FURTHER ON THE PROBLEM OF QUANTITATIVE DETERMINATION OF CHOLESTEROL IN CONCREMENTS

Communication II

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Biliary concrements contain 90% cholesterol at the average, occasionally reaching up to 99%. Along with cholesterol, sedimentation is likewise observed of biliary pigments, fats, calcium phosphate and proteins. The universally accepted division of gallstones in pure cholesterol stones, pigment stones and calcium carbonate (chalk) stones might be assumed as conditional as pure cholesterol, non-pigmented concretions are met comparatively seldom. Adequate methods for quantitative determination of the composition of biliary concrements have not been described in literature to date (5, 6, 7). It is generally proposed to extract the cholesterol with ether and further elaborate the dried residue, following evaporation of the ether for cholesterol according to some of the well known methods for serum cholesterol. The Libermann — Burchard reaction is chiefly employed. In a previous report (1), we published the results of cholesterol determination in concrements both in the presence and absence of bilirubin. It is evident from the data presented that removal of the bilirubin is rather expedient, for otherwise it accounts for high cholesterol values. With the method proposed by Babson (2), by rule, a good possibility is secured for the elimination of bilirubin from the serum by way of adsorption. Against the background of personal experience, it was established that even when the gallstone did not contain bilirubin, high cholesterol values were likewise obtained in some cases, obviously due to larger quantities of accompanying pigments. This finding of ours fully coincides with the data submitted by Harrison (5), proving that in some cases various oxidative products of bilirubin: biliverdin, bilifuscin and bilihumin are contained in pigment biliary concrements. It is thereby concluded that elimination in due course of the pigments is mandatory in order to obtain the purest possible cholesterol extracts.

In gallstones containing minimum quantities of pigment, the utilization of the Klungsoyr mixture (9): absolute alcohol — ethyl acetate in proportion 1 : 1 and subsequent adsorption on $Al(OH)_3$ (2) yields good results. However, in instances of higher pigment content, an increase of the $Al(OH)_3$ quantity is necessary, thus creating conditions for the cholesterol adsorption. The most suitable solvent appears to be the pure ether, by virtue of the fact that in the latter the bilirubin is virtually unsolved, whereas its derivative pigments are hardly soluble. In the course of personal investigations however, it was established that in the process of filtration the extracts get concentrated and the cholesterol accordingly cristalized on the filter on account of

the high volatility of ether. The loss of cholesterol in these instances was obvious. It was found that the mixture absolute alcohol — ether in a proportion 1 : 1 yields optimal results. The ether component reduces the solubility of pigments, whereas the alcoholic component, due to its limited volatility, interferes with cholesterol cristallization. In addition, the possibilities were studied, in experiment, for the complete elimination of minimal quantities passed into the solution, undesired pigments on various adsorbents and under varying conditiors — on column, by centrifugation and by mixing and filtration. All investigations were carried out with parallel control samples of the same concrement, with pigments extracted through hot dilution in M/10 Na_2CO_3 solution. The insoluble residue, after full dryness, was further treated for cholesterol, parallel with the sample for direct cholesterol determination.

1. Preliminary Investigations

Standard cholesterol solutions were prepared mixing one part absolute alcohol and one part ether (solvent 1), and mixture of absolute alcohol — ethyl acetate (solvent 2) in the same proportion with 1 mg/ml concentration. Next saturated solution of bilirubin was added in the respective solvent in a fashion to provide for model solutions, containing cholesterol and bilirubin in priorly determined concentrations. The solutions thus obtained were further treated for cholesterol according to the Kiliani method (2) with $Fe\,Cl_3$. The following results were obtained:

Table 1

Level of Cholesterol (100 gamma) Determined in the Presense of Bilirubin Different Number Gamma

Cholesterol quantity in gamma	Bilirubun quantity in gamma	Determined cholesterol in gamma	
		in solvent 1	in solvent 2
100	0,4	105,0	—
100	1,0	108,3	109,2
100	1,6	114,6	—
100	2,0	114,9	116,4
100	2,4	116,7	—
100	3,0	120,0	122,0
100	3,5	120,8	—
100	4,0	122,8	127,2
100	5,0	—	130,1

From the data presented in the table, it is evident that even minimal pigment concentrations exert an influence on cholesterol determination.

With a view to investigating the adsorption capacity of $Al\,(OH)_3$ and Al_2O_3, adsorption columns were prepared in glass tubes (50 ml burets without plugs), containing accordingly 4 gr substance freely poured on 1 cm glass

felt layer. Standard cholesterol solutions in solvent 1 and 2 were passed through part of the columns, whereas solutions containing 1 mg/ml cholesterol and 0.1 mg/ml bilirubin were passed through the remaining part of the columns. The cholesterol in the columns was eluated with CCl_4, $CHCl_3$, solvent 1 and solvent 2. The following results were received:

Table 2

Cholesterol retention on adsorption columns containing Al (OH)$_3$ and Al_2O_3

Poured 5 ml standart cholesterol solution in concentration 1 mg/nl	Al (OH)$_3$ 4 gr	Al_2O_3 4 gr
1	2	3
I. Cholesterol passed through	2,90 mg	0,40 mg
Eluated with 10 ml CCl_4	1,65 „	2,55 „
total quantity passed through	4,55 „	2,95 „
cholesterol retained	0,05 „	2,05 „
		„
II. Cholesterol passed through	3,00 „	0,60 „
I elution with 5 ml $CECl_3$	1,20 „	0,40 „
II * „ „ „ „	0,00 „	1,90 „
total quantity passed through	4,20 „	2,90 „
cholesterol retained	0,80 „	2,10 „
III. Cholesterol passed through	3,00 „	0,82 „
I elution with 5 ml solvent 1	1,94 „	3,81 „
II „ „ „ „ „ „	0,00 „	0,32 „
total quantity passed through	4,94 „	4,96 „
cholesterol retained	0,06 „	0,04 „
IV. Cholesterol passed through	2,65 „	0,83 „
I elution with 5 ml solvent 2	2,30 „	2,98 „
II „ „ „ „ „ „	0,00 „	0,50 „
total quantity passed through	4,95 „	4,31 „
cholesterol retained	0,05 „	0,69 „
Poured standard solutions, containing 1 mg cholesterol and 0.1 bilirubin		
I. Cholesterol passed through	0,58 mg	0,31 mg
eluated with $CHCl_3$	0,58 „	0,39 „
total quantity passed through (determined as for cholesterol)	1,16 „	0,70 „
difference	+0,16 „	—0,30 „
II. Cholesterol passed through	0,51 „	0,42 „
eluated with solvent 2	0,50 „	0,29 „
total passed through quantity	1,01 „	0,72 „
difference	+0,01 „	—0,28 „
III. Cholesterol passed through	0,50 „	0,50 „
eluated with solvent 1	0,52 „	0,57 „
total quantity passed through	1,02 „	1,07 „
difference	+0,02 „	+0,07 „

The data in table 2 show that adsorption on Al_2O_3 does not meet the requirements by virtue of the fact that large quantities of cholesterol are retained, hardly susceptible to subsequent elution. The cholesterol retained on $Al(OH)_3$ is eluated as early as with the first 5 ml solvent, whereas Al_2O_3 cannot be fully liberated even after repeated elution with additional 5 ml.

With a view to cutting the purification time, we performed adsorption by means of thoroughly mixing up the working solution with the adsorbent. The separation could be effected in two possible routes: by centrifugation or by filtration through quantitative filter and subsequent washing of the adsorbent on the filter, collecting the filtrate and the washed liquids in a graduated flask, poured until a determined volume is reached. During computation of the results the dilution is considered. The average results of 10 comparative determinations are illustrated in table 3, compared with control samples of the same concrement.

Table 3

Tthe cholesterol level in a control sample freed from pigments through treatment of the concrement with M/10 $NaCO_3$ and in two parallel samples for direct cholesterol determination with elimination of adsorbent by centrifugation and filtration

Control sample % cholest.	By centrifugation			By filtration		
	% cholesterol	mean arith. difference	σ	% cholesterol	mean arith. difference	σ
97,55	93,25	± 4,53	± 1,94	97,40	± 1,45	± 0,76

The data in table 3 demonstrate that during adsorption through mixing the solution of the concrement with Al $(OH)_3$ and subsequent filtration and washing on a filter uniform results are obtained as compared to control sample.

We carried out a number of experiments for the adsorption of pigments through mixing dehydrated $CaCl_2$ or $BaCl_2$. The latter revealed certain degree of adsorptional capacity, but the minimal quantities Ca^{2+} and Ba^{2+} passing through into the solution interfere with photometration carried out at a later stage, due to the turbidity of the samples caused by the formation of $CaSO_4$ and $BaSO_4$ resp.

The preliminary investigations show that the most suitable solvent for cholesterol from biliary concrements proves to be the mixture absolute alcohol — ether in a proportion 1 : 1. It provides for determination of the cholesterol after either methods of Kiliani (with $FeCl_3$) without previous evaporation and subsequent dissolving in a different solvent. Moreover, the solubility of pigments in this mixture is minimal. To the end of definitive elimination of pigments, mixing of the sample with $Al(OH)_3$ in a quantity 0.2 gr 10 ml sample proves most suitable. The closest to control sample results are obtained during filtration of sediments and washing the residues over the filter.

On the basis of the preliminary investigations just described we set ourselves the task to elaborate an adequate method for quantitative determination of cholesterol in concrements. The latter method consists in:

A weighed sample of previously thoroughly dried and tritiated concrement is dissolved in a mixture (1 : 1 proportion) of absolute alcohol and ether. Adsorption with $Al(OH)_3$ is resorted to for the complete elimination of pigments passed through into the solution. In the comparatively pure extract thus obtained, the cholesterol is determined after the Kiliani reaction. With a view to avoiding volume alterations, brought about by vaporization of the ether subsequent to the heating of the mixture during the addition of staining slovent, the weighed sample of cholesterol solution is filled up to the necessary for staining purposes volume with a mixture of ethyl acetate — absolute alcohol in proportion 1 : 1. If determination of the cholesterol fractions is needed, the free cholesterol is precipitated after the known fashion — with digitonin; in the filtrate the esther bound cholesterol is determined in an analogous pattern.

Materials

1) Mixture of absolute alcohol — ether in proportion 1 : 1.

2) Mixture of absolute alcohol — ethyl acetate in proportion 1 : 1.

For staining the reagent was used, employed by Rosenthal and Ind (12) and Bowman and Wolf (4) in determination of serum cholesterol, namely:

a) basic reagent: 2.5% solution of $FeCl_3 \cdot 6H_2O$ in 87% H_3PO_4 p.a.

b) staining reagent — 8 ml of the basic solution is poured up to 100 ml with concentration H_2SO_4 p.a.

The basic solution is of unlimited durability, whereas the working solution is stored in a refrigerator and in case of discolouring or becoming turbid, it is renewed.

Adsorption means — $Al(OH)_3$ p.a.

Standard cholesterol solution with concentration 1 mg/ml in solution 1.

Method of Work

From the thoroughly washed with warm water, dried and tritiated (grinded to fine powder) concrement, a sample is obtained weighing 0.01 gr and accordingly dissolved in 5 ml of the solvent 1. 0.1 to 0.2 gr $Al(OH)_3$ is added to the solution, it is thoroughly stirred with a galls stick and filtrated through a quantitative filter. The adsorbent is quantitatively transferred and the filter undergoes triple washing with 1 ml of solvent 1; thereupon the solution is poured up to 10 ml.

Working sample — 0.2 ml of the solution for cholesterol in a test-tube is filled up with 1.8 ml of solvent 2.

Standard sample — 0.2 ml of the standard solution of cholesterol is placed in the test-tube and filled up with 1.8 ml solvent 2.

Empty sample — 0.2 ml of solvent 1 in a test tube is poured with 1.8 ml of solvent 2.

Table 4

Level of cholesterol and cholesterol esthers in mixed cholesterol concrements

Clinical case	Control sample	After the Kiliani method			After Liebermann — Burchard				
		according to method suggested:			Babson	Bowman Wolf	Mrskos Tovarek	Pearson	Bloor
		total cholesterol	difference	esthers					
H. Z.	98,6	99,0	+0,4	—	104	106	102	106	126
J. V.	84,0	—	—	—	103,5	99,9	—	110	—
J. S.	98,2	—	—	—	108,3	106,5	—	—	—
J. M.	92,0	—	—	—	88,3	90,8	—	—	—
438	91,0	92,5	+1,5	2,8	104,2	100,8	—	—	127
438 mean	92,8	91,5	—1,3	0,0	100,0	98,3	—	—	117,6
123	99,6	100,1	+0,5	6,1	109,4	101,5	109,0	115,6	—
129	94,5	96,2	+1,9	7,6	100,0	100,0	117,5	108,0	—
A. A.	96,6	94,9	—1,7	4,5	107,6	97,7	124,6	—	—
G. D.	42,0	41,2	—0,8	2,1	47,2	47,5	40,5	—	—
P. M.	85,9	82,3	—3,6	1,2	100,2	94,8	100,9	—	—
K. P.	98,2	98,5	+0,3	8,0	99,6	—	—	—	—
E. I.	77,5	76,2	—1,3	2,5	—	—	—	—	—
T. G.	78,3	77,7	—0,6	3,2	—	—	—	—	—
352	32,0	31,3	—0,7	0,0	—	—	—	—	—
111	97,8	98,4	+0,6	1,5	—	—	—	—	—
113	99,6	102,9	+3,3	5,0	—	—	—	—	—
114	93,8	92,9	—0,9	2,4	—	—	—	—	—
Average	85,68	85,04 $\sigma = 0,420$							

Staining — 2 ml of the tinging reagent is added to each of the three samples (work is carried out with microburette), next they are thoroughly stirred and plugged and left for further 20 min in dark. Thereafter photometration is carried out with Pulfrich photometer with thickness of the layer 0.5 cm and filter S_{57} against the empty sample.

The volume of the working sample (standard and empty samples respectively) should be by all means fully identical to the volume of the staining reagent. Otherwise stining is not produced.

Author's notice: Determination of the standard solution extinction is performed once daily and it is mandatory when new staining reagent is prepared.

Computations:

$$\% \text{ of total cholesterol} = \frac{\text{ml of standard solution} \times \text{E of the sample}}{\text{ml sample} \times \text{E of standard solution}} \times 100$$

Results

Table 4 illustrates the results of the parallel determinations of cholesterol in the concrements, carried out according to personal and various other methods, and compared with the control sample for each individual conrement.

Table 5

Errors due to ether evaporization from solvent 1 during sample development

Samples treated with solvent 1 only	Samples supplemented with solvent 2 up to 2 ml	Difference in excess during work only with solvent 1 as referred to samples supplemented with solvent 2
108,00%	97,80%	3,00%
98,80%	97,80%	1,00%
101,20%	100,00%	1,20%
102,00%	102,00%	0,00%
102,20%	100,00%	9,20%
79,80%	65,40%	7,40%
76,50%	71,50%	5,00%
50,00%	47,00%	3,00%
50,00%	47,20%	2,80%
53,15%	50,00%	3,15%
84,20%	81,70%	2,50%
61,45%	55,20%	6,25%
59,50%	55,70%	3,75%

mean arithmetical difference = 2,94
σ = 1,30

Table 6

Accuracy of the method suggested, determined after the method of standard supplementation

Initial concentration	Added	Determined	Difference from available concentration
	A. To standard cholesterol solutions		
0.100 mg	0.100 mg	0.200 mg	0.000 mg
0.100 „	0.150 „	0.243 „	— 0.007
0.100 „	0.180 „	0.288 „	+ 0.008
0.100 „	0.200 „	0.300 „	0.000 „
0.100 „	0.250 „	0.353 „	+ 0.003
0.100 „	0.300 „	0.401 „	+ 0 001 „
			± 0.002 „
	B. To working concrement solutions		
0.356 mg	0.100 mg	0.451 mg	— 0.005 mg
0.361 „	0.100 „	0.451 „	— 0.010 „
0.372 „	0.100 „	0.450 „	+ 0.008 „
0.152 „	0.100 „	0.254 „	+ 0.002 „
0.143 „	0.100 „	0.254 „	+ 0.012 „
0.170 „	0.100 „	0.253 „	+ 0.017 „
0.132 „	0.100 „	0.230 „	+ 0.002 „
			± 0.0028

Note: Data in the first column are from parallel determinations.

t = 0.09 (student Fischer) shows that no statistically significant differences exist between the values for the cholesterol level, determined by the control sample and according to the method proposed by the authors.

Table 5 demonstrates the need of employing two types of solvents: solvent 1 for the extraction of cholesterol and solvent 2 as a medium for staining and photometration.

Verification as to accuracy of the method suggested was carried out after the method of standard supplementation, equally to pure cholesterol standard solutions and to working concrement solutions. The results thus obtained are presented in table 6.

Discussion

It is obvious from the data in table 4 that the method herein suggested for determination of cholesterol in biliary concrements provides for closest to the real values of cholesterol level as compared to values obtained in control samples. The results obtained with the method of Babson (2) and the method of Bowman & Wolf (4) are similar and up to a certain extent resemble those obtained with our method, a phenomenon anticipated as all three methods are based on the Kiliani reaction (8). The values obtained according to methods based on the Lieberman — Burchard reaction are substantially higher.

In the course of determination of cholesterol fractions it was established that in some of the concrements there was no esther bound cholesterol at all, whereas in others — only in limited quantity. Merely in four concrements did we find esther bound cholesterol exceeding 5%. Thus, the inference was reached that determination of total cholesterol is absolutely sufficient, with the only exception of instances when determination of individual cholesterol fractions was aimed at in particular.

The accuracy of the method suggested by the authors, proved by standard supplementation of cholesterol, is completely sufficient for clinical as well as for research purposes. The possibility of carrying out the determination on the original solution itself, without need of preliminary vaporization and dissolution in an other solvent, is time-saving and serves economy of materials. The utilization is avoided of the strongly irritating the mucosa acetic anhydride and chloroform, which, particularly in serial parallel samples, are hardly tolerated by the working personnel. The previous mixture of the $FeCl_3$ with sulphuric acid makes possible the achievement of prompt staining of samples and with highprecision employment of the microburette. The time needed for determination of cholesterol in the prepared tritiated concrement is 40 minutes. The method provides for simultaneous determination of a great number of samples.

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К ВОПРОСУ О КОЛИЧЕСТВЕННОМ ОПРЕДЕЛЕНИИ ХОЛЕСТЕРИНА В КОНКРЕМЕНТАХ

2-ое сообщение

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РЕЗЮМЕ

Проведены изучения над мешающим влиянием желчных пигментов — билирубин и его производные — на определение количества холестерина в пигментированных желчных камнях. Предлагается метод для количественного определения холестерина в подобных камнях, при котором влияние упомянутых пигментов практически элеминировано. Для доказательства точности метода проведена проверка по методу стандартной надбавки, как к стандартным растворам холестерина, так и к рабочим растворам конкрементов. Значения уровня холестерина в разных конкрементах, полученные по предлагаемому методу, сравниваются, как с контрольной пробой, полностью освобожденной от наличия пигментов, так и с параллельными определениями при помощи других методов для определения содержания холестерина в сыворотке крови. Самые близкие к контрольной пробе результаты получаются при применении предлагаемого метода.

Метод быстрый, безвредный, позволяющий проведение параллельного определения большего числа проб.