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An Ultra Micromethod for the Determination of Total Cholesterol in Bile Based on the Tschugaeff Color Reaction

KLAVS H. JØRGENSEN and HENRIK DAM

Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen, Denmark

In the present method all operations, including the development of the color, take place in one and the same vessel (a centrifuge tube). The final volume of the reaction mixture is small (0.5 ml). Hence the method permits the determination of as little as 2 μg cholesterol. When used on a hamster bladder bile extract the Tschugaeff reaction gives rise to a yellow color besides the red color due to cholesterol. A correction for this yellow color is described.

The method was developed for the purpose of determining cholesterol in bladder bile of syrian hamsters where the volume obtainable from one animal usually is between 5 and 50 microliter. The amount of cholesterol present in such a sample may be as low as 2 μg. In principle it may be possible to use the method on other biological fluids than bile, such as blood plasma.

EXPERIMENTAL

Reagents

Standard cholesterol, prepared from crude cholesterol by purification through the dibromide,

Aqueous KOH solution, 1 M.

Chloroform, Ph.Dan.

Acetyl chloride, analytical grade (E. Merck, A.G.). This reagent should not be used for a longer period than 1 month after the first opening of the ampoule, and should be stored at a temperature below 5°C.

Glacial acetic acid, analytical grade (E. Merck, A.G.).

$ZnCl_2$, anhydrous, in sticks, analytical grade (M & B, Ltd.).

A 20 % solution (w/w) of $ZnCl_2$ in glacial acetic acid was prepared by heating on a water bath at 65°C under occasional shaking. The solution which is turbid is clarified by standing or centrifugation. It can be used for at least one month.

Procedure

A. Extraction. 0.003 to 0.010 ml hamster bladder bile * is introduced by means of a Carlsberg pipette [1] into a centrifuge tube having a standard taper female joint 10/15 (shown in Fig. 1). The shape and size of the tube is so chosen that with a content up to

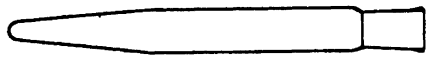

Fig. 1. The vessel in which all operations take place. It is furnished with a standard taper female joint 10/15. Total length: 116 mm, max. diameter: 12 mm, volume up to the joint: about 7 ml.

1 ml nothing will escape from the vessel during violent shaking on a "Microid Flask Shaker" (Griffin & George Ltd.). 0.5 ml of a 1 M KOH solution is added and mixed with the bile by shaking on the just mentioned shaker. The bile alkalized in this way is quite clear. 0.3 ml of chloroform is added and the tube is shaken violently on the shaker for 1/2 min, whereby an emulsion is formed. The tube is then centrifuged (3 000 r.p.m.). In order to ensure extraction of that part of the layer which at the beginning of the shaking may have been thrown up on the upper interior surface of the tube, the latter is again shaken 1/2 min and centrifuged until a yellow, clear aqueous layer is completely separated from a bottom layer which consists of an upper white emulsion and a lower colorless chloroform part. As much as possible of the clear aqueous layer is carefully removed by suction by means of a Carlsberg pipette equipped with a rubber cap. 1/2 ml of water is poured down along the inner wall of the tube. The tube is shaken on the shaker for about 10 sec, centrifuged, and the clear supernatant sucked off. This washing procedure is carried out 4 times in all. The last aqueous layer which is sucked off shall be of neutral reaction, and the emulsion layer shall have disappeared or be reduced to a thin white film between the chloroform and water layers. After the last sucking off, 0.3 ml chloroform is poured down along the inner wall of the tube.

Simultaneously with two samples of bile, a blank and a known amount of cholesterol, 4 μg for instance, serving as a standard, are treated similarly. The cholesterol standard is introduced into the tube by pipetting, say 0.5 ml of a chloroform solution, which is thereafter evaporated.**

B. Evaporation. The evaporation of the 0.3 + 0.3 ml chloroform and about 0.050 ml water (the rest of the washing liquid) is carried out in the following way: The tube is placed in one of the clamps of the shaker and furnished with a standard taper male joint which through a rubber tube fixed in an other clamp of the shaker can be connected with a water suction pump or an oil pump. First the water suction pump is connected with the tube and the chloroform evaporated under shaking while the tube is immerged in a water bath of room temperature. When the pressure has fallen to 15 – 20 mm Hg the temperature of the water bath is raised to 40 – 50°C. Hereby the water in the tube is evaporated. The last trace of water is removed by means of the oil pump. The evaportion procedure requires only a few minutes for each tube.

C. The color reaction. To the residue in each of the tubes (2 with cholesterol from bile, one with standard cholesterol and one with the blank) is added 0.100 ml freshly prepared reagent mixture *** consisting of chloroform, acetyl chloride and 20 % $ZnCl_2$ acetic solution in the proportion 3 : 1 : 1 (by vol.). The tubes are sealed with polyethylene stoppers coated on the upper part of the conical surface with a thin layer of starch-glycerol

* After puncture of the gallbladder of the killed animal the bile is taken out in a capillary tube by suction (not directly with the Carlsberg pipette).

** Concerning the technique of evaporation, see under B.

*** For the pipetting of $CHCl_3$ and reagent mixture Carlsberg pipettes equipped with rubber caps were used.

grease [2]. The stoppers are turned down into the grounds until they fit quite tightly *. The whole inner surface, but not the stopper, is brought in contact with the reagent mixture by suitable gentle turning of the tube. The tubes are then placed in a water bath of 65°C and remain there for exactly 6 min, whereafter they are cooled in ice water for some minutes and are then warmed to room temperature. The stoppers are removed, and the contents of the tubes are diluted with 0.400 ml of the reagent mixture. The stoppers are again inserted and the tubes shaken on the shaker, whereafter the contents are transferred by pipettes to semimicro cuvettes (internal measures: height 40 mm, width 4 mm, and length (ligth path) 10 mm). These cuvettes may be used for contents of about 0.5 ml in a Beckman Spectrophotometer DU when the slit of the instrument is covered with a metal plate furnished with a small opening and the cuvettes are raised about 5 mm from the bottom of the holder. Finally, the extinctions ($E = -\log (I/I_0)$) are measured within 15 min against the blank at 522 mμ and at 440 mμ.

CALCULATION OF THE CHOLESTEROL CONTENT

As mentioned later, the red color developed in the Tschugaeff reaction will be accompanied by a yellow color when the reaction is applied to a hamster bladder bile extract. This yellow color will absorb to a certain extent at the maximum of the red color (522 mμ). It is possible, however, to compute the extinction E'^{bile}_{522} caused by cholesterol alone, by making use of the observed extinctions E^{bile}_{522} and E^{bile}_{440} for the bile sample examined, and the corresponding values E^{st}_{522} and E^{st}_{440} for the standard:

$$E'^{\text{bile}}_{522} = E^{\text{bile}}_{522} \frac{1-0.43\ E^{\text{bile}}_{440}/E^{\text{bile}}_{522}}{1-0.43\ E^{\text{st}}_{440}/E^{\text{st}}_{522}}$$

Thereby the cholesterol content m_{bile} of the sample may be calculated from the amount of standard cholesterol m_{st}:

$$m_{\text{bile}} = m_{\text{st}}\ E'^{\text{bile}}_{522}/E^{\text{st}}_{522}$$

or

$$m_{\text{bile}} = \frac{E^{\text{bile}}_{522} - 0.43\ E^{\text{bile}}_{440}}{E^{\text{st}}_{522} - 0.43\ E^{\text{st}}_{440}}\ m_{\text{st}}$$

DISCUSSION OF THE METHOD

Experiments with standard cholesterol

The colorimetric determination of as little as 2 μg of cholesterol with a reasonable accuracy in cuvettes with a light path of 1 cm and a final volume of the reaction mixture of 0.5 ml requires the use of either the Tschugaeff or the Zlatkis reaction [3]. The Tschugaeff reaction is preferred because of the relative unspecificity of the Zlatkis reaction as pointed out by Lovern [4].

Several methods based on the Tschugaeff reaction are described in the literature, but they all use a final volume of 5 ml or more.

* Polyethylene stoppers fitting to standard taper joints are now commercially available.

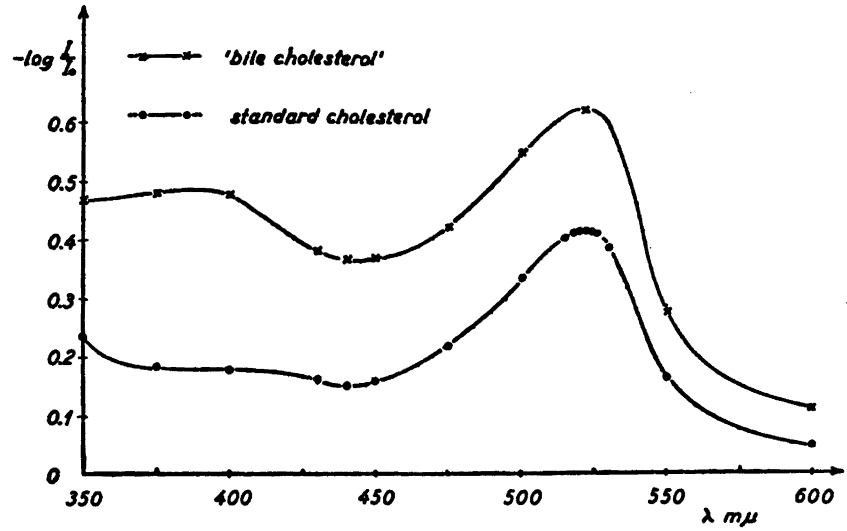

Fig. 2. Absorption curves for the Tschugaeff reaction on 8 μg standard cholesterol and "bile cholesterol" from 0.025 ml hamster bladder bile. Total volume of reaction mixture: 1/2 ml.

Recently, Schön and Gey [5] have described a method in which the color is developed by standing 3 h at room temperature. We could, however, not obtain the agreement with Beer's law reported by these authors.

The same applies to methods using a reaction temperature of 65°C, *e.g.* that of Hanel and Dam [6], when only a few μg of cholesterol shall be determined. The reason for these discrepancies is not clear.

If the reaction mixture is diluted 5 fold after the color has been developed (at 65°C and at a ratio by vol. between chloroform, acetyl chloride and $ZnCl_2$ reagent of 3 : 1 : 1) we have found that Beer's law will be followed in the extinction interval 0.1 to 1.7.

With a final volume of 0.5 ml it was necessary to use primarily 0.100 ml reagent mixture which is later diluted with 0.400 ml of the reagent mixture.

Table 1. Extinctions for various amounts of standard cholesterol per 0.5 ml total volume.

cholesterol μg	E_{522}	E_{522} per 2 μg	E_{440}	E_{440} per 2 μg
0.8	0.036	0.090	0.014	0.035
2	0.105	0.105	0.035	0.035
4	0.215	0.108	0.070	0.035
8	0.420	0.105	0.145	0.036
16	0.860	0.108	0.295	0.037
32	1.750	0.109	0.552	0.035

In order to prevent the escape of part of the volatile reaction mixture the afore-mentioned small tubes with polyethylene stoppers are required. When such stoppers are greased with starch-glycerol grease and turned firmly into the standard taper joints, they can withstand the vapor pressure during the heating of the reaction mixture.

Fig. 2 shows the results of measurements at different wavelengths for a cholesterol standard. A maximum occurs at 522 mμ and a minimum at 440 mμ.

The results obtained for various concentrations of cholesterol at the maximum and the minimum are shown in Table 1.

Beer's law is followed with sufficient accuracy in the concentration interval 2 to 32 μg per 0.5 ml total volume.

If the solution of $ZnCl_2$ had been stored for several weeks deviation from Beer's law occurred. However, the solution could be used at least 1 month after its preparation. Deviation from Beer's law was also found if the acetyl chloride had been stored for a longer time after the first opening of the bottle. If stored cool the acetyl chloride could be used for at least 1 month.

The red color developed in the reaction was not absolutely stable. However, the decrease in intensity during 15 min was low (3—4 %) and nearly proportional to the concentration of cholesterol. Thus, the instability of the color is not an important source of error if only the photometer reading is carried out within 15 min after the development of the color and as simultaneously as possible for the bile sample and the reference standard.

Experiments with hamster bladder bile

As postulated by Zuckerman and Natelson [7], diluted alkali will split cholesterol from protein without heating in the presence of chloroform. This is confirmed by the results presented in Table 2.

Hydrolysis by heating with KOH is, therefore, superfluous. It is possible that esters of cholesterol will not be hydrolysed by the treatment chosen in our method, but cholesterol esters are not known to occur in bile, and, further, in the Tschugaeff reaction esters give the same extinction on a molar basis as free cholesterol [4].

In order to examine the purity of the "Tschugaeff color" when the reaction is applied to bile, the formerly described procedure was carried out on a bile sample and the extinction was measured at different wavelengths (Fig. 2.).

Table 2. Analytical results for bile heated for various lengths of time on steam bath with 0.5 ml 1 M KOH before the extraction with $CHCl_3$.

Sample No. 1 0.005 ml bladder bile		Sample No. 2 0.005 ml bladder bile	
Heating time, h	E_{522}	Heating time, h	E_{522}
0	0.138	0	0.238
0.25	0.143	0.5	0.242
0.5	0.139	1	0.239

Table 3. Extinctions at 522 mμ and 440 mμ for the colors obtained with the 1st, 2nd and 3rd extracts of alkalized bile.

Sample No.	Extraction No.	E_{522}	E_{440}	E_{522}/E_{440} Extract No. 1	2	3
I	1	0.137	0.093	1.47		
0.010 ml	2	0.012	0.028		0.429	
bile	3	0.003	0.003			—
II	1	0.622	0.366	1.70		
0.025 ml	2	0.058	0.137		0.424	
bile	3	0.029	0.067			0.433
III	1	1.08	0.541	2.00		
0.025 ml	2	0.076	0.168		0.452	
bile	3	0.035	0.086			0.407
IV	1	>3	1.60	—		
0.025 ml	2	0.114	0.235		(0.485)	
bile	3	0.090	0.205			0.439
				Average:	0.435	0.426

The absorption curve for "bile cholesterol" has another shape than the curve for standard cholesterol. In both cases the maximum is found at 522 mμ and the minimum at 440 mμ, but the absorption at the lower wavelengths is relatively higher for "bile cholesterol" than for standard cholesterol. Thus, $E^{st}_{522}/E^{st}_{440}$ is about 2.7, whereas $E^{bile}_{522}/E^{bile}_{440}$ is about 1.7. Therefore, the color obtained with "bile cholesterol" has a yellow tinge.

Several attempts to eliminate the yellow color were carried out such as heating of the alkalized bile before the extraction, further washing of the chloroform extract with water, alkali or acid, or the use of other solvents than chloroform, but without success.

In order to isolate the chromogenic impurities, and to ensure that the proposed procedure gives a complete extraction of cholesterol the following experiment was carried out: bile+0.5 ml 1 M KOH were shaken on the shaker with 0.3 ml chloroform and centrifuged in the usual way. The first supernatant was pipetted into another tube with 0.3 ml chloroform. After shaking for 1 min and subsequent centrifugation the supernatant was pipetted into a third tube with 0.3 ml chloroform. After renewed shaking for 1 min and subsequent centrifugation the supernatant was discarded. The 3 portions of chloroform were washed as usual and the analytical procedure was carried out as formerly described on all three samples and a blank. The colors given by the second and third extracts were not visibly red, but in most cases distinctly yellow. The results are presented in Table 3.

The experiments show that the yellow color given by the second and third extracts gives a certain extinction at 522 mμ. Therefore, the same must be the

Table 4. Experiment showing that the yellow color and the red "Tschugaeff color" do not interfere with each other.

Sample	No.	E_{522}	E_{440}
"Bile cholesterol" from 0.0125 ml bile	1	0.189	0.114
The same amount of "bile cholesterol" + 8 μg standard	2	0.595	0.261
8 μg standard	3	0.404	0.146

case for the yellow color included in the color from the first extract. Thus, a correction for the yellow color is necessary.

The table shows that the values E_{522}/E_{440} are increasing with increasing cholesterol concentration: this means that the relative contamination by the yellow color decreases.

Excluding the high value E_{522}/E_{440} for the second extract from sample IV, which apparently is extremely rich in cholesterol, the average values of E_{522}/E_{440} for the second and third extracts are nearly identical: about 0.43. This suggests that the cholesterol is completely extracted by the first extraction as a small amount of cholesterol in the second extract would have caused a significant increase of E_{522}/E_{440} compared with the corresponding value for the third extract.

To see whether the red color produced by cholesterol and the yellow color from the impurities interfered with each other, the following experiment was made: The extraction and evaporation procedures were carried out on 0.050 ml bile. The residue was dissolved in 1 ml chloroform. From this solution 0.250 ml was pipetted into a tube. 0.250 ml of the same solution was pipetted into another tube containing 8 μg of the standard cholesterol. A third tube contained only 8 μg of standard cholesterol. Thereafter, the rest of the procedure was carried out on the three samples and the blank, *cf.* Table 4.

Table 5. Double analyses of some samples of bile.

Sample No.	Bile for analysis ml	Cholesterol per ml bile μg	Mean	Deviation between single determinations in % of mean
I	0.005	765	779	3.6
	0.010	793		
II	0.003	1360	1290	10.9
	0.006	1220		
III	0.005	645	633	3.8
	0.010	621		
IV	0.005	571	597	8.9
	0.010	624		
V	0.005	540	537	1.1
	0.010	534		
			Average:	5.7

It is seen that the sum of the extinctions for samples 1 and 3 is equal to the extinction for sample 2 at 522 mμ as well as at 440 mμ. Thus, the yellow color which is included in the color for sample 1 does not interfere with the "Tschugaeff color" but is directly superposed on the latter.

Since, further, Beer's law is followed for the "Tschugaeff color" both at 522 and 440 mμ it is possible to correct for the yellow color in the following way:

With the designations

E^{bile}_{522} and E^{bile}_{440} = the observed extinctions for bile cholesterol,

E'^{bile}_{522} and E'^{bile}_{440} = the corrected extinctions, *viz.* the extinctions obtained if the yellow color were not present in the bile,

E^{st}_{522} and E^{st}_{440} = the observed extinctions for the standard,

and supposing that E_{522}/E_{440} for the yellow color is 0.43 (*cf.* Table 3), the following equation is obtained:

$$E'^{bile}_{522} = E^{bile}_{522} - (E^{bile}_{440} - E'^{bile}_{440})\ 0.43.$$

Introducing $E'^{bile}_{440} = E'^{bile}_{522} \frac{E^{st}_{440}}{E^{st}_{522}}$, the equation is transformed to:

$$E'^{bile}_{522} = E^{bile}_{522} \frac{1-0.43\ E^{bile}_{440}/E^{bile}_{522}}{1-0.43\ E^{st}_{440}/E^{st}_{522}}$$

The ratio $E'^{bile}_{522}/E^{bile}_{522}$ is usually between 0.8 and 0.9.

The content of cholesterol in the bile sample is thereafter calculated as formerly described (p. 1203).

In addition to the afore-mentioned recovery experiment (*cf.* Table 4), three recovery experiments were carried out in which the added cholesterol was introduced already at the beginning of the procedure. The results showed full recovery of the added cholesterol, *viz.* 102, 102, and 99 %, respectively.

In order to evaluate the accuracy with which cholesterol can be determined in hamster bile, a series of double estimations were carried out (Table 5).

To introduce as great a variation in the experimental conditions as possible, different amounts of the two samples corresponding to one double analysis were used. Further, the two samples were treated at different times, and the colors developed were measured together with different amounts of standard.

The average deviation between the individual determinations corresponding to a double analysis is 5.7 %.

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