QUANTITATIVE DETERMINATION OF THE TOTAL CHOLESTEROL IN CONCREMENTS

Communication I

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Numerous methods have been proposed for cholesterol determination in biological materials, most of them being applied for establishing mainly serum cholesterol. They are divided in two large groups. The first one is based on the green staining, obtained during interaction of cholesterol with acetanhydrid (4, 11), acetylchloride (8, 5), trichloracetic acid (6) or glacial acetic acid (9) in the presence of concentrated sulphuric acid. Very probably, the green colouring is due to the cholesterilen produced. The second group of methods is based on the violet tinge, appearing during the interaction of cholesterol with ferrichloride, always in the presence of concentrated sulphyric acid (13, 3, 10, 12).

With all methods for proving cholesterol in the serum, based on the *Liebermann — Burchard* (5, 8), and *Killiani* (14) reactions, the possibility has not been considered for the higher cholesterol levels in the instances when the material studied contained bilirubin. The reason is the easy oxidation of the bilirubin into biliverdin. For the first time *Babson* et al., *Leffler* et al. (10), *Ramanathan* et al. (12) propose methods for determination of seral cholesterol, in which measures are provided for avoiding the effect of the bilirubin upon the cholesterol values.

Referring to our earlier studies on the content of concrements (1), we set ourselves the task to follow-up the quantity of the total cholesterol in biliary, renal and bladder concrements. Bearing in mind that concrements, and more particularly biliary ones, contain bilirubin occasionally, we considered necessary to look among the known methods for serum cholesterol determination for the most suitable one for establishing of cholesterol in concrements. In the present report the results are given from the application of the methods of *Authenrieth* (2) and *Babson* et al. (3) in the determinations performed by us, of cholesterol in concrements of various nature.

In the course of studies it was established that the method of *Authenrieth* secures adequately reproducible results (table 2). However, in the cases when the concrements contain bilirubin as well, the levels are elevated. In this respect the *Babson* method reveals the following advantages:

1. It allows for cholesterol determination in serum being accomplished in one stage, therefore being time saving.

2. It enables the preliminary elimination of bilirubin, which otherwise brings about higher levels of cholesterol. This is particularly valid for the methods founded on the *Li bermann — Burchard* reaction.

Reagents

1. According to *Authenrieth* — alcohol-ether mixture 1 : 2; chloroform p. a.; acenanhydrid p. a.; concentrated sulphuric acid p. a.

2. According to *Babson* — ethylacetate p. a., freshly open packing (those kept open for a longer time will absorb up to 3% water, which will slowly hydrolize the esther, whereas traces of acetic acid deter the adsorptional capacities of the aluminum hydroxide (3). Absolute alcohol; 0,1% solution of ferrichloride in ethylacetate; concentrated sulphuric acid p. a.; aluminum hydroxide p. a. inactive, with a low sulphate content (3), cholesterol — 4 time cristalized from acetone.

Method of work

1. According to *Authenrieth*: the concrement[1] washed with warm water in advance and dried at 105°C, is thoroughly tritiated (grinded to fine powder). A sample is weighed of 0,01 gr for biliary or 0,1 gr for renal and bladder concrements, next it is poured with 15—20 ml alcohol-ether mixture and is heated under water bath for 5—10 min., with periodical stirring. The insoluble residual is filtrated, the sediment washed up with alcohol-ether mixture and the total filtrate and washing waters evaporated in water bath until drying. The rest is dissolved in chloroform, transferred into a graduated test-tube with refined stopper and then up to 10 ml chloroform is added. If needed, a greater dilution is resorted to, always bearing in mind that in concentrations beneath 0,03 mg/ml or above 2 mg ml the method is relatively inaccurate on account of difficulties in readings. In a separate test-tube similar to the former one 3 ml of this solution, 2 ml acetanhydrid and 0,1 ml concentrated sulphuric acid are mixed up, the tube is plugged, thoroughly stirred and left for 15 min in dark, and thereafter photometered on *Pulfrich* photometer with thickness of the layer 1 cm and filter S_{66} against chloroform-acetanhydrid-concentrated sulphuric acid in the same proportions. The results are recorded on the basis of the standard curve formed with the aid of chloroform solution of cholesterol, treated according to the method just described.

2. According to *Babson*: the concrement is thoroughly washed with hot water, dried at 105°C, tritiated and a sample is weighed from the biliary stones — 0,01 gr, and from the renal and bladder — 0,1 in centrofugated test-tubes with plugs (not rubber). 50—100 mg aluminium hydroxide is added for adsorption of the bilirubin. It is poured with 10 ml mixture of ethylacetate and absolute alcohol, proposed as extraction means by *Klunrsoyr* et al. (7). It is plugged, mixed up thoroughly for extraction of the cholesterol. Next it is centrifuged for 3 minutes at medium revolutions. From the solution thus obtained samples are taken from 0,1 ml to 0,5 ml depending on the cholesterol concentration. Staining is achieved with the aid of the *Ztatkis* reagent (13) — 2,5 ml ferrichloride reagenz are added and 2 0 ml concentrated sulphuric acid (during serial determination the microburet is preferable) with constant stirring. It is cooled up and photo-

[1] Concrements containing and not containing bilirubin were obtained from the Surgical and Internal Clinics.

metration carried out with *Pulfrich* photometer, at 1 cm thickness of the layer, filter S-55 or S-57 against ethylacetate, absolute alcohol, ferrichloride reagent, concentrated sulphuric acid — in analogous proportions. The results are recorded by the standard curve formed with the aid of a constant solution of cholesterol in ethylacetate-absolute alcohol, treated in accordance to the method employed.

Results

The results of the investigation are illustrated in tables. The data in table I show the composition of the cholesterol and bilirubin in the concrements investigated. The data concerning the cholesterol are according to the *Authenrieth* method, whereas for the bilirubin — according to our personal method (yet unpublished).

Table II shows the accuracy of the processes with the *Authenrieth* method in concrements not containing bilirubin.

Table III illustrates the comparative evaluation of the two methods in the analysis of concrements, not containing bilirubin.

Table IV demonstrates the cholesterol level in concrements, containing bilirubin, established according to both methods.

Table I

The composition of cholesterol and bilirubin in the concrements investigated

Product	Sample	Insoluble in HCl in %	Cholesterol in %	Bilirubin in %
1 renal	0,2018	53,22	9,34	—
2 „	0,0991	5,03	1,56	2,39
3 „	0,0443	30,65	0,36	—
4 „	0,3003	22,96	1,50	1,40
5 bladder	0,2695	91,36	0,09	—
6 „	0,1658	99,15	0,14	—
7 „	0,2796	97,03	0,07	—
8 biliary	0,2000	91,25	34,69	—
9 „	0,1014	99,20	95,61	—
10 „	0,1036	98,15	80,40	—
11 „	0,3060	100	83,24	0,20
12 „	0,3010	79,33	60,80	0,012
13 „	0,2031	90,01	82,34	0,07
14 „	0,2156	99,09	75,87	0,19
15 unknown	0,2007	95,07	13,75	—

Note: The data represent mean arithmeticals from 4 determinations.

Table 2

The accuracy of cholesterol determination in concrements according to Authenrieth

No	Sample	Soluble in $CHCl_3$	Cholesterol in %		Mean square deviation
			as regards sample	as regards solutes	
1	0,1040	0,0860	82,93	97,17	∓ 0,215
2	0,1010	0,0880	87,33	97,40	∓ 0,19
3	0,1060	0,0840	78,46	97,13	∓ 0,26
4	0,2000	0,1620	81,43	89,80	∓ 0,027
5	0,0223	0,0185	87,64	92,70	∓ 0,07
6	0,0246	0,0194	79,06	95,54	∓ 0,09
7	0,0200	0,0139	68,87	82,86	∓ 0,04

Note: The data are the result of 4 to 10 parallel determinations.

Table 3

Cholesterol level in biliary concrements not containing bilirubin

No	According to Authenrieth in %	Mean square deviation	According to Babson in %	Mean square deviation
1	52,43	± 0,14	52,50	± 0,25
2	46,62	± 0,15	46,00	± 0,205
3	69,58	± 0,94	72,00	± 0,14

Note: The data are the result of nine parallel determinations.

Table 4

Cholesterol level in biliary concrements containing bilirubin

No	According to Authenrieth in %	Mean square deviation	According to Babson in %	Mean square deviation	Difference in %
1	70,97	± 0,255	60,16	± 0,105	10,81
2	80,21	± 0,245	70,28	± 0,145	9,93
3	83,80	± 0,156	75,14	± 0,105	8,66
4	84,78	± 0,15	77,50	± 0,11	7,28
5	92,54	± 0,22	85,15	± 0,03	7,39

Note: The data are result of nine parallel determinations.

Discussion

Against the background of the data reported in tables II and III it becomes obvious, that both methods lead to rather similar results in concrements not containing bilirubin. From the data presented in table IV it is evident that in instances when the concrement does contain bilirubin, according to the *Authenrieth* method, the results obtained are higher as virtually anticipated. This is exactly the point making the significance of the methods more obvious, always considering the influence of the bilirubin and providing for adequate measures for its elimination (3, 10, 12). This fact justifies the application and comparison of the methods for determination of the cholesterol in concrements containing bilirubin. The *Authenrieth*'s method and the modification proposed by Babson et al., suggested for determination of cholesterol in the serum was adapted for studying the cholesterol in the concrements.

The classical methods for determination of cholesterol in biological materials require 1—3 hours time. The methods for cholesterol determination in which the bilirubin is eliminated (3, 12) allow to carry out determination in one session, without resorting to supplementary manipulations, which is a time-saving procedure. For cholesterol determination in concrements according to *Babson*, 20 min. are sufficient without affecting the accuracy of the results obtained. The latter fact makes it particularly suitable in serial determinations in clinical practice, as well as in scientific research work for the analysis of concrements.

Owing to the fact that the cholesterol detemination in concrements was carried out according to methods for serum cholesterol, and therefore the influence of bilirubin was not taken into consideration, it is assumed that the literature data concerning cholesterol level in concrements in the presence of bilirubin are the least increased and if needed, they should be verified by resorting to new methods. In the light of the facts herein reported, the conclusion is reached that in all cases of cholesterol determination in concrements, regardless of whether or not they contain bilirubin, it is advisable to resort to a method ploviding for the bilirubin elimination.

Studies are in progress aimed at investigating the adsorptional capability of various other sediments with respect to bilirubin, as well as comparative studies on other methods for determination of serum cholesterol, adopted for concrements.

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КОЛИЧЕСТВЕННОЕ ОПРЕДЕЛЕНИЕ ОБЩЕГО ХОЛЕСТЕРОЛА В КОНКРЕМЕНТАХ

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РЕЗЮМЕ

Исследовано определение холестерола в конкрементах при наличии и отсутствии билирубина. Полученные результаты утверждают, что уровень холестерола в конкрементах, не содержащих билирубин, определяемый классическим методом (без устранения билирубина) и по модификации Babson показывают хорошую согласованность. При конкрементах содержащих билирубин, определение холестерола классическим методом дает более высокие показатели холестерола из-за влияния билирубина. Считается, что до определения холестерола во всех случаях должны быть приняты меры для устранения билирубина. Определение холестерола по прилагаемой модификации выполняется приблизительно за 20 мин, тогда как для определения другими методами необходимы от 1 до 3 часов.