A COMPARATIVE STUDY OF METHODS FOR THE DETERMINATION OF CHOLESTEROL IN BLOOD

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A COMPARATIVE STUDY OF METHODS FOR THE DETERMINATION OF CHOLESTEROL IN BLOOD.

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CONTENTS

HISTORICAL REVIEW

The chemical literature contains many methods, with various modifications, for the quantitative determination of cholesterol in blood. One of the earliest procedures was suggested by Windaus[1] in 1910. This was based on the precipitation of cholesterol by digitonin in alcohol solution. The cholesterol digitonide was then determined gravimetrically. Since 15 to 20 cc. of blood was necessary, this method was not well suited to clinical work.

In 1910 also, Grigaut[2] reported a method which determined the cholesterol by the Liebermann-Burchard reaction. This reaction[3] was developed in 1885, but it was not applied to quantitative work previous to 1910. The majority of methods devised since that time have utilized this reaction. Grigaut's precedure required 5 to 10 cc. serum and ether extraction in a Soxhlet extractor for 3 to 5 hours. This was later adapted to clinical use by Autenrieth and Funk[4], who used only 2 cc. serum and reduced extraction time to two hours.

Bloor[5], in 1915, further simplified this method so that it could be applied in his system of analysis of blood lipoids. These colorimetric methods involved saponification of esters, including the cholesterol esters. In 1916, Bloor[6] reported that this saponification was unneeessary. Although in 1922, Bloor, Pelkan, and Allen[7]

published a revised method again using saponification, in 1928, Bloor's latest directions[8] omit this step.

Bloor has undoubtedly done the most extensive work in the determination of cholesterol, but many of his contemporaries have submitted other methods which have been widely accepted.

All of these methods have certain features in common. They involve: first, the extraction of cholesterol by some fat solvent, and second, the treatment of the extract obtained for the quantitative estimation of cholesterol.

The methods differ in the manner in which the cholesterol is extracted. Some methods employ dry extraction. In the procedures of Myers and Wardell[9], the blood is absorbed on several grams of plaster of Paris and dried before extraction. Leiboff[10] and Ling[11] use fat-free filter discs to absorb the blood. They claim this prevents caking often experienced with plaster of Paris. It also eliminates any loss due to changing the plaster of Paris from the crucible to the extraction thimble and further loss when the extract is transferred to a volumetric flask. Abrahamson[12] recommends absorbent cotton to replace the filter paper. This method sometimes shows incomplete extraction probably due to caking of the blood during drying. Other workers have used permutit and washed sea sand.

In this study cheesecloth was used to absorb the

blood*. Besides being much less expensive than most of the above absorbents, it is easy and convenient to handle. Microscopic examination shows that the blood is absorbed in the cotton fibers and is dispersed so the solvent can readily extract lipoid substances.

In nearly all cases, the absorbed blood is dried before extracting. Ling says this step is unnecessary in his method but it can be done. Chloroform is the solvent usually employed because the Liebermann-Burchard color can be developed in the chloroform extract.

In the wet extraction methods, preliminary drying is unnecessary. The blood is run into the solvent, and the mixture is heated and filtered to remove the precipitated proteins. Bloor[5,6,7,8] uses a mixture of absolute alcohol and ether (3:1). The alcohol precipitates the proteins and mixes with the water in the blood. The ether extracts the lipoid substances more completely. Schoenheimer and Sperry[13] in their digitonin precipitation method use a mixture of equal parts of acetone and absolute alcohol for extraction. Autenrieth and Funk[4], like Grigaut[2], use only ether which requires longer extraction time. When alcohol and ether or alcohol and acetone are used, it is necessary to heat on a water bath at the boiling point for only a few minutes

*The use of cheesecloth was originated in this laboratory by E.C. Tabor in an attempt to find an inexpensive , convenient substitute for the filter discs for use in Biochemistry classes.

before filtering to obtain complete extraction.

The various methods also differ widely in the treatment of the extract to determine the cholesterol. Most methods employing chloroform as the initial solvent utilize the Liebermann-Burchard color reaction. The green color produced can be compared with standards in the colorimeter. It can also be measured with photometer or photelometer using a suitable filter.

Ohyama[14] has devised a new color reaction. The chloroform solution is treated with salicylaldehyde, water, and sulfuric acid. After shaking for two hours, the red-violet color of the chloroform layer is determined colorimetrically.

A red color is produced in a glacial acetic acid solution of cholesterol when heated with acetyl chloride and anhydrous zinc chloride. Although the color is given by several other sterols, Bernoulli[15] claims it is applicable to the determination of cholesterol in blood. The advantage of this reaction is that the color is more stable than that given by the Liebermann-Burchard reagents. However, these two color reactions have not as yet been very widely accepted.

The alcohol-ether extract in Bloor's method may or may not be subject to saponification with sodium ethylate or sodium hydroxide and later acidified. In either case, it is evaporated to dryness, extracted with chloroform, and the Liebermann-Burchard reaction carried out. The initial

extraction with alcohol and ether eliminates the necessity for drying and extracts the lipoids more completely.

The alcohol-acetone extract of Schoenheimer and Sperry is saponified by potassium hydroxide and then treated with a solution of digitonin. The digitonide, after washing and drying, is dissolved in 1 cc. acetic acid, which is substituted for the 5 cc. chloroform ordinarily used in the Liebermann-Burchard reaction. The color in this solvent requires about twice as long to develop.

Szent-Gyorgyi[16] has modified the Windaus method so that the digitonide precipitate can be determined on a micro scale. He also reports a titrimetric method in which the digitonide is oxidized by potassium chromate. The excess chromate is determined by adding potassium iodide and titrating with a standard thiosulfate solution. Ruth Okey[17], working in Bloor's laboratory has simplified certain parts of this procedure. The latter methods are receiving increasing attention in the literature and many modifications are reported.

The very fact that there are so many and varied methods indicates that probably no one is entirely satisfactory. The literature contains contradictory claims as to which gives the highest or most consistent results.

EXPERIMENTAL PROCEDURE

Although gravimetric and titrimetric methods have received some attention, the colorimetric methods seem to be used most frequently because they are less time consuming and just as accurate.

For this study only colorimetric methods were used. Leiboff's[10] method, as given in the Journal of Biological Chemistry, 61, 177, was chosen as a representative of the dry extraction method. Three-inch squares of cheesecloth were substituted for the fat-free filter paper discs.

Bloor's[4] method was used as an example of wet extraction. It is one most frequently found in text books for the determination of cholesterol. The method was used without saponification as given in the Journal of Biological Chemistry, 77, 53. The extract was made up to 50 cc. after 2.5 cc. blood had been added and the mixture heated. Aliquots of 5 cc. of this extract, after filtration, were taken so that the quantity of blood in each was 0.25 cc. This is the same quantity employed in the Leiboff method and allowed the use of the same standard. Aliquots of this extract were saponified with concentrated sodium hydroxide according to the directions by Bloor, Pelkan, and Allen[2] in the Journal of Biological Chemistry, 52, 191.

The digitonin method was also chosen since it is being rather widely accepted. The method was followed as outlined by Schoenheimer and Sperry[13] in Journal of Biological Chemistry,

106, 745. A 2 cc. fraction of the original extract was taken in place of 1 cc. to give a deeper color and thus remain within the more sensitive range of the photolometer. Subsequent reagents except the acetic anhydride and sulfuric acid were also doubled. An alcohol solution of digitonin was used instead of the water solution. This is recommended by Sturges Knudson Schoenheimer and Sperry (18) in a later publication. The potassium chloride which is formed when the saponification mixture is neutralized does not dissolve when this alcohol solution of digitonin is added. A few drops of distilled water must be added till the solution is clear. This does not interfere with the precipitation of the cholesterol digitonide.

Rat's blood was used in all the work. Since samples vary slightly, only results obtained on the same samples can be compared. The extractions were made as soon as possible after the samples were obtained.

In the first work the colorimeter was used to compare the color obtained from the blood with that given by standard solutions. This did not prove very satisfactory because of the yellow tints developed in the unknown making comparison difficult. This coloration was especially marked in runs by the Leiboff and saponified Bloor methods and was worse in some tubes than others. In nearly all cases the unsaponified Bloor extracts gave colors which could easily be matched with that of the standard.

The color produced by the digitonin method did not have a yellow tinge but rather a gray appearance which made comparison by the colorimeter almost impossible.

In this case much better results were obtained with the photelometer using a red filter. The center cell of the photelometer was filled with a blank solution containing the solvent, acetic anhydride, and sulfuric acid in the same proportions as in the samples. The instrument was set at 100 when this cell was in place. Then this blank was replaced by the cell containing the sample and the reading was recorded. A reference curve was prepared by making up several standards of varying cholesterol content. The photolometer readings were plotted against the cholesterol content on semi-logarithmic paper and resulted in a straight line. The cholesterol content was recalculated to give milligrams per 100 cc. blood or plasma directly from the graph. This was done by using the following formula:

$$\frac{\text{mg cholesterol in standard}}{\text{cc. blood (or plasma)}} \times 100 = \text{mg cholesterol per 100 cc.}$$

The dilution of the standard was the same as that of the unknown made later. A different graph was required for the chloroform solution than for the acetic acid solution. The line given by the chloroform standard had a greater slope.

The results obtained by these methods using whole blood are contained in Table I. Table II contains the results given by plasma treated in the same ways.

DATA

Table I

Results using whole blood

Sample	Leiboff	Bloor		Digitonin
		Saponified	Unsaponified	
I	160 160		132 128 128	124 121 115
II	160 160 157		137 138 136 140 137	140 135 130 132 132
III	150 150		115 110 115 120	120 130 134
IV	160 160 160		140 140	123 130 123
V	164 164 164	132 126	141 142 141 137.	126 122
VI	132	115 111	115 111	110 105
VII	148	124 122	127 125	127 125
VIII	133 133	98	109 109 108	

TABLE II

Results using plasma

Sample	Leiboff	Bloor		Digitonin
		Saponified	Unsaponified	
I	60 60	70 74	77 77	80 77
II	80 80	100	105	103 105
III	80 82	94 92	107 100 100	94 92 92
IV	73 70 71		115 120	100 96 96

DISCUSSION OF RESULTS

With whole blood the Leiboff method gave the highest results and the digitonin precipitation method the lowest in most cases. Duplicate samples determined by the Leiboff method usually agreed better than those by the other methods. This is probably due to the fact that the entire process is carried out in the same container.

Variations between the different methods may be explained as follows: Although the first directions given by Bloor are for plasma, serum, or whole blood, he states in 1928[8] that the cholesterol is not entirely extracted from whole blood by alcohol and ether. This undoubtedly accounts for the lower results obtained by this method. The values obtained corroborate Bloor's statement that saponification is unnecessary since both gave practically the same results. Moreover the color was not improved and so this step seems a waste of time and materials.

In the first tests saponification caused very low results. According to the directions, the sodium sulfate obtained when the saponification mixture is acidified is porous and allows complete extraction. The directions also state, "If the drying and distribution of the salt have been carefully carried out, very little of the salt will break loose from the bottom of the flask." An attempt was made to find a cause for the lower results. After extraction according to directions, the salt was broken up with a stirring rod and

further extracted. This extract gave considerable green color when treated with acetic anhydride and sulfuric acid, showing that extraction had been incomplete. Therefore, in subsequent tests the salt was stirred and broken during extraction. The salt was allowed to settle and the chloroform extract decanted through a small filter into the graduated cylinder. This avoided the use of large amounts of chloroform which must first be evaporated down in a larger container and then transferred to the graduated cylinder with further danger of loss. Determinations made using this modification agreed quite closely with values obtained without saponification.

Careful neutralization and drying are important in Bloor's procedure. Complete neutralization liberates free fatty acids which are soluble in the chloroform. These acids may be a contributing factor to the yellow color later developed. Failure to neutralize may result in the decomposition of the cholesterol. Heating on the steam bath after the alcohol has disappeared is apt to cause oxidation of some of the more unstable compounds. There is equal danger at this point in the unsaponified extract. On the other hand, if the drying is incomplete, small amounts of moisture may be extracted by the chloroform. Unless the extract and reagents are completely anhydrous, the color will not develop completely.

The digitonin method is also described for whole blood, as well as for serum and plasma. Probably the same difficulty is involved here as in Bloor's method in securing complete ex-

traction from the whole blood. The directions of Schoenheimer and Sperry call for heating the acetone-absolute alcohol mixture to the boiling point before adding the blood. This causes formation of large clots which are difficult to break up so that complete extraction can be made. It was found more satisfactory to run the blood into the cold mixture and heat to boiling later. This causes a flocculent precipitate which does not form large clots on heating if the tube is kept in motion. This motion also prevents superheating.

Schoenheimer and Sperry claim, "The isolation of cholesterol as digitonide entirely avoids the errors which are introduced into other colorimetric methods by other chromogenic substances present in fatty extracts." This absence of "other chromogenic substances" may account for the slightly lower results obtained. The term "entirely avoids the errors" is not quite correct since later in the same report they state that "digitonin precipitates practically all natural sterols, not all of which give color reactions." Those sterols which give the Liebermann-Burchard reaction are likely to interfere in all methods.

As stated in the previous section, the yellow color often developed in the samples makes comparison in the colorimeter very difficult. This difficulty with the colorimetric determinations has been recognized since Grigaut first devised his method. The cause of the yellow color has been explained in several ways all of which may be partially correct. Bloor(16)

showed that temperature control is important. A temperature of 22° C. is most satisfactory. At higher temperatures the color assumes a yellowish tinge. At lower temperatures the samples, which contain some cholesterol esters, develop faster than the standard which contains only pure cholesterol. This results in high values.

The yellow cast may be due to other color-producing substances in the blood. Luden[20] states that bile derivatives may be a contributing factor. Mueller[21] suggests that oxycholesterol may interfere, but too little is known of it to make a positive statement.

Prolonged heating of the residue after the alcohol and ether are evaporated from the Bloor extract may cause oxidation and decomposition of some of the less stable fatty acids. The oxidation products, if extracted by chloroform, may give that extract a yellow color even before the Liebermann-Burchard reagents are added.

Several attempts have been made to improve the color in the unknown. It was thought that saponification would remove some of the interfering substances, but the color after such treatment still had a yellow tinge. Mueller suggested washing the chloroform extract with water and dehydrating with anhydrous sodium sulfate. This process requires a great deal of time, and it is difficult to know when this extra step is necessary because the extract seldom shows a yellow color before it is treated with acetic anhydride and sulfuric acid. The use of

a red glass filter with the colorimeter has been suggested by Bloor. This is a simple expedient if such a filter is available. The red glass filter in the photelometer accomplishes the same results by allowing only certain rays to be transmitted. The red filter is also used in the photelometer with the digitonide color. Schoenheimer and Sperry studied the absorption of the color produced by cholesterol digitonide and found it was the same as that given by pure cholesterol at all wave lengths.

The determinations were made using plasma because Bloor admits his method is not entirely satisfactory for whole blood. These results are shown in Table II. Here the Leiboff method gave the lowest results in contrast with the highest for whole blood. Since this method was devised for whole blood, the literature contains no information on values given by it for plasma. It is possible that the more complete extraction of cholesterol from plasma by the alcohol and ether causes that method to give higher results in this case. Using plasma, the Bloor method gave values slightly higher than those obtained with digitonin. This was also the case when whole blood was used. It is probable then that wet extraction is more satisfactory with plasma while dry extraction gives getter results for whole blood.

In evaluating the methods, there are advantages and disadvantages to each. In the class laboratory when only one or two determinations are to be made, the Leiboff method would probably give the most satisfactory results. There is

little technique involved beyond that of accurate pipetting and proper use of the colorimeter or photelometer. The entire procedure is carried out in one container and takes a relatively short time. The main disadvantage is the yellow color which makes it difficult to compare the unknown with the standard in a colorimeter. The photelometer overcomes this difficulty, but it is not worth while to standardize it unless a series of determinations is to be made.

Bloor's method involves at least three containers, two extractions, and two filtrations. Moreover, the evaporation of the alcohol and ether requires great care, and makes it necessary for the operator to have considerable experience before the results are likely to be dependable. More blood is required than for either the Leiboff or digitonin methods. However, after the preliminary steps are carried out, the color is better.

In the digitonin method the centrifuge tube into which the extract is pipetted is used throughout the remainder of the determination. This is an advantage over Bloor's method. However, the necessary washings and decantations require careful manipulation to avoid loss. Duplicate determinations require at least three hours more time than duplicates by the Leiboff method. However, the determination is more specific for cholesterol. In this method, as in Bloor's, the extract is filtered before being used. With such volatile solvents as alcohol, ether, and acetone it is

impossible to avoid some evaporation which may affect the concentration of the filtrate.

Regardless of the procedure used preliminary to developing the color, the final step requires great care. The reaction is a delicate one. The color develops to a maximum and then soon starts to fade. Therefore, it is necessary to read the color within a specific time after the sulfuric acid is added. This is 15 to 25 minutes for chloroform solutions and 27 to 35 minutes for acetic acid solution. The speed with which the color develops depends on the temperature. As previously stated 22° C. has been found most satisfactory. The amount of sulfuric acid is a very important factor, a 0.1 cc. pipette being most satisfactory for adding the acid.

In conclusion, it is impossible to say which method gives the most accurate results. It is not necessarily the method giving the highest results since the color is not entirely specific for cholesterol. Per cent recovery of added cholesterol does not give much information as to accuracy, since there are claims of high per cent recovery by all the methods. It seems probable that added cholesterol would be extracted more readily than cholesterol and its esters which are mixed with other blood lipoids. Consequently the methods can only be compared and the differences recognized when they are used. Any value for cholesterol content should be accompanied by specific directions as to the method used.

SUMMARY

1. In the estimation of cholesterol in whole blood, the Leiboff method gave the highest results and the digitonin method of Schoenheimer and Sperry the lowest results. Values by Bloor's method were intermediate, being only slightly higher than those by the digitonin method.

2. Using plasma in place of whole blood, Bloor's method gave the highest values. Results by Schoenheimer and Sperry's method were slightly lower while the Leiboff method gave much lower values.

3. The color produced by extraction according to Leiboff's method was frequently distinctly different from that of the standard. Colors by Bloor's method, especially when saponification was omitted, were easier to match with a standard. The digitonin precipitation method gave a very light green color which appeared gray when compared with the standard.

4. The Leiboff procedure was found to be much simpler than the other methods used in this study. However, it is doubtful whether absolute values are obtained by any of the methods now employed.

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