

## Determination of cholesterol in human serum by an enzymatic method with the application of DAOS reagent

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A sensitive and specific enzymatic method has been developed for the determination of the total and free cholesterol in human serum based on the application of the newly-synthesized *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, (DAOS) Trinder's reagent. Using the proposed method, cholesterol could be determined in the concentration range of 0–15 mmol/L with a relative standard deviation of up to 1.1%. In order to find the optimal experimental conditions for the application of the DAOS reagent, the influence of its concentration on the linearity of the method, the influence of pH, as well as the influence of the activities of cholesterol esterase and cholesterol oxidase were examined. The obtained results of the determination of the total and free cholesterol were compared to the results obtained by the application of the most frequently employed enzymatic method in clinical practice, based on phenol as a reagent. The sensitivity of the method was 0.070 A/mmol/L, the limit of detection  $DL=0.03$  mmol/L and the limit of quantification  $QL=0.09$  mmol/L of cholesterol.

**Keywords:** cholesterol, enzymatic determination, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (DAOS) - modified Trinder's reagent.

### INTRODUCTION

Cholesterol is an important component of cellular membranes and a precursor of physiological products, steroidal hormones and bile acids. It is found in plasma partially in the free form, but mostly esterified with fatty acids.

The most commonly employed methods for cholesterol determination can be divided into chemical<sup>1–4</sup> and enzymatic.<sup>5–8</sup> The chemical methods are based on cholesterol oxidation in an acid medium with the addition of iron salts (Salkowski reaction) or by the application of the Lieberman-Burchard<sup>9</sup> reagent in non-aqueous solutions. The drawback of these methods, is that they are not specific. In order to raise the specificity of the cholesterol determination methods, more attention has been paid to the development of enzymatic methods.

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The enzymatic methods are based on the hydrolysis of cholesteryl esters at C-3 into free cholesterol and fatty acids under the influence of the enzyme cholesterol esterase (CHE). In addition, cholesterol reacts with  $O_2$ , catalysed by cholesterol oxidase (CHOD), resulting in cholest-4-ene-3-one and  $H_2O_2$ .<sup>9</sup>

The presented reactions are common to all enzymatic methods. However, the methods differ in the way the reaction products, usually hydrogen peroxide, are measured. The suggested method uses the formation of a chromogen by the oxidative condensation of the hydrogen donor (DAOS) with a coupling reagent 4-aminoantipyrine (4-AA) and hydrogen peroxide, in the presence of peroxidase (POD). The advantage of the application of DAOS lays in the fact that the formed chromogen has a bathochromic shift of the absorption maximum at 591.7 nm compared to the method that uses phenol instead of DAOS, as a hydrogen donor, when the formed chromogen has an absorption maximum at 500 nm. The most frequent interfering substances in serum, like bilirubine, the products of hemolysis and serum lipemia, absorb slightly at 591.7 nm and so, with the choice of DAOS, any interferences in the course of the determination of cholesterol are annulled.

#### EXPERIMENTAL

##### *Apparatus*

The absorbance measurements were made using a CINTRA 40, double-beam UV-VIS spectrophotometer, GBC (Australia) with a thermostat unit  $\pm 0.01$  °C. The pH measurements were made with a Corning 250 pH-meter with an Orion sure-flow electrode (USA). An ABBOTT - Spectrum (USA) diode array automatic analyzer was used for determination of cholesterol.

##### *Reagents*

Redistilled water and analytical grade reagents were used for the preparation of the solutions. Cholesterol standard (3 $\beta$ -hydroxy-5-cholestene), cholic acid Na salt, cholesterol esterase (CHE), cholesterol oxidase (CHOD), peroxidase (POD), were products of Serva (FRG); PIPES (1,4-piperazinediethanesulfonic acid), cholesteryl linoleate, hydroxypolyethoxydodecane were products of Sigma (USA); *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, sodium salt (DAOS) were obtained from DOJINDO, Lab (Japan); hydrogen peroxide from Zorka - Šabac (Yugoslavia). The control serum with a declared amount of cholesterol was a product of Boehringer Mannheim-Precinorm (Austria). A serum pool with a known amount added of added cholesterol was used for the determination of the precision of the method.

The composition of the reagent for the determination of the CHOD activity was CHOD 75 U/L, 150 U/L, 300 U/L, 600 U/L, PIPES buffer pH 6.9, 50 mmol/L; DAOS, 0.2 mmol/L, POD 2.0 KU/L, 4-AA, 0.3 mmol/L; hydroxypolyethoxydodecane, 0.2 %. A primary standard solution of cholesterol with a concentration of 15.6 mmol/L was used as the analyte. An amount of 10  $\mu$ L of the sample was added into 1.00 mL of the reagent and the absorbance was measured at 592 nm using the reagent solution as a blank at the temperature of 37.0 °C.

The composition of the reagent for the determination of the CHE activity was CHE 500 U/L, 250 U/L, 125 U/L, 67 U/L; CHOD 1000 U/L; PIPES buffer pH 6.9, 50 mmol/L; DAOS 0.2 mmol/L; POD 2 KU/L; 4-AA 0.3 mmol/L; hydroxypolyethoxydodecane, 0.2%. Cholesterol linoleate at a concentration of 15.0 mmol/L was used as the analyte. The same analytic procedure was used as for the determination of CHOD activity.

The composition of the reagent for determination of the total cholesterol was PIPES buffer pH 6.9 mmol/L; DAOS, 0.2 mmol/L; CHOD, 300 U/L; CHE, 250 U/L; 4-AA, 0.3 mmol/L; POD, 2 KU/L; Na-holat, 10 mmol/L; hydroxypolyethoxydodecane, 0.2 %.

The reagent for the determination of free cholesterol contained all the components listed for the reagent for the total cholesterol determination, except CHE.

#### *Analytical procedure*

The total cholesterol was determined using a serum sample of 10  $\mu\text{L}$  volume which was added into 1 mL of the reagents. The solution absorbance was measured at 592 nm after 5 min incubation at the temperature at 37  $^{\circ}\text{C}$  using reagent solution as the blank.

The free cholesterol and the total cholesterol in the serum were determined using the same analytical procedure.

### RESULTS AND DISCUSSION

In order to apply DAOS as an analytical reagent for cholesterol determination, the absorption spectra of DAOS and the chromogen were recorded at pH 6.9. The

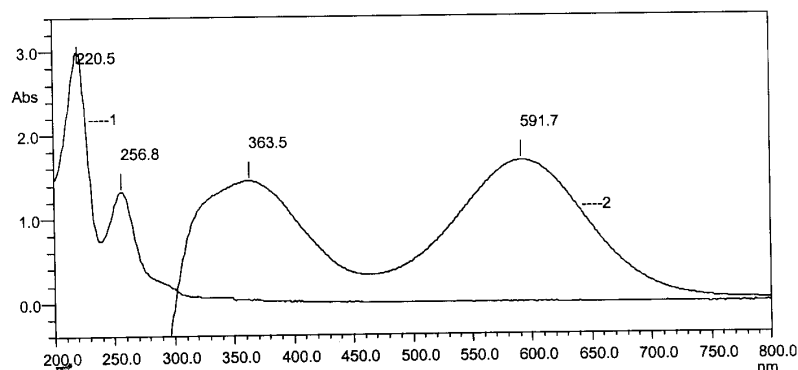


Fig. 1. Absorption spectra of DAOS (curve 1;  $1 \times 10^{-4}$  mol/L, pH 6.9) and the chromogen (curve 2;  $1 \times 10^{-4}$  mol/L, pH 6.9).

obtained spectra of DAOS (Fig. 1, curve 1) has two absorption bands with maxima at 220.5 nm and at 256.8 nm, while the maxima of the chromogens are bathochromically shifted at 363.5 nm and to 591.7 nm (Fig. 1, curve 2). The molar absorptivity of the chromogen at 591.7 nm was  $1.7 \times 10^4$  L mol $^{-1}$  cm $^{-1}$ . The influence of pH on the formed chromogen was examined in the pH region 6.0–8.0, since most enzymes important in the determination of cholesterol have the highest stability and activity

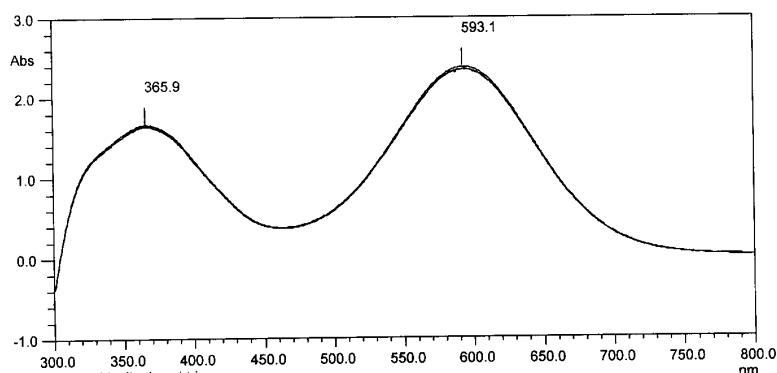


Fig. 2. Absorption spectrum of the chromogen ( $1 \times 10^{-4}$  mol/L) in the pH region 6.0–8.0.

in this pH range. The obtained spectra (Fig. 2) show that there are no significant changes in the positions of the maxima of the absorptions bands, nor in the intensity of the absorption. PIPES buffer pH 6.9 was selected as the most suitable for the biological material in this pH region.

It was experimentally shown that using a DAOS concentration of  $2 \times 10^{-4}$  mol/L, cholesterol could be optimally determined in accordance with Beer's law in the cholesterol concentration range of 0–15 mmol/L.

#### Kinetic studies

CHOD and CHE were kinetically studied for the rate of the hydrolysis reaction of cholesteryl esters, as well as for the rate of cholesterol oxidation.

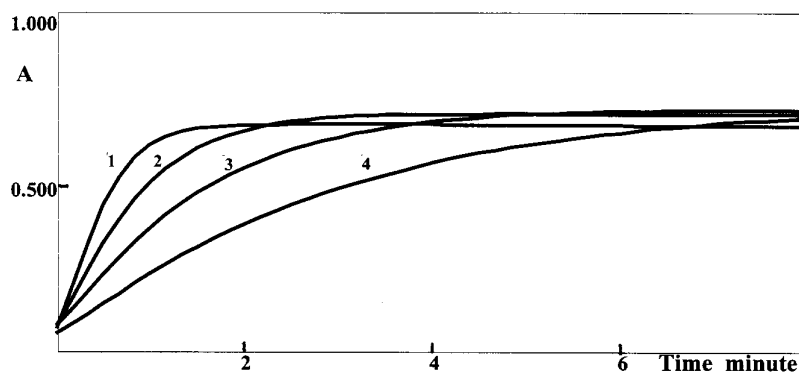


Fig. 3. The influence of the CHOD activity on the rate of cholesterol oxidation (CHOD activity: curve 1–600 U/L, curve 2–300 U/L, curve 3–150 U/L, curve 4–75 U/L).

*Determination of the optimum CHOD concentration.* The influence of CHOD activities on the rate of cholesterol oxidation up to cholest-4-en-3-one is shown in Fig. 3.

The CHOD activity in the analytical reagent for cholesterol determination ranged from 75 U/L to 600 U/L, while the concentrations of the other components

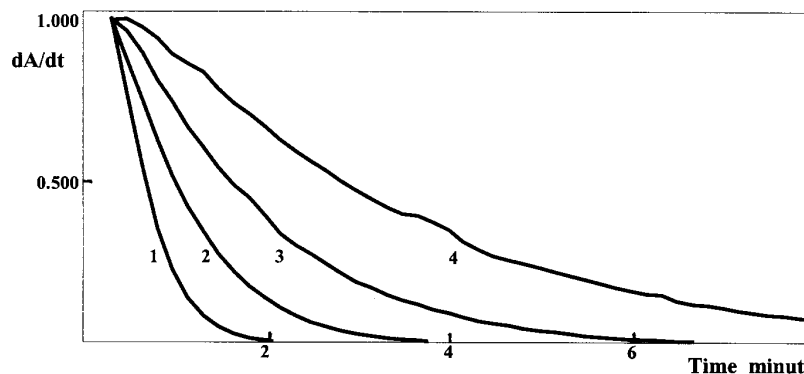
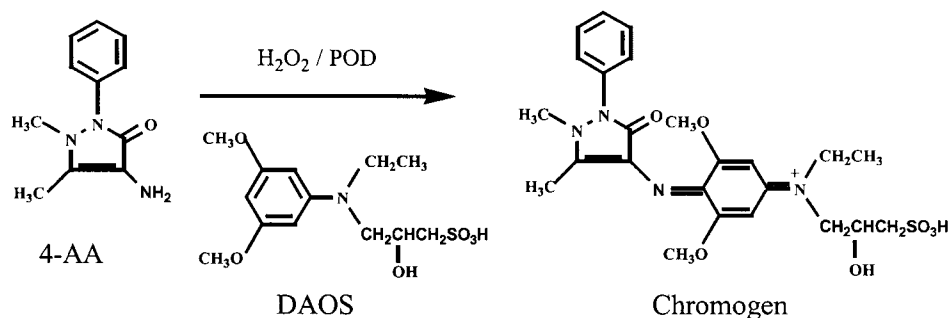
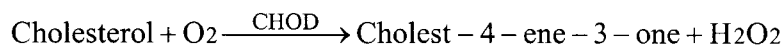
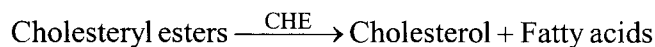


Fig. 4. CHOD activity shown as  $dA/dt$  vs. time (curve 1–600 U/L; curve 2–300 U/L; curve 3–150 U/L; curve 4–75 U/L).

were the same. The reaction was considered as finished when there were no changes in the absorptions during a certain time interval, *i.e.*, when  $dA/dt \rightarrow 0$ .

In further work with the cholesterol determination reagent, an incubation time of 5 minutes with a CHOD activity of 300 U/L was used.

*Determination of the optimum CHE concentration.* CHE hydrolyses the ester bond in the position 3 of cholesterol with the formation of cholesterol and fatty acids. Hydrolysis of cholesteryl esters and the reaction of the cholesterol and the formation of the chromogen are shown in the following equations:



If the rate of cholesteryl esters hydrolysis is given by  $v_1$ , and the rate of cholesterol oxidation by  $v_2$ , then  $v_2$  must be greater than  $v_1$  in order to oxidize cholesterol quantitatively. Practically, this means that when determining the total cholesterol, the CHOD activity in the reagent must not be less than the CHE activity. The initial CHE activity in the determination of the optimum CHE activity was 500 U/L. In a sequence of solutions, the CHE activity was continually decreased (250 U/L, 125 U/L and 67 U/L), while the concentrations of other reagent components remained constant. Since the CHOD activity (1000 U/L) was greater than the CHE activity, the rate of the reaction depended only on the CHE activity.

Cholesteryl linoleate was used as the substrate for the study of the influence of the CHE activity on the rate of hydrolysis, and the same analytical procedure was applied as in the determination of the influence of CHOD activity. It was experimentally determined that the optimum CHE concentration is 250 U/L. Thus, in the subsequent work, for the determination of the total and free cholesterol, CHOD and CHE with activities of 250 U/L were used.

The determination of the total and the free cholesterol was performed using a calibration curve obtained by the determination of the cholesterol in a series of standard solutions of cholesterol in the concentration range 2.6–15.6 mmol/L of cholesterol ( $y = 0.070x + 0.001$ ;  $r = 0.99997$ ,  $n = 7$ ). On basis of the obtained calibration curve, the total and free cholesterol were determined in samples from 35 patients.

In order to check the obtained results, in the same samples of human serum, the total and free cholesterol were determined by the standard enzymatic method based on the use of phenol as the hydrogen donor instead of DAOS.

The obtained results indicated a high level of correlation ( $a = 0.022$ ,  $b = 0.9992$ ,  $r = 0.9993$ ) for the total cholesterol and for the free cholesterol ( $a = 0.016$ ,  $b = 0.9992$ ,  $r = 0.9987$ ).

The precision of the method was tested by a recovery procedure. A known amount of the standard cholesterol solution was added into the serum pool. The obtained results of the cholesterol determinations are shown in Table I.

TABLE I. Application of the proposed method to the analysis of the total cholesterol

Added (mmol/L)	Found <sup>a</sup> (mmol/L)	Recoveries $\pm$ RSD (%)
3.00	2.91	97 $\pm$ 1.1
6.00	6.11	102 $\pm$ 0.9
9.00	9.10	101 $\pm$ 0.7

<sup>a</sup>Mean value of five determinations

#### *Sensitivity, detection limit and quantification limit*

The sensitivity of the method was determined on the basis of the calibration curve ( $a = 0.070$  A/mmol/L) while the detection limit,  $DL = 0.03$  mmol/L and the quantification limit,  $QL = 0.09$  mmol/L were calculated using the recommendations of IUPAC.<sup>10</sup>

The suggested method is simple, fast and, owing to the reduction of the interference of the serum, more accurate when compared to the method used so far for the determination of cholesterol in human serum.

### ИЗВОД

#### ОДРЕЂИВАЊЕ ХОЛЕСТЕРОЛА У ХУМАНОМ СЕРУМУ ЕНЗИМСКОМ МЕТОДОМ ПРИМЕНОМ DAOS РЕАГЕНСА

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Предложена је осетљива и специфична ензимска метода за одређивање укупног и слободног холестерола у хуманом серуму, заснована на примени новосинтетисаног Trinder-овог реагенса, *N*-етил-*N*-(2-хидрокси-3-сулфопропил)-3,5-диметоксианилин (DAOS). Холестерол се предложеном методом може одређивати у концентрационом опсегу од 0–15  $\mu$ mol/L са релативном стандардном девијацијом до 1,1 %. У циљу изналажења оптималних услова за примену DAOS реагенса, испитан је утицај његове концентрације на линеарност методе, затим утицај pH, као и утицај активности холестеролестеразе и холестеролоксидазе. Добијени резултати одређивања укупног и слободног холестерола поређени су са резултатима добијеним применом најчешће коришћене ензимске методе у клиничкој пракси, базиране на фенолу као реагенсу. Осетљивост методе је 0.070 A/mmol/L, граница детекције износи  $DL=0,03$  mmol/L и граница квантификације  $QL=0,09$  mmol/L холестерола.

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