Serum cholesterol. Comparative investigations on the reliability of various methods of determination

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SUMMARY

In the light of our own investigations and a review of the literature, we believe that thanks to their high degree of specificity, their good precision and accuracy, and the simple, non-hazardous manipulations involved in the routine test laboratory the enzymatic methods of cholesterol determination will displace direct photometric methods with their high susceptibility to interference. The availability of reliable test kits and the fact that enzymatic methods can be adapted to all automatic analyzers also plays a major role. The lower normal ranges of serum cholesterol obtained by enzymatic methods must be taken in stride as a temporary disadvantage.

Rudel and Morris' o-phthalaldehyde method or a gas chromatographic procedure are recommended as reference standards.

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Hypercholesterolemia occupies an important place among the arteriosclerosis risk factors. Reliable determination of blood cholesterol levels is therefore of eminent clinical importance.

Cholesterol is found in the blood at relatively high concentrations. Normally it is esterified with fatty acids to about 70%. Due to its poor water solubility, cholesterol is bound to lipoproteins, a fact which must be taken into consideration in its determination.

In view of the eminent diagnostic significance of cholesterol levels in the blood, numerous assays have been developed for the determination of total cholesterol, the parameter of foremost clinical interest. Table 1 summarizes the basic principles of the most important methods used in the determination of blood cholesterol levels. Most determinations are based on cholesterol color reactions or on its enzymatic conversion to 4-cholestenone. More recently gas chromatographic procedures are also drawn upon for the quantitative determination of cholesterol.

1. Photometric Methods

The numerous photometric methods are based on one of two principles: (1) the reaction of cholesterol in an acetic acid-acetic anhydride-sulfuric acid medium (Liebermann-Burchard reaction; 5, 12) and (2) the reaction of cholesterol with iron-(III)-chloride, acetic acid and sulfuric acid described by Zlatkis, Zak and Boyle (28). According to findings elicited in more recent investigations (6) both reactions give rise to resonance stabilized, polyunsaturated oxidation products of cholesterol: the Zlatkis reaction gives rise to a tetraenic cation (with $\lambda_{max}$ at 563 nm), the Liebermann-Burchard reaction to a pentaenic cation ($\lambda_{max}$ at 620 nm). The reaction scheme proposed by the authors (6) is illustrated in Fig. 1.

In 1969 Zlatkis and Zak (29) proposed a method using the reaction of o-phthalaldehyde with cholesterol dissolved in a sulphuric acid medium for the determination of cholesterol. The color reaction was used by Rudel and Morris (19) to determine blood cholesterol levels.
Table 1: Basic principles of the various methods for the determination of serum cholesterol.

<table>
<thead>
<tr>
<th>Jahr</th>
<th>Reagentien</th>
<th>Autoren</th>
</tr>
</thead>
<tbody>
<tr>
<td>1872</td>
<td>Chloroform + H₂SO₄</td>
<td>Salkowski (20)</td>
</tr>
<tr>
<td>1885</td>
<td>Essigsäureanhydrid + H₂SO₄</td>
<td>Liebermann (12)</td>
</tr>
<tr>
<td>1890</td>
<td>Chloroform + Essigsäureanhydrid + H₂SO₄</td>
<td>Burchard (5)</td>
</tr>
<tr>
<td>1953</td>
<td>p-Toluolsulfonsäure + Essigsäureanhydrid + H₂SO₄</td>
<td>Pearson (14)</td>
</tr>
<tr>
<td>1953</td>
<td>Essigsäure + Eisen-(II)-chlorid + H₂SO₄</td>
<td>Zlatkis et al. (28)</td>
</tr>
<tr>
<td>1960</td>
<td>Dimethylbenzolsulfonsäure + Essigsäure + H₂SO₄</td>
<td>Watson (28)</td>
</tr>
<tr>
<td>1969</td>
<td>o-Phthalaldehyd + Eisessig + H₂SO₄</td>
<td>Zlatkis, Zak (29)</td>
</tr>
</tbody>
</table>

2. Enzymatische Verfahren
- 1973: Verseifung/Cholesterinoxidase
- 1974: Cholesterinesterase/Cholesterinoxidase + Kageyama-Reaktion (11)
- 1974: Cholesterinesterase/Cholesterinoxidase + Trinder-Reaktion (24)
- 1976: Cholesterinesterase/Cholesterinoxidase + Katalase (Athanol) + Aldehyddehydrogenase

3. Gaschromatographische Verfahren
- 1960: Beerthuis, Recourt (4)

Table 2: Control of Accuracy (Precilip Control Serum)

<table>
<thead>
<tr>
<th></th>
<th>Enzymatisch</th>
<th>Enzymatisch</th>
<th>Watson</th>
<th>o-Phthalaldehyd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kageyama</td>
<td>PAP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sollwert</td>
<td>130</td>
<td>130</td>
<td>154</td>
<td>130</td>
</tr>
<tr>
<td>mg/100 ml</td>
<td>3.35</td>
<td>3.35</td>
<td>3.96</td>
<td></td>
</tr>
<tr>
<td>mmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mittelwert</td>
<td>127</td>
<td>131</td>
<td>146</td>
<td>130</td>
</tr>
<tr>
<td>mg/100 ml</td>
<td>3.27</td>
<td>3.39</td>
<td>3.77</td>
<td>3.35</td>
</tr>
<tr>
<td>mmol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abweichung des Mittelwerts vom Sollwert (%)</td>
<td>-2.3</td>
<td>+0.77</td>
<td>-5.2</td>
<td></td>
</tr>
</tbody>
</table>

A = enzymatic Kageyama; B = enzymatic PAP; 1 = theoretical value; 2 = mean value; 3 = deviation of mean value from theoretical value (%).
None of these color reactions is strictly specific; a major disadvantage is that they are so sensitive to humidity that even low amounts of humidity already interfere with the reaction. Moreover, all of these color reactions have the disadvantage that strongly caustic reagents, such as concentrated sulfuric acid, acetic anhydride or glacial acetic acid are used.

The first reliable photometric methods of determination required saponification of the cholesterol ester and subsequent extraction of total cholesterol before the color reaction could be carried out. The most frequently used indirect method, i.e. a method using saponification and extraction, is the classic method of Schoenheimer and Sperry (21) and Sperry and Webb (22) and the modification of Abell and Kendall (1) which is usually considered the standard method in the United States.

Attempts to obviate the time consuming saponification and extraction steps and to develop direct methods were already made at an early date. At first, the Liebermann-Burchard reaction was also utilized for this purpose, but the results were too unreliable. In 1953 - at about the same time - two new direct methods for the determination of cholesterol were published.

The method described by Zlatkis et al. (28), based on the reaction of cholesterol with iron-(III)-chloride, acetic acid and sulfuric acid, already mentioned above, has been modified repeatedly, but has not found general acceptance for the manual determination of cholesterol here. On the other hand, until recently the method for the determination of cholesterol first described by Pearson, Stern and McGavack (14), and later modified by Watson (26) was considered the method of choice. According to Watson, glacial acetic acid, dimethylbenzene sulfonate, acetic anhydride and sulfuric acid are added directly to the serum and the evolving color is measured at 550 nm. Dimethylbenzene sulfonate disperses the proteins, and acetic anhydride extracts the cholesterol, precipitates the proteins and assures an anhydrous milieu.
2. **Enzymatic Methods**

The use of specific enzymes for the determination of cholesterol represented a major advance. At first, Richmond (16) used a cholesterol oxidase from *Nocardia* sp. which oxidizes cholesterol to $\Delta^4$-cholestenone, a process in which hydrogen peroxide is formed. A prerequisite for this method is saponification of the cholesterol ester with ethanolic potassium hydroxide solution. Later Röschlau et al. (18) replaced saponification by enzymatic hydrolysis with a cholesterol esterase.

The following parameters may be used to determine the cholesterol concentration in the enzymatic methods (cf. Fig. 2):

a) **Measuring the increase in extinction at 240 nm:** Measuring the increase in extinction at 240 nm, due to the formation of cholestenone, is used mostly for the determination of cholesterol oxidase activity in the purified system (18).

b) **Measuring the hydrogen peroxide formed:** Measuring the hydrogen peroxide formed with the aid of a peroxidase acceptor system, a method which had already yielded good results in the enzymatic determination of blood sugar and uric acid, is particularly suitable for the enzymatic determination of cholesterol in routine laboratory analysis. At first, the Kageyama reaction (11), already developed for the enzymatic determination of uric acid, was used as acceptor: in this method hydrogen peroxide reacts with methanol to give rise to formaldehyde. The formaldehyde formed, reacted with acetylacetone in the presence of ammonia, gives a 3,5-diacetyl-1,4-dihydrolutidine, whose color intensity is measured at 405 - 415 nm (18). Furthermore, 4-aminoantipyrine and phenol (Trinder reaction, 29) may also be used as acceptor: this reaction gives rise to a stained quinone imine whose color intensity is measured at 500 nm (PAP reaction according to Allain et al., 2). Tarbutton and Gunter (23) recommend o-dianisidine as acceptor and measure the color intensity of the quinone imine formed at 450 nm. In a more recent study, Richmond (1?) used the reaction with titanium oxide, xylenol orange and sulfuric acid to determine the hydrogen per-

? = should probably read 16. Translator
Fig. 2:

1. *spektrofotometrische Verfahren* (12)

13. **Akzeptoren:**
   a) Methanol-Acetylaceton-Ammoniak (Kageyama-Reaktion)
   b) 4-Aminophenazon-Phenol (PAP)
   c) o-Dianisidin
   d) Titanoxid-Xylenolorange-H₂SO₄

14. 2. *fluorimetrische Verfahren*

15. **Akzeptor:**
   Homovanillinsäure

1 = cholesterol ester; 2 = cholesterol esterase; 3 = fatty acid; 4 = cholesterol oxidase; 5 = cholesterol + O₂; 6 = Δ¹-cholestenone + H₂O₂; 7 = polarography; 8 = measuring increase in extinction at 240 nm; 9 = measuring O₂ consumption; 10 = photometric test; 11 = peroxidase + acceptor; 12 = spectrophotometric methods; 13 = acceptors; 14 = fluorimetric methods; 15 = acceptor; 16 = homovanillic acid.
oxide produced; the color is measured at 550 nm. Huang et al. (8) react the hydrogen peroxide formed with homovanillic acid and measure changes in the fluorescence at 425 nm ($\lambda_{ex} = 315$ nm) for 5 – 6 min. Haeckel and Perlick (7a) have proposed the cholesterol oxidase reaction coupled with catalase and aldehyde-dehydrogenase for the quantitative determination of cholesterol concentrations. The most important advantage of this method – by comparison with other cholesterol oxidase methods – is the short reaction time and the use of NADP$^+$ which permits direct calculation of the cholesterol concentration from the absorbance values.

Clarke (quoted in 15) has recently outlined a method for the enzymatic determination of cholesterol in which the hydrogen peroxide produced is determined by polarography. In principle it is also possible to determine oxygen consumption directly by polarography.

3. **Gas chromatographic Methods**

Recently gas chromatographic methods have also been used for the determination of cholesterol (cf. 4,7,9,10,13). Although gas chromatographic methods are distinguished by their high specificity and accuracy, they are only of limited usefulness in routine tests.

**RELIABILITY OF CHOLESTEROL DETERMINATIONS IN AUSTRIA**

The reliability of cholesterol determinations in Austria is constantly checked by control experiments of the Austrian Society for Clinical Chemistry in collaboration with the Institute for Medical Chemistry of the University of Vienna. In each experiment, the laboratories participating in the control experiments receive two samples with different cholesterol concentrations; these are to be assayed under routine conditions. The results received are arranged according to methods (photometric determinations, enzymatic determinations) and evaluated statistically. First mean value, standard deviation and coefficient of variation are calculated. Subsequently, after values outside the 2s range of the entire collective have been eliminated, corrected characteristic values ($\bar{x}, s, VK$) are calculated. Fig. 3 shows first of all that the use of enzymatic methods of determination has been on the rise since the beginning of last year:
**Fig. 3:**

![Graph showing percentage change over time with months labeled in German.](image)

**Fig. 4:**

- **ENZYMATIC**
- **LIEBERMANN - BURCHARD**

![Bar chart showing comparison between ENZYMATIC and LIEBERMANN - BURCHARD methods with months labeled in German.](image)

Mai = May; 
Feb = February; März = March; Juni = June; Okt = October.
while only about 25% of the laboratories determined cholesterol enzymatically in the control experiment of February 1975, the proportion of enzymatic determinations in the total collective had risen to approximately 60 - 70% by September 1976. The increasing use of enzymatic methods is primarily attributable to the availability of reliable reagent kits.

Fig. 4 illustrates the coefficients of variation for enzymatic and non-enzymatic determinations for the period February 1975 to September 1976. Although one is struck by the fact that the reliability of the results in cholesterol determinations at first showed no principal improvement, and that the photometric, non-enzymatic methods originally yielded better results, the two last control experiments (June and September 1976) show improvement in the results obtained by both methods; no difference between non-enzymatic and enzymatic determinations was in evidence in the two last experiments.

RELIABILITY OF VARIOUS METHODS OF CHOLESTEROL DETERMINATION UNDER STANDARDIZED CONDITIONS

Since objective statements on the reliability of methods on the basis of these control experiments can only be made with some reservations, serum cholesterol was assayed in 163 serum samples by four different methods. Sera with high levels of cholesterol and sera with normal values from routine test laboratories were available for this comparison, and were assayed by the following methods:

1. Enzymatic color test in conjunction with the Kageyama reaction (Boehringer test kit).
2. Enzymatic color test in conjunction with the Trinder reaction (CHOD-PAP method; Boehringer test kit).
3. Photometric determination according to Watson (Boehringer test kit).
4. The o-phthalaldehyde method as modified by Rudel and Morris (24).

According to Richterich the following factors characterize the reliability of a method: specificity, accuracy, precision and practical performance. Below we shall discuss the results of our comparison of methods in the light of these criteria.
1. Specificity

With respect to their specificity enzymatic color tests are clearly superior to photometric, non-enzymatic methods because total cholesterol is present as free cholesterol after enzymatic hydrolysis, and cholesterol oxidase specifically only determines cholesterol. In Watson's (26) direct photometric determination other steroids present in the serum are to some extent codetermined as cholesterol. Furthermore, even low amounts of humidity in the sulfuric acid used will cause interference; and when strongly hemolytic or icteric sera are used the values obtained are too high. In the o-phthalaldehyde method no interfering substances are present in the hexane extract following ethanolic saponification and extraction with hexane. The only serum steroid that can cause any interference is dehydroepiandrosterone.

2. Accuracy

The deviation of a measuring value from the actual value of the sample is a measure of the accuracy of the method. We checked the accuracy of various methods with Boehringer's control serum 'Precilip'. For the enzymatic color tests the theoretical value of the control serum is $3.35 \text{ mmol/liter} (= 130 \text{ mg/100 ml})$, while a theoretical value of $3.96 \text{ mmol/liter} (= 154 \text{ mg/100 ml})$ is given for the Watson method. The results of our investigation have been compiled in Table 2. Contemplating the mean values obtained with the four different methods, one notes that the theoretical value of the control serum is attained by the enzymatic PAP color test. The mean value obtained with the enzymatic Kageyama color test is 2.3% below the theoretical value of the control serum. The mean value obtained with Watson's direct photometric method is 5.2% lower than the theoretical value of the control serum. The results of cholesterol determination with the o-phthalaldehyde method yield a mean value of $3.35 \text{ mmol/liter}$. This mean value is below the theoretical value given for the Watson method but is in agreement with the theoretical value given for the enzymatic procedures. It may therefore be concluded that the true cholesterol content of the sample can be detected by this expensive extraction method.

Table 3 lists the correlations (regression equation, scatter and coefficient of correlation) between the values obtained
Table 3: Correlation between the results of various methods of cholesterol determination related to the results the enzymatic determination (Kageyama).

<table>
<thead>
<tr>
<th>Methode</th>
<th>Regressionsgleichung</th>
<th>Streuung</th>
<th>Korrelationskoeffizient</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Phthalaldehyde enzymatisch (PAP)</td>
<td>$Y = 1,00 X - 5,2$</td>
<td>14,43</td>
<td>0,962</td>
</tr>
<tr>
<td>Liebermann-Burchard-Reaktion (direkte Methode n. Watson)</td>
<td>$Y = 0,98 X + 10,27$</td>
<td>13,58</td>
<td>0,965</td>
</tr>
</tbody>
</table>

A = method; B = regression equation; C = scatter; D = coefficient of correlation; 1 = enzymatic (PAP); 2 = Liebermann-Burchard reaction (direct method according to Watson).

Table 4: Day to day precision on ten test days.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Enzymatisch Kageyama</th>
<th>Enzymatisch PAP</th>
<th>Enzymatisch Watson</th>
<th>o-Phthalaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/100 ml mmol/l</td>
<td>mg/100 ml mmol/l</td>
<td>mg/100 ml mmol/l</td>
<td>mg/100 ml mmol/l</td>
</tr>
<tr>
<td>Precip</td>
<td>127</td>
<td>3,27</td>
<td>3,39</td>
<td>3,77</td>
</tr>
<tr>
<td>s</td>
<td>6,53</td>
<td>0,17</td>
<td>0,21</td>
<td>0,23</td>
</tr>
<tr>
<td>VK%</td>
<td>5,15</td>
<td>6,10</td>
<td>6,22</td>
<td>3,76</td>
</tr>
<tr>
<td>Patient 1</td>
<td>236</td>
<td>6,1</td>
<td>6,31</td>
<td>6,38</td>
</tr>
<tr>
<td>s</td>
<td>9,19</td>
<td>0,24</td>
<td>0,26</td>
<td>0,53</td>
</tr>
<tr>
<td>VK%</td>
<td>3,89</td>
<td>4,11</td>
<td>8,37</td>
<td>3,76</td>
</tr>
<tr>
<td>Patient 2</td>
<td>186</td>
<td>4,77</td>
<td>4,83</td>
<td>5,04</td>
</tr>
<tr>
<td>s</td>
<td>4,32</td>
<td>0,11</td>
<td>0,12</td>
<td>0,27</td>
</tr>
<tr>
<td>VK%</td>
<td>2,34</td>
<td>2,48</td>
<td>6,41</td>
<td>1,98</td>
</tr>
<tr>
<td>Patient 3</td>
<td>495</td>
<td>12,8</td>
<td>13,21</td>
<td>13,99</td>
</tr>
<tr>
<td>s</td>
<td>16,66</td>
<td>0,43</td>
<td>0,43</td>
<td>0,99</td>
</tr>
<tr>
<td>VK%</td>
<td>3,36</td>
<td>3,22</td>
<td>7,10</td>
<td>2,88</td>
</tr>
</tbody>
</table>

A = enzymatic method (Kageyama); B = enzymatic method (PAP).
with the different cholesterol determination methods, related to the enzymatic Kageyama color test.

3. Precision

To check the precision of the four methods, the control serum 'Precilip' and three patient sera were analyzed on ten consecutive working days, and the mean values, standard deviations and coefficients of variation calculated. The direct photometric determination according to Watson gave the highest scatter around the mean value over the entire range of cholesterol concentrations (3.27 - 12.8 mmol/liter or 127 - 495 mg/100 ml respectively).

The level of precision from day to day is evidently much higher in the results obtained by enzymatic determination, and those elicited with the o-phthalaldehyde method; this is true for the control serum as well as patient sera. Results have been compiled in Table 4.

4. Practical Performance

Contemplating the pipetting steps required for the various methods (cf. Table 4), one is struck by the simplicity of enzymatic cholesterol determination in conjunction with the PAP reaction: one pipetting of the serum and one other step: adding the reagent, is all that is required. Standard and blank sample values are dispensed with. At 37°C the incubation period is 15 min., and the color developed remains stable for 2 hrs. The method is also particularly suitable for automatic analyzers: Auto-Analyzer II (16), ABA-100 (13), Vickers D-300 (15), Centrifichem (39). Separate determination of free and esterified cholesterol is not feasible.

Two additional pipetting steps are required for the enzymatic color test used in conjunction with the Kageyama reaction: to wit, adding cholesterol oxidase to the sample and the blank sample value. The working instructions give a period of incubation of 60 min. at 37°C. Yet, experience has taught us that a period of incubation of 75 min. is required to achieve exact values. The use of a standard is dispensed with. The concentration of free cholesterol can be determined by altering the medium slightly.
Since the reagent and sulfuric acid have to be added to the sample, Watson's direct cholesterol determination requires three pipetting steps. Concentrated sulfuric acid must be added to develop the color; as a result the medium is heated. To keep this process as constant as possible, the medium must be mixed and cooled at once. The use of caustic reagents, interference by hemoglobin and bilirubin, the fact that the analysis depends on the degree of purity of the sulfuric acid, and the low specificity, are major disadvantages of this method.

Despite its sensitivity and low susceptibility to interference, in view of the cumbersome manipulations required, the o-phthalaldehyde method of Rudel and Morris (19) is not a suitable technique for routine tests, and remains the preserve of the research laboratory.

**************

REFERENCES

Translation of foreign literature titles:

3. Serum cholesterol. Comparison between an enzymatic method of determination and the extraction method on the Automatic Analyzer II.

5. A contribution on cholesterols.

7. Gas chromatographic determination of serum cholesterol.


18. Enzymatic determination of total serum cholesterol.

20. Reacting cholesterol with sulfuric acid.

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