic KOH with a 0.2 ml. pipet graduated in hundredths.¹ The blank consists of 1 ml. of KOH.

CALCULATION RESULTS

The fluorometer readings from the standards and blank are plotted graphically against the initial volume of cholesterol standard used.

¹Although graduated pipets have been used throughout, micro constriction pipets (3) are as satisfactory and convenient.
The volume corresponding to the reading obtained with a serum sample is read from the graph. This figure multiplied by 2000 gives the "total" cholesterol content of the serum in mg. per 100 ml. When sera contain so much cholesterol that readings exceed the top of the standard curve, 1 ml. in place of 2 ml. of the petroleum ether extract can be used. If this is still inadequate, then 2 ml. of extract can be diluted to 10 ml., an aliquot taken, and appropriate changes in the method of calculation made.

RESULTS AND DISCUSSION

RESPONSE TO CHOLESTEROL STANDARD

As expected (1), this was linear in the range indicated, provided that the incident light in the fluorometer was adjusted to its lowest level (i.e., aperture size 6). With the sensitivity adjusted so that the highest cholesterol standard (giving a final concentration of 15 µg./ml. in the fluorometer tube) gave a reading of approximately 90, the blank reading was usually in the range 6-9 after completion of the whole procedure. This might have been further reduced by redistillation of reagents (1).

SPECIFICITY

The specificity of the fluorescence has already been studied (1). Of the other steroids giving fluorescence, none is expected to occur in serum in quantities which would yield misleading results. This is confirmed by the agreement of the results for sera with those obtained by the Abel method for which it has been demonstrated that not less than 99 per cent of the values obtained with sera are specifically due to cholesterol (2).

CRITICAL VARIABLES

The results are not affected by a wide range of laboratory temperatures or by carrying out the determinations in sunlight. Obviously, any errors in pipetting (except for alcoholic KOH) will be reflected in changes in final serum concentrations. Similar dilution errors will follow from failure to prevent evaporation of petroleum ether before the aliquots are taken, or from incomplete evaporation in the water bath. The procedure can be interrupted just prior to evaporation of the petroleum ether (2).

The final addition of 0.08 ml. sulfuric acid is accomplished by using a 0.2 ml. graduated pipet. This step is particularly critical. In test
runs the addition of 0.07, 0.075, 0.085, and 0.09 ml. have given 94, 99, 103, and 105 per cent, respectively, of the fluorescence obtained with 0.08 ml. of acid.

RESULTS WITH RAT SERA

Thirty-two serum samples with a range of 60 to 300 mg. per 100 ml. cholesterol were taken from female rats of approximately 250 Gm. weight that had been on experimental diets with and without cholesterol and methionine supplements for 6 weeks. They were analyzed for total cholesterol by the micro method described with complete replication (i.e., with the replicate analyses carried through on different days and, therefore, with different standard curves), and also by the Abell method (2) in which duplicate aliquots of petroleum ether from a single extraction were used.

The individual results are presented in the form of a scatter diagram (Fig. 1). The over-all mean for the Abell determination on all the sera was 126 mg. per ml.; the micro method gave an over-all mean 3 mg. per 100 ml. lower, and the standard deviation of this difference for the individual sera was ±8 mg. per 100 ml. The standard deviation due to error within the micro method for a single value determined in duplicate (as calculated from the differences between replicates) was ±5 mg. per 100 ml.

RESULTS WITH HUMAN SERA

A second similar trial was conducted with 43 samples of human serum (obtained during routine examination of apparently normal subjects) covering a range from 160 to 380 mg. per 100 ml. in their cholesterol content.

The results were similar to those obtained for rat sera. The over-all mean for the series was 253 mg. per 100 ml. by the Abell method, and 1 mg. per 100 ml. lower by the micro method. The standard deviation of the difference between the macro- and micro-method results for individual sera was 11 mg. per 100 ml. The standard deviation within the micro-method was ±4 mg. per 100 ml. for this series, when calculated in the same way as for the results with rat sera.

ROUTINE APPLICATION

The findings of the special trials and the general results obtained during routine use in the laboratory indicate that the method is suit-

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able for the estimation of total serum cholesterol from both human subjects and experimental animals.

There seems to be no advantage in using quantities less than 0.02 ml. With lipemic sera, particularly, even shaking to break up clots might be inadequate to give good sampling with a lower volume. At the 0.02 ml. volume, the fluorescence developed is sufficiently intense so that regular apparatus and cuvets can be used. Modification of the colorimetric methods of cholesterol estimation for smaller samples has involved the use of micro equipment (4).

It has been confirmed that the fluorescence remains constant for the period from 15 to 60 minutes after the addition of the final reagent (sulfuric acid) (1), and this has allowed about 60 tubes to be carried
through the reagent stage and then read in one batch, even though the
two stages take rather different times.

The method used routinely for collection of rat and mouse sera was
to bleed 0.2 to 0.3 ml. from the tail into a 2 ml. centrifuge tube. After
standing for an hour, this was centrifuged and 0.02 ml. aliquots were
taken directly from the tube with hemoglobin pipets. The bleeding
and taking of serum aliquots may have to be done the same day since
storage overnight of whole blood significantly changed values for
lipemic samples with cholesterol values of 1000 mg. per 100 ml. or
more. However, it has proved possible for two people to bleed 60-70
rats and complete the estimations in duplicate, with a full standard
curve, in a working day.

Since the adoption of this method, several thousand samples have
been carried through without any apparent difficulties. Different
batches of trichloroethane have given significantly different slopes to
the standard curves, but the values obtained for check samples of
serum have been unchanged.

SUMMARY

A method has been described for the estimation of total cholesterol
in 0.02 ml. of serum without the need for micro apparatus. It involves
the measurement of the fluorescence developed after the addition of
sulfuric acid to a solution of cholesterol in a mixture of 1,1,2-trichloro-
ethane and acetic anhydride (1).

The results obtained with both human and rat sera have been in
good agreement with those obtained with a standard colorimetric
method (2), and the standard deviation for an estimation carried
through in duplicate, owing to error within the method, has been
±4-5 mg. per 100 ml. under routine conditions.

REFERENCES

   (1952).