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Medical-scientific Department of Boehringer Mannheim GmbH

Determination of cholesterol in serum

Comparison of chemical methods and a new enzymatic method

H. Forstmeyer

Abstract

Determination of cholesterol in the serum

The regular control of the lipid metabolism is an essential element in preventive diagnostics with regard to coronary sclerosis as well as to peripheral and cerebral vascular diseases. While an enzymatic test for triglycerides is already available, cholesterol had to be determined hitherto either by a chemical method which is very susceptible to errors or by expensive extraction methods. In the meantime one has succeeded in developing a comparatively simple enzymatic test for cholesterol. This new method is not only simpler to handle than the usual chemical methods with numerous possibilities of error, but also has the advantage of higher specificity.

Framingham and similar long-term epidemiological studies have called attention to the importance of a number of risk factors for generating ischemic heart illnesses [8]. High serum cholesterol level leads the risk factors for coronary sclerosis. Not only for this reason but also because of its connection to other risk factors, total fat metabolism diagnostics is at the focal point of medical prevention [18]. Since high cholesterol and triglyceride values are rarely accompanied by clinical symptoms, reliable laboratory methods for the diagnosis of hyperlipoproteinaemia are indispensable. Ninety-five percent of all hyperlipoproteinaemia can be detected by the combined determination of cholesterol and triglycerides [25].

The enzymatic method has generally been used in clinics and laboratories for the determination of triglycerides[1]. On the other hand, chemical methods have always been used in the case of cholesterol. However, after the enzymes, cholesterol esterase and cholesterol oxydase, were isolated and enzymatic reactions were linked with the colour reaction known from the enzymatic colour test for uric acid[2], an enzymatic colour test specific for cholesterol[3] has also become available. This test is also suitable for routine investigations.

The heretofore most common methods measure the colour reaction of cholesterol with sulphuric acid and acetic acid photometrically (colour reaction according to Salkowski, modified by Liebermann,

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[1]Combination test neutral fats (triglycerides). Boehringer Mannheim GmbH

[2]Combination test Urica-quant®. Boehringer-Mannheim GmbH

[3]Combination test cholesterol, enzymatic colour test, Boehringer-Mannheim GmbH

Burchard et al). Halochromes are formed in the acetic acid medium under action of sulphuric acid for dehydration, dehydrogenation and formation of multiply unsaturated high-molecular hydrocarbons.

Since the composition of the reaction products is not known, the molar extinction coefficient cannot be determined. Therefore, in cholesterol determinations, which are based on this reaction, a standard must always be used for comparison for the concentration calculation [19].

The Liebermann-Burchard reaction is strongly temperature-dependent. Water contained in serum in combination with the colour reagent leads to heat tones which, depending on the water content, influence the reaction rate and colour formation in various samples and in the standard solution differently.

For example, the influence of the temperature manifests itself in the following manner [10]: 1 mg of cholesterol was dissolved in 10 ml of chloroform and reacted with acetic anhydride and sulphuric acid. If it is measured at 430 nm, the colour develops very rapidly, depending on the incubation temperature. In addition, greater extinctions are also obtained at higher temperatures. The conditions are different if it is measured at 690 nm: here, the colour intensity decreases after given periods, depending on the incubation temperature (Fig. 1).

Furthermore, colour complexes which are formed in the Liebermann-Burchard reaction are light-sensitive, and thus, are not stable. It is therefore important to watch the reaction times in order to achieve higher possibility of an exact tempering [7].

Acetic anhydride used in the Liebermann-Burchard reaction serves primarily to dilute sulphuric acid and to reduce the water in the

reaction mixture (serum sample). But the colour which develops is influenced by the proportion of sulphuric acid/acetic anhydride. With high ratios, a cherry red Salkowski colour develops; with very low ratios, that is, at very high dilution of sulphuric acid with acetic anhydride, a blue-green Liebermann-Burchard colour develops.

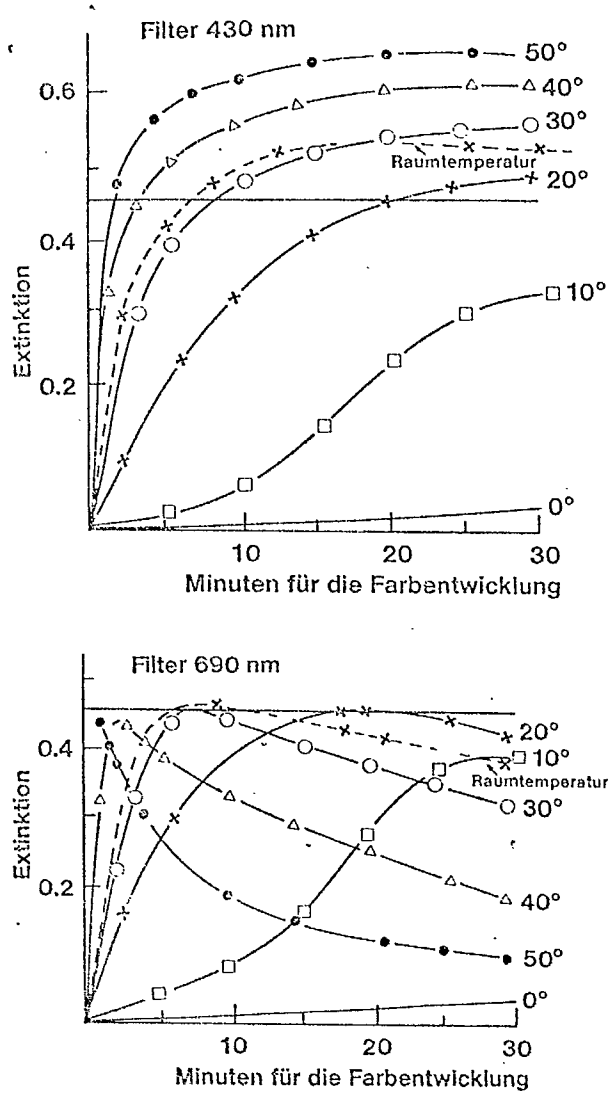

Fig. 1. Colour development in the Liebermann-Burchard reaction [10]

Minuten für die Farbentwicklung = minutes for colour development;
Extinktion = extinction; Raumtemperatur = room temperature

In addition to the difficulties produced by different heat tones, the action of light and the presence of water, turbidities appear occasionally in the mixture in the presence of proteins. Moreover, it has been shown that serum albumins with sulphuric acid and acetic anhydride can enter a colour reaction such that, for example, serum albumin with a content of 6 g/100 ml simulates about 8 mg/100 ml cholesterol [16].

Difficulties appear if the chemical cholesterol determination is carried out without prior separation of cholesterol in strongly lipemic, hemolytic or jaundiced serum. Erroneously high values are simulated through interferences of hemoglobin and bilirubin. For example, according to the modification of the Liebermann-Burchard method, 4-10 mg/100 ml cholesterol is simulated by 1 mg/100 ml bilirubin.

In addition, it has been shown that sulphuric acid and acetic acid with free cholesterol and cholesterol ester do not produce the same colour intensities [22]. Serum was extracted with petroleum ether once without pretreatment, the other, only after saponification of the cholesterol ester. After evaporating off the organic solvent, cholesterol was detected in the residue through its colour reaction with Liebermann-Burchard reagent. Comparing the residue containing only free cholesterol with the unsaponified residues containing free cholesterol and cholesterol ester, values differing up to 10% were obtained. Depending on the amount of cholesterol ester, higher as well as lower colour intensities can be measured by the Liebermann-Burchard reaction and also by the method of Zak et al [26] in comparison to unesterified cholesterol [11].

Another indicator system for the determination of cholesterol is used in Zak's method. The basis of the measurement here is the

Lifschütz colour reaction. The reagent consists of a highly diluted iron(III) chloride solution in concentrated sulphuric acid which is added to the sample dissolved in glacial acetic acid. A red colour measurable at 560 nm is produced. The higher colour intensity compared with the Liebermann-Burchard reaction allows it to repress the reaction of possibly interfering serum constituents in the sample by suitable dilution. At the absorption maximum of 560 nm, other steroids show smaller extinctions in comparison to cholesterol than is the case in the Liebermann-Burchard reaction. In the Lifschütz reaction, however, compounds with phenolic groups participate in the colour development. Besides, the colour reaction is disturbed by nitrite, nitrate and other N-containing inorganic compounds [14] which are often contained in sulphuric acid as impurities. For these reasons, the specificity of the method given by Zak et al is insufficient.

Because of the possibilities of error in the direct determination of cholesterol in serum, there have been attempts to develop methods which allow an exact determination of cholesterol. Depending on the number of steps involved in separating the interfering substances, one speaks of one-, two-, three- and four-step methods. The multi-step methods are laborious and not suitable for routine investigations.

One of these extraction methods, Kendall-Abell's modification [1], is considered to be a reference method in the USA and has also been established in epidemiological long-term studies, such as the Framingham study. 699

The individual steps are as follows:

1. Cholesterol esters are saponified with alcoholic potassium hydroxide

directly in the serum sample (60 min at 45° C).

2. The free cholesterol is extracted with petroleum ether.
3. Aliquots of the petroleum ether extract are withdrawn and evaporated to dryness under nitrogen.
4. The free cholesterol contained in the residue is measured photometrically with the Liebermann-Burchard reagent (acetic anhydride, acetic acid, sulphuric acid).

Significant use of this laborious method is made normally only in research studies.

While the semi-enzymatic methods published very recently [4,15] all still have the drawback of alkaline saponification which is laborious and also susceptible to interferences (reducing substances are formed in treating serum with potassium hydroxide), enzymatic reaction is now successfully carried out in these first steps.

Not only for the saponification step but also for the following indicator reaction, strong reagents are no longer used. Now there is particular relief in that the concentrated sulphuric acid is no longer pipetted and odour annoyances by acetic acid-acetic anhydride mixtures are eliminated.

That this enzymatic method correlates excellently with the common reference method of Kendall-Abell, as studies by Röschlau et al [17] have shown, is estimated to be of very great benefit (Fig. 2).

Principle of the enzymatic colour test

Cholesterol esters present in serum are cleaved hydrolytically by cholesterol esterase. The resulting free cholesterol is oxidized

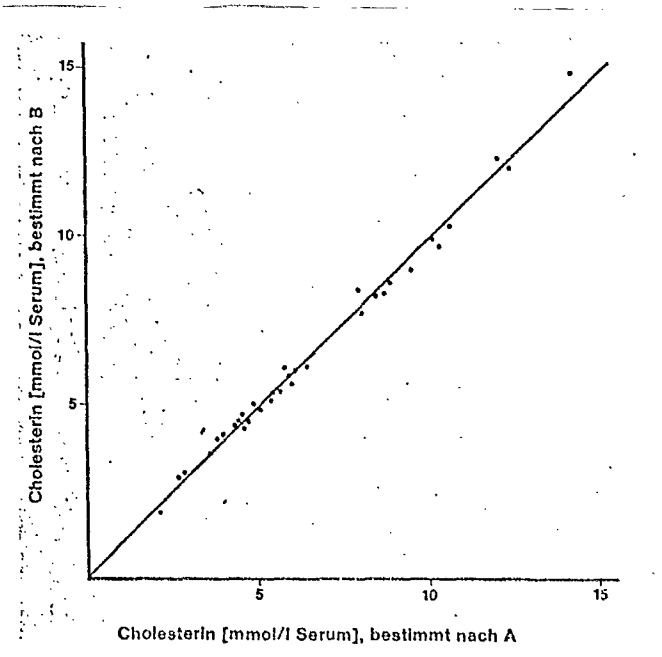

Fig. 2. Correlation between the cholesterol determination according to Abell (A) and the enzymatic cholesterol colour test (B).
Regression: $y = 0.999\ x - 0.01$; $r = 0.996$; $S_{y,x} = 0.26$; $N = 34$

Cholesterin [mmol/l Serum], bestimmt nach A = cholesterol [mmole/l serum], determined according to A; Cholesterin [mmol/l Serum], bestimmt nach B = cholesterol [mmole/l serum], determined according to B

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to Δ^4-cholestenone by cholesterol oxydase in the presence of O_2 (Fig. 3). Hydrogen peroxide which forms in this process is then used in forming the colour indicator, as already known from the enzymatic colour test for uric acid. The resulting lutidinic derivative is a definite substance [13] which allows calculation by means of a factor [20]. The colour intensity can be measured at 405-415 nm.

The H_2O_2 which forms in this series of reactions can also be measured by other already known colour reactions, for example, by transforming with o-dianisidine [21] or peroxidase and subsequent colour coupling with 4-aminoantipyrine and phenol [2]. However, the Kageyama

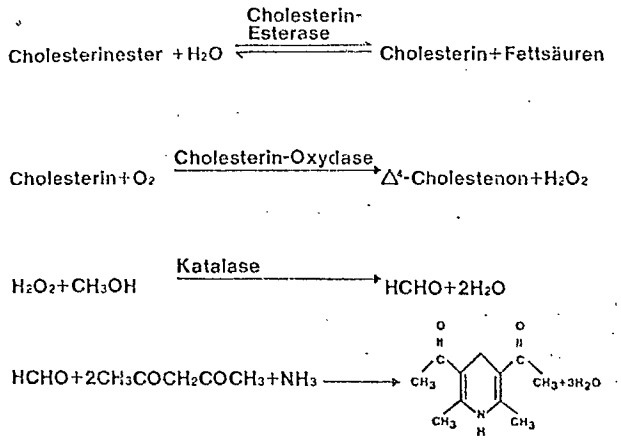

Fig. 3. Reaction principle of the enzymatic cholesterol determination according to Röschlau et al.

Cholesterinester = cholesterol ester; Cholesterin-Esterase = cholesterol esterase; Cholesterin = cholesterol; Fettsäuren = fatty acids; Cholesterin-Oxydase = cholesterol oxydase; Δ^4-Cholestenon = Δ^4-cholestenone; Katalase = catalase

reaction is preferred since catalase transfers oxygen specifically to methanol under the given test conditions, and thus, this colour indicator reaction remains insusceptible towards interfering influences, for example, of reducing agents.

Test run: concentration and composition of the solutions:

Solution 4: cholesterol oxydase 4 U/ml

Solution 5: 0.57 M ammonium phosphate buffer, pH 7; 1.7 M methanol; 0.02 M acetylacetone; 0.1% hydroxypolyethoxydodecane; catalase 670 U/ml; cholesterol esterase 26 mU/ml.

The test is carried out simply and requires only a few pipetting steps (Table 1). Wavelengths: Hg 405 nm, 410 nm. Glass cuvette: 1-cm thickness. A sample blank value is determined in order to eliminate

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sources of error caused by the serum background (for example, effects of drugs) [20].

Table 1. Pipetting scheme for the cholesterol determination in the enzymatic colour test

Pipette into the bottom of the reagent glass:	blank	sample
undiluted sample	0.05 ml or 0.02 ml	-
solution 5	5.00 ml	-
Mix well contents of the reagent glass.		
Solution 4	-	0.02 ml
Pipette from the reagent glass of the blank (Return to reagent glass remainder in pipette)	-	2.50 ml
Mix well. Incubate blank and sample at least 60 min at 37° C. Measure extinction of sample against blank: E_{sample}		

Calculation: From the extinction difference of the sample compared with the blank, the cholesterol concentration at Hg 405 nm can be determined as follows:

with 0.02 ml sample

$c = 1311 \times E_{Probe}$ (mg/100 ml) bzw.
$33{,}9 \times E_{Probe}$ (mmol/l)

$E_{probe} = E_{sample}$; bzw. = or
mmol/l = mmole/l

with 0.05 ml sample

$c = 528 \times E_{Probe}$ bzw.
$13{,}6 \times E_{Probe}$ (mmol/l).

The direct correlation between colour intensity and cholesterol

concentration is given in the case of 0.05 ml serum in the range of 0-500 mg/100 ml. Checks in several large hospital laboratories have shown that cholesterol concentrations over 500 mg/100 ml occur only rarely.

Accuracy: Accurate cholesterol values are found with the new method [17]. Cholesterol added to various human sera was recovered quantitatively. Bilirubin (up to 20 mg/100 ml serum), hemoglobin (up to 500 mg/100 ml serum) and anticoagulants in usual concentrations did not interfere with the analysis. An interference by drugs could not be ascertained [20]. The test is not affected by high triglyceride values.

Precision: In human sera in the normal range, a variation coefficient (VC) of about 3% results from day to day with 0.02 ml serum. With a pathological cholesterol value, the VC is lower.

Example [20]:

Precision from day to day

$n = 10$	$n = 10$		
$\bar{x} = 192$ mg/100 ml	$\bar{x} = 480$ mg/100 ml		
VK = 3,1%	VK = 1,8%	VK = VC = 3.1%	1.8%

The following example shows that with an 0.05-ml sample volume, the precision can be clearly improved:

Precision from day to day

0.05 ml sample	0.02 ml sample		
$n = 8$	$n = 8$		
$\bar{x} \pm 2s = 131,0$	$\bar{x} \pm 2s = 131,1$		
$\pm 2,8$ mg/100 ml	± 10 mg/100 ml		
VK = 1,06%	VK = 3,9%	VK = VC = 1.06%	3.9%

The determination of free cholesterol is also possible with the enzymatic colour test if the determination is carried out in the absence of cholesterol esterase.

Clinical interpretation

With the introduction of a new enzymatic test for cholesterol, the question naturally arises of how wide consequences for the clinical interpretation are.

In the enzymatic cholesterol determination, the results obtained in the epidemiological studies in Framingham can be accepted unconditionally since the 4-step method used in these studies following Kendall-Abell yields excellently correlating values with the new enzymatic test (Fig. 2). Cholesterol values determined only enzymatically, and thus specifically, can be placed in relation to the results of these studies, for it is now guaranteed that methodical differences between the values found following Kendall-Abell and the enzymatic method are eliminated.

As threshold value for an increased heart infarct risk, a value of 220 mg/100 ml applies, according to the results of the Framingham study.

As diagnostic precaution for the prevention of arteriosclerosis, the following is recommended according to present knowledge [24]:

Limiting ranges for "suspicious" and "high" measured values in the early recognition of risk factors:

Cholesterol: Suspicious from 220 mg/100 ml, high from 260 mg/100 ml.

On the basis of the investigation of sera of 489 samples, which were regarded as "healthy" based on clinical studies, normal (reference) ranges were determined with the test combination of cholesterol, enzymatic colour test (Table 2) [3].

A total of 465 employees of an industrial firm in the age group of 18-65 years were used for this study.

Table 2. Normal (reference) ranges determined for cholesterol in mg/100 ml and mmole/l in enzymatic determination as a function of age and sex.

	Männer	Alter	Frauen
mg/100 ml	100—230	bis 20	100—220
	130—250	21—30	130—240
	150—270	31—45	130—250
	160—280	46—60	150—280
	170—320	über 60	170—330
mmol/l	2,6—6,0	bis 20	2,6—5,7
	3,4—6,5	21—30	3,4—6,2
	3,9—6,9	31—45	3,4—6,5
	4,1—7,2	46—60	3,9—7,2
	4,4—8,3	über 60	4,4—8,6

Männer = men; Alter = age; Frauen = women; mg/100 ml = mg/100 ml; mmol/l = mmole/l; bis = up to; über = over

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The limiting ranges recommended by Schettler [24] are in part considerably below the normal values which are based on studies pre- 701
viously cited of a group considered clinically healthy. However, it is noted in these investigations that in samples used for a normal value determination in which manifest arteriosclerotic vascular illnesses are absent, this group could include persons whose arteriosclerosis eludes clinical detection and whose cholesterol level is therefore considered as belonging to the norm.

A conclusive statement of whether the samples used for the normal value determination were really healthy can be made exactly with risk factors of arteriosclerosis, dependent on the occurrence of corresponding pathological symptoms in the course of a certain life period, as followed in epidemiological long-term studies.

"Generally, it is considered that normal ranges established in

the highly industrialized countries are by no means identical with ideal ranges, as would be desirable to prevent vascular complications" [9].

Similar results were obtained [23] in statistical normal value determination following the semi-enzymatic method [15]:

men: 203-318 mg/100 ml cholesterol

women: 196-295 mg/100 ml cholesterol

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