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A New Enzymatic Determination of Cholesterol

The Use of Aldehyde Dehydrogenase to Measure H_2O_2 Producing Reactions, II.

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Summary: Cholesterol oxidase, coupled with the catalase and aldehyde dehydrogenase, is proposed for the determination of cholesterol. The main advantages of this procedure over comparable methods employing cholesterol oxidase are its short reaction time and the use of NADP which permits the direct calculation of the cholesterol concentration from the absorbance value.

Neue enzymatische Methode zur Bestimmung der Cholesterinkonzentration. Verwendung von Aldehyddehydrogenase zur Indikation H_2O_2-bildender Reaktionen, 2. Mitteilung
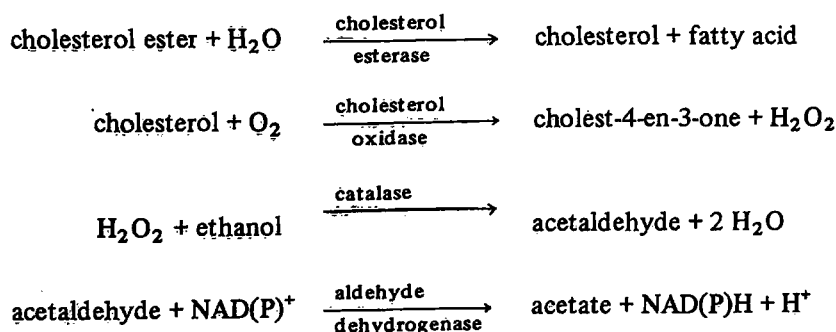
Zusammenfassung: Die mit Katalase und Aldehydrogenase gekoppelte Cholesterinoxidase-Reaktion wird zum Nachweis der Cholesterinkonzentration vorgeschlagen. Die wichtigsten Vorteile dieses Verfahrens gegenüber vergleichbaren Cholesterinoxidase-Methoden sind eine kurze Reaktionszeit und der Einsatz von NADP, der eine direkte Berechnung der Konzentration aus den Absorptionswerten ermöglicht.

Introduction

Recently we have proposed the use of catalase coupled with aldehyde dehydrogenase to measure the action of H_2O_2 producing oxidoreductases (1, 2). Meanwhile this principle has been extensively evaluated for the determination of uric acid in human serum and urine samples (3). The main advantages over comparable methods are: the reaction is completed in a few minutes and the use

of NAD+ or NADP+ (3) permits the direct calculation of the substrate concentration from the absorbance value without reference to a standard solution. This is especially relevant if the preparation of primary standard solutions is problematical.

In the following study the cholesterol concentration was determined with cholesterol oxidase (4) using the aldehyde dehydrogenase principle:

$$\text{cholesterol ester} + H_2O \xrightarrow[\text{esterase}]{\text{cholesterol}} \text{cholesterol} + \text{fatty acid}$$

$$\text{cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{cholesterol}} \text{cholest-4-en-3-one} + H_2O_2$$

$$H_2O_2 + \text{ethanol} \xrightarrow{\text{catalase}} \text{acetaldehyde} + 2\,H_2O$$

$$\text{acetaldehyde} + NAD(P)^+ \xrightarrow[\text{dehydrogenase}]{\text{aldehyde}} \text{acetate} + NAD(P)H + H^+$$

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Materials

Most reagents required are listed in table 1. Cholesterol, Moni-Trol I and II were obtained from Merz & Dade (D-8000 Munich), Precilip from Boehringer Mannheim (D-6800 Mannheim), Seronorm from Molter GmbH (D-6900 Heidelberg) and Hyland control serum from Travenol GmbH (D-8000 Munich).

Reagents

1. *KCl-diphosphate buffer* (pH = 8.5): dissolve 3.75 g KCl (Merck No. 4936) and 22.25 g tetrasodium-diphosphate-10-hydrate (Merck No. 6591) in approximately 300 g/l glycerol, adjust the pH-value to 8.5 with HCl and add 300 g/l glycerol to 1000 ml.

2. *300 g/l glycerol*: 300 g glycerol p. a. (87%, Merck No. 4094) and bidist. H_2O to 1000 ml.

3. *NAD⁺ or NADP⁺ solution* (10 g/l): 100 mg NAD⁺ (Boehringer Mannheim No. 127 329) or 100 mg NADP⁺ (Boehringer Mannheim No. 127 353) and bidist. H_2O to 10 ml.

4. *Reaction mixture* (according to tab. 1). All reagents can be mixed together: 500 ml KCl-diphosphate buffer, 50 ml ethanol, 50 ml NADP solution, 0.5 ml catalase, 50 mg aldehyde dehydrogenase, 10 ml cholesterolesterase, 1 ml Triton X 100 (5 ml cholesteroloxidase). The reaction mixture should be prepared immediately before use; it is stable approximately 6 hours at 4°C. The final concentrations of the reaction mixture are listed in table 1.

The aldehyde dehydrogenase can also be obtained from Boehringer Mannheim (D-6800 Mannheim).

Tab. 1. Final concentrations in the assay for the determination of the cholesterol content of human sera.

$Na_4P_2O_7 \cdot 10\ H_2O$ (Merck No. 6591)		45	mmol/l
KCl (Merck No. 4936)		45	mmol/l
NADP⁺ (Boehringer Mannheim No. 127 353)		1.35	mmol/l
Glycerol (Merck No. 4094)	approx.	2.5	mol/l
Ethanol (Merck No. 972)		1.54	mol/l
Catalase (Boehringer Mannheim No. 106 810) Hydrogen Peroxide: Hydrogen Peroxide Oxido-reductase (EC 1.11.1.6)		910	kU/l
Cholesterol oxidase (EC 1.1.3.6) (Boehringer Mannheim No. 126 934)		400	U/l
Cholesterol esterase (Boehringer Mannheim No. 161 772)		40	mg/l
Triton X-100 (Serva No. 37240) (Octylphenol-Polyethyleneglycolether)		1	ml/l
Aldehyde: NAD(P) oxidoreductase (EC 1.2.1.5) (K⁺-activated from Baker's yeast, Sigma No. A 6758)		500	U/l
pH-value		9.5	

Methods

Manual assay procedure: extrapolation method using a recorder

Reaction mixture (without cholesteroloxidase)	1000 μl (2000 μl)
Sample volume (serum)	10 μl　(20 μl)

Mix; record absorbance for 1–2 minutes at 334 or 340 nm (at room temperature)

Cholesterol oxidase	10 μl　(20 μl)

Mix; record absorbance after reaction has come to the end and extrapolate to absorbance value before the addition of cholesterol oxidase (A_1)

Cholesterol oxidase	10 μl　(20 μl)

Mix and read absorbance difference (A_2) immediately.

Calculation: $(A_1 - A_2) \cdot 16.5$ (mmol/l)[1].

In each series the cholesterol oxidase absorbance (A_2) has to be determined just once. This value can be used for all samples; it can usually be neglected.

Mechanized procedure (or for manual measurements in series): sample blank method (if necessary multiply all volumes by any factor required)

	A_1	A_2
Reaction mixture (with cholesterol oxidase)	500 μl	
Reaction mixture (without cholesterol oxidase)		500 μl
Sample	5 μl	5 μl

Mix; read absorbance A_1 against A_2 after 20 minutes (room temperature)

Calculation: $(A_1 - A_2) \cdot 16.34$ (mmol/l)[1]

For the manual procedure instruments from Eppendorf Gerätebau GmbH (D-2000 Hamburg) were used: System 5085 with Phillips recorder. The mechanized procedure was performed with an Eppendorf endpoint analyzer 5030.

For an intermethod comparison the original procedure of *Abell* et al. (6) was applied and referred to the cholesterol standard solution from Merz & Dade (D-8000 Munich).

Regression analysis: the parameters a (intercept with the abscissa) and b (slope of the regression line) were calculated with *Deming's* procedure which considers the random errors of the two methods compared (7).

Results

Reaction velocity

Under the conditions listed in table 1, the reaction is completed after approximately 5 minutes in a standard solution, after 8 minutes in a control or patient serum with a "normal" cholesterol concentration and after 12–14 minutes in sera with an elevated cholesterol content at room temperature (fig. 1). The endogenous reaction observed in the reaction mixture is independent of the sample. It must be accounted for either by the extrapolation or by the blank subtraction procedure.

Precision

The precision data are listed in table 2. They are satisfactory with respect to the requirements of the College of American Pathologists (precision from day to day: coefficient of variation less than 4.9%) and the Guidelines of the Medical Society of West Germany for Statistical Quality Control and Collaborative Surveys (precision from day to day: coefficient of variation less than 10%) (8, 9).

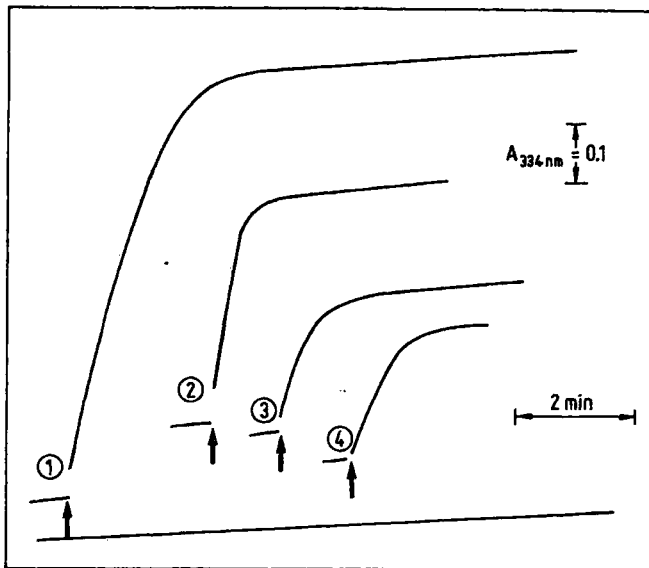[1]) Coefficient of absorbance: $\varepsilon_{334\,nm} = 6.18 \cdot 10^6\ cm^2/mol$ (4).

Fig. 1. The reaction kinetics of the procedure proposed for the determination of the cholesterol concentration at room temperature (21 °C). The reaction was started by adding cholesterol oxidase as indicated by arrow. (1) human serum with an elevated and (2) with a normal cholesterol concentration; (3) cholesterol solution (Merz & Dade, no cholesterolester present); (4) Precilip (Boehringer Mannheim, containing cholesterol ester).
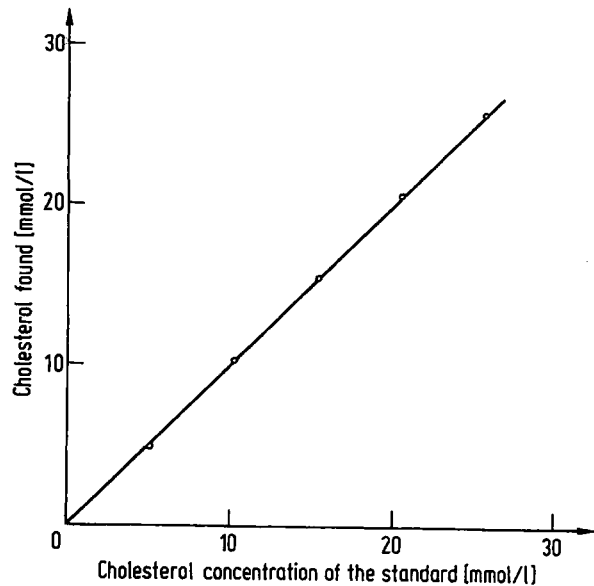

Fig. 2. Relation between cholesterol concentration of standard solutions (Preciset from Boehringer Mannheim) and the results found by the method proposed. Each point is a mean of 2 determinations. Concentrations above 10 mmol/l were obtained by adding 20 or 50 μl (instead of 10 μl) of the sample to the reaction mixture. In these cases, the alteration of the end volume was taken into consideration. Regression analysis: y = 1.01 x − 0.24, $\bar{x}$ = 15.5, $\bar{y}$ = 15.4, r = 0.999, n = 5.

Tab. 2. Precision from day to day of the determination of the cholesterol concentration in various control sera.

Control serum (batch No.)	assigned value (range)	$\bar{x}^{1)}$	s	CV	n
Precilip (440 A)	3.4 (3.05−3.73)	3.01	0.13	4.4	13
Cholesterol (CT − 271)	4.76 (4.51−5.01)	4.66	0.33	7.1	11
Seronorm (126)	2.07 (1.81−2.22)	2.03	0.06	2.9	12
Hyland (4 A 1)	4.3	3.65	0.12	3.4	8
Moni-Trol I (LTD 130 A, B)	3.52 (3.16−3.88)	3.26	0.11	3.5	9
Moni-Trol II (RTD 35 A, B)	3.1 (2.78−3.38)	2.56	0.07	2.7	9

[1]) mean value (mmol/l) with standard deviation, coefficient of variation and number of contributing values.

Tab. 3. The effect of diluting patients' sera on the cholesterol concentration determined with the method described.

Serum No.	cholesterol (mmol/l) undiluted	diluted 1 + 1	1 + 2
1	10.8	−	10.8
2	7.2	−	7.4
3	13.3	−	13.6
4	10.6	10.9	10.4
5	9.9	10.0	10.2
6	10.7	10.8	10.8
7	12.6	13.8	13.7

amount of oxygen dissolved in the final reaction mixture is sufficient for a serum concentration of 23 mmol/l cholesterol.

Intermethod comparison

The cholesterol concentration was determined in several sera randomly selected from hospital patients with the method proposed above using an endpoint analyzer 5030 and the manual procedure of Abell et al. (6). In recent years many authors have used this method as a reference procedure for the determination of the cholesterol concentration in human sera.

The results obtained show a good correlation (fig. 3). The intercept of the regression line on the abscissa can be explained as a positive bias in Abell's method according to Allain et al. (10). Several authors reported similar

Linearity

The linear range for the method described was verified with solutions of cholesterol for values up to 20 mmol/l using an Eppendorf analyzer 5030 (fig. 2). No attempt to find the limit of linearity by applying higher concentrations was undertaken. These data are not very meaningful, since the esterase reaction is not considered. Therefore, patients' sera with a relatively high cholesterol content were diluted and reanalyzed (tab. 3). No significant deviation from linearity was observed up to a concentration of 11 mmol/l total cholesterol. The theoretical
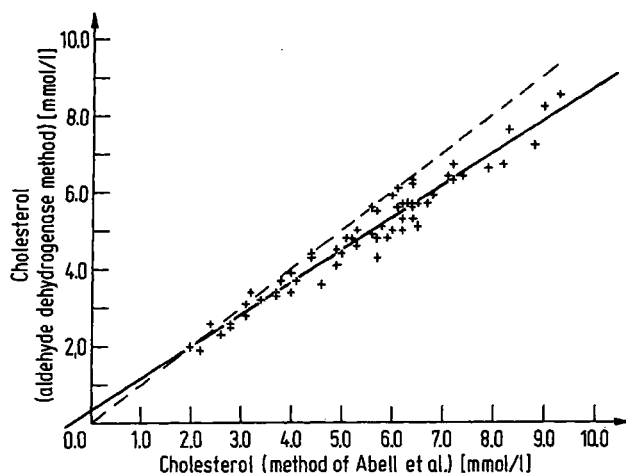
Fig. 3. Comparison of the cholesterol concentration determined with the method described (aldehyde dehydrogenase method) and with that of *Abell* et al. in various patients' sera. Regression analysis: $\bar{x}$ = 5.42 ± 1.75, $\bar{y}$ = 4.85 ± 1.50, n = 64; classical procedure: y = 0.84x + 0.32; *Deming's* procedure: y = 0.86x + 0.19; dotted line: y = x.

Interferences

In 2 recent studies 52 representative drugs commonly used were investigated for possible interferences with another cholesterol oxidase method (13) and with a procedure which also applies the catalase-aldehyde dehydrogenase reaction sequence to determine the uric acid concentration in biological materials (3). In the latter assay the sample volume is diluted 1 + 10, whereas the procedure described above uses a 1 + 100 sample dilution. Therefore, no interference is to be expected from all 52 drugs studied in 1. c. (3) and (8).

A high absorbance of the sample matrix could limit the photometric reading at 340 nm or Hg 334 nm. This limit is not reached by a bilirubin concentration of 680 μmol/l, a hemoglobin concentration of 25 g/l or a triglyceride concentration of 20 mmol/l.

As already mentioned several non-cholesterol sterols react with cholesterol oxidase (4, 10, 11), and therefore interfere with all methods employing this enzyme.

Homogentisic acid (above 25 mg/100 ml) or high activities of alcohol dehydrogenase can disturb the cholesterol determination with the present indicator system. The former interference can be avoided by lowering the pH-value of the reaction mixture, the latter one by using NADP as coenzyme (3). However, these concentrations are not expected to occur in human sera.

results when comparing an enzymatic procedure with that of *Abell* et al. (4, 10, 11). This effect is caused by differing reactivities of naturally occurring non-cholesterol serum sterols. The normal serum content of these sterols leads to an average 0.5 mmol/l-error (20 mg/ 100 ml) in enzymatic procedures using cholesterol oxidase and an average 1.0 mmol/l error (38 mg/100 ml) in the *Abell* procedure (10, 11).

Detection limit

The detection limit determined by a series of 15 cholesterol-free samples according to *Kaiser* (12) was 0.2 mmol/l.

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