[Chem. Pharm. Bull. 28(10)3131—3134(1980)]

A Sensitive Enzymatic Method for the Fluorimetric Assay of Cholesterol in Serum and High-Density Lipoprotein

CHIZUKO HAMADA, MASATAKE IWASAKI,[1a] YUTAKA MIBUCHI,[1b] KIYOSHI ZAITSU and YOSUKE OHKURA[1c]

Daiichi College of Pharmaceutical Sciences,[1a] Japan Medical Laboratory-Kyushu,[1b] and Faculty of Pharmaceutical Sciences, Kyushu University 62[1c]

(Received May 19, 1980)

A highly sensitive fluorimetric method is described for the enzymatic assay of free and total cholesterol in serum and of cholesterol in serum high-density lipoprotein. Cholesterol is oxidized by means of the cholesterol oxidase-mediated reaction to give hydrogen peroxide, which is measured by a fluorimetric technique using the peroxidase-tyramine system. Cholesterol ester is hydrolyzed by means of the cholesterol ester hydrolase-catalyzed reaction. High-density lipoprotein is separated by the dextran sulfate–Mg^{2+} method. The method is simple, with good precision, and is extremely sensitive, requiring less than 1 μl of serum for free or total cholesterol assay and less than 5 μl of serum for high-density lipoprotein cholesterol assay.

Keywords——free and total cholesterol in serum; cholesterol in high-density lipoprotein; fluorimetry; enzymatic assay; cholesterol oxidase-peroxidase system; cholesterol ester hydrolase; tyramine; dextran sulfate-magnesium ion method; ultramicro analysis

Several colorimetric and fluorimetric methods have been reported for the enzymatic assay of free and total cholesterol (total of free and esterified forms) in serum. These methods use the cholesterol oxidase(COD)-catalyzed reaction; hydrogen peroxide generated is determined by the peroxidase(POD)-mediated oxidation of a chromogenic[combination of 4-aminoantipyrine with phenol,[2] *p*-hydroxybenzoic acid[3] or *p*-(N,N-diethyl)-aniline,[4] or benzidine derivatives such as *o*-anisidine[5]] or fluorogenic (homovanillic acid[6]) substrate, or by the catalase-mediated conversion of methanol to formaldehyde, which is measured by means of the Hantzsch reaction.[7] In the assay of total cholesterol, the ester is subjected to the cholesterol ester hydrolase(CEH)-catalyzed reaction. Colorimetric methods have been used for the assay of cholesterol in serum high-density lipoprotein(HDL),[8–10] but no fluorimetric method has so far been reported.

Recently, we found that tyramine was a much more effective fluorogenic substrate for POD than homovanillic acid when used in the COD-POD system. This paper describes a fluorimetric method for the precise assay of free and total cholesterol in only 1 μl of serum and in HDL separated from 5 μl of serum by the dextran sulfate—Mg^{2+} method,[11] based on the above finding.

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1) Location: *a) Tamagawa, Minami-ku, Fukuoka, 815, Japan*; *b) Ogura Kasuga-shi, 816, Fukuoka, Japan*; *c) Maidashi Higashi-ku, Fukuoka, 812, Japan*. To whom correspondence should be addressed.
2) C.C. Allain, L.S. Poon, C.S.G. Chan, W. Richimond, and P.C. Fu, *Clin. Chem.*, **20**, 470 (1974).
3) F. Meiattini, L. Prencipe, F. Bardelli, G. Giannini, and P. Tarli, *Clin. Chem.*, **24**, 2161 (1978).
4) G.S. Rautela and R.J. Liedtke, *Clin. Chem.*, **24**, 108 (1978).
5) P.N. Tarbutton and C.R. Gunter, *Clin. Chem.*, **20**, 724 (1974).
6) H. Huang, J.W. Kuan, and G.G. Guilbault, *Clin. Chem.*, **21**, 1605 (1975).
7) P. Röschlau, E. Bernt, and W. Gruber, *Z. Klin. Chem. Klin. Biochem.*, **12**, 226 (1974).
8) F. van der Harr, C.M. van Gent, F.M. Schouten, and H.A. van der Voort, *Clin. Chem. Acta*, **88**, 469 (1978).
9) K.O. Ash and W.M. Hentschel, *Clin. Chem.*, **24**, 2180 (1978).
10) R.J. Liedtke, B. Busby, and J.D. Batjer, *Clin. Chem.*, **24**, 161 (1978).
11) P.R. Finley, R.B. Schifman, R.J. Williams, and D.A. Lichti, *Clin. Chem.*, **24**, 931 (1978).

Experimental

Apparatus——Fluorescence spectra and intensities were measured with a Hitachi MPF-4 spectrofluorimeter using quartz cells of 10×10 mm optical path-length. The slit widths in terms of wavelengths were set at 10 nm in both the exciter and analyzer. The spectra are uncorrected. pH was measured with a Hitachi -Horiba M-7 pH meter at 25°.

Reagents——All chemicals were of reagent grade unless otherwise noted. Double-distilled H_2O and isopropanol were used.

CEH-COD-POD solution (78 mU[12]/ml, 6.9 mU/ml and 5 purpurogallin units[13]/ml, respectively): Dissolve 6 mg of CEH (1.3 U/mg, from *Schizophylum commune*, Miles), COD solution (23 U/ml, from *Nocardia*, Miles) and 1.7 mg of POD (300 purpurogallin units/mg, from horseradish, Toyobo) in 100 ml of 0.3 M K-K phosphate buffer (pH 7.5). The solution is usable for more than 30 days when stored at 4°.

COD-POD solution: Prepare in the same way, but omit CEH.

Tyramine solution (5 mg/ml as its hydrochloride): Dissolve 500 mg of tyramine–HCl (recrystallized from ethanol–ether) in 0.3 M K-K phosphate buffer (pH 7.5) and store at 4°. Use within a week.

Dextran sulfate-Mg^{2+} solution (0.1% as its Na salt and 0.1 M, respectively): Mix 0.2% Na dextran sulfate 500 (Nakarai) solution in H_2O and 0.2 M $MgCl_2$ solution in saline in the volume ratio of 1:1. The solution is usable for 4 days when stored at 4°.

Procedure for Serum Cholesterol Assay——For total cholesterol assay, place 3.0 ml of 0.3 M K-K phosphate buffer (pH 7.5) containing 0.025% Triton X-100 (Wako) in a test tube, add 1 μl of serum and 0.1 ml each of tyramine solution and CEH-COD-POD solution, and incubate at 37° for approximately 20 min. Prepare a blank by treating 1 μl of H_2O in place of 1 μl of serum. For a standard curve, replace 1 μl of serum with 1 μl of cholesterol standard solution (50—400 mg/dl in isopropanol). Measure the fluorescence intensities at 404 nm with excitation at 317 nm.

For free cholesterol assay, carry out the same procedure except that 0.1 ml of CEH-COD-POD solution is replaced with 0.1 ml of COD-POD solution.

Procedure for HDL Cholesterol Assay——Place 5 μl of serum in a centrifuge tube, add 5 μl of dextran sulfate-Mg^{2+} solution and allow to stand at room temperature for 10 min. Centrifuge at room temperature and approximately $1000 \times g$ for 15 min. Treat 2 μl of the supernatant in the same way as for total cholesterol assay. For a standard curve, replace 2 μl of the supernatant with 2 μl of cholesterol standard solution (10—50 mg/dl in isopropanol).

Results and Discussion

The fluorescence excitation (maximum, 317 nm) and emission (maximum, 404 nm) spectra for the final mixtures in both serum and HDL cholesterol assays are identical to with those for the final mixtures obtained through the procedure with cholesterol standard solutions. This indicates that standard curves can be constructed in the absence of serum and HDL. The standard curves for serum and HDL cholesterol are linear up to at least 400 mg/dl, and pass through the origin.

The optimal pHs for the enzyme reactions are at 4—8 (CEH) and approximately 7 (COD and POD) and the product from tyramine fluoresces most intensely at pH 10—11. At pH 7.5, the fluorescence intensity increases with increasing concentrations of the enzymes in the range tested (26—130 mU/ml CEH, 2.3—11.5 mU/ml COD and 2—8 purpurogallin units/ml POD) when examined with serum containing a very high concentration of cholesterol (approximately 500 mg/dl). Thus, pH 7.5 and concentrations of 78 mU/ml (CEH), 6.9 mU/ml (COD) and 5 purpurogallin units/ml (POD) were selected as optima for our final procedure.

The activities of CEH and COD employed in the procedure are greatly enhanced by Triton X-100 at pH 7.5 in 0.3 M phosphate buffer. Buffer which contains 0.025% Triton X-100 gives a maximum rate of fluorescence development (Fig. 1).

Reagent solution containing more than 4 mg/ml tyramine hydrochloride gives a maximum and constant fluorescence; a 5 mg/ml solution was used. Although the overall reaction is most rapid at 40°, 37° was used for conveninence. The reaction is completed within 20 min.

12) U represents the International Unit of enzyme activity.

13) B. Chance, A.C. Maehly, "Methods in Enzymology," Vol. II, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, Inc., New York, 1955, p. 773.

The fluorescence developed is stable for more than 2 hr at room temperature in room light and for more than 1 day at 4° in the dark.

The dextran sulfate-Mg^{2+} method was employed with some modification for the separation of HDL. In the original procedure a mixture of serum and the reagents is centrifuged at 4°. We found that the cholesterol concentration in the supernatant was not dependent on the temperature of centrifugation (10—30°). Thus, centrifugation at room temperature was used. Other methods examined (heparin-Mn^{2+}-EDTA,[14] heparin-Ca^{2+} [15] and phosphotungstate-Mg^{2+} [16] methods) could be coupled to our procedure for total cholesterol assay, but required centrifugation at 4°. The heparin-Mn^{2+} method[17] could not be applied because the enzyme reaction mixture became turbid.

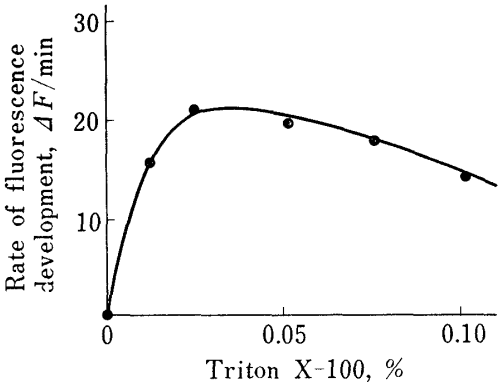

Fig. 1. Effect of Triton X-100 Concentration in the Phosphate Buffer on the Rate of Fluorescence Development

Portions (1 μl) of four-fold water-diluted serum were mixed with 3.0 ml of 0.3 M phosphate buffer (pH 7.5) containing 0.1 mg/ml tyramine hydrochloride and various concentrations of Triton X-100. The mixtures were preincubated at 30° for 3 min, then incubated again after addition of 50 μl of the enzyme solution (0.26 U/ml CEH, 4.6 mU/ml COD and 3 purpurogallin units/ml POD), and the increases in fluorescence intensities were recorded for about 5 min.

Bilirubin and uric acid in serum do not affect the value of serum cholesterol at concentrations less than approximately 10 and 5 mg/dl, respectively, but higher concentrations cause apparent decreases (1.4 mg/dl per 1 mg/dl bilirubin and 2.3 mg/dl per 1 mg/dl uric acid). Ascorbic acid at concentrations less than approximately 10 mg/dl in serum does not interfere with the method for serum cholesterol assay, but at higher concentrations, the measured value is dependent on the serum and the conditions of its storage. Glucose and hemoglobin added to serum do not affect the value of serum cholesterol at concentrations of 400 and 100 mg/dl, respectively. These compounds do not affect the values of HDL cholesterol even when present in serum at unusually high concentrations. Heparin and EDTA used as anticoagulants for blood have no effect on the values of serum and HDL cholesterol at the concentrations usually employed.

The recovery of cholesterol (100 and 200 mg/dl) added to serum with 101.8 mg/dl total cholesterol was 99.5±1.7% (mean ±SD, $n=10$ each), and that of cholesterol (25 and 50 mg/dl) added to the HDL fraction with 35.4 mg/dl cholesterol was 98.5±1.8% ($n=10$ each). The lower limits of detection for cholesterol in serum and HDL are 6.9 and 5.2 mg/dl, respectively; these values give fluorescence intensities of twice the blanks. The sensitivity may permit the assay of free and total cholesterol, and of HDL cholesterol in only 0.1 and 0.2 μl of serum, respectively.

The precision was established with respect to repeatability and reproducibility. The coefficients of variation in repeatability were 1.5% ($n=20$) for a mean serum total cholesterol value of 189.6 mg/dl and 1.7% ($n=20$) for a mean HDL cholesterol value of 46.2 mg/dl, and those in reproducibility (the assay being repeated for 10 days on serum stored at −20°) were 2.1% ($n=20$) for a mean total cholesterol value of 201.5 mg/dl and 1.9% ($n=20$) for a mean HDL cholesterol value of 43.5 mg/dl.

14) B.W. Steele, D.F. Koehler, M.M. Azar, T.P. Blaszkowski, K. Kuba, and M.E. Dempsey, *Clin. Chem.*, **22**, 98 (1976).
15) A. Noma, K. Nezu-Nakayama, M. Kita, and H. Okabe, *Clin. Chem.*, **24**, 1504 (1978).
16) M. Burnstein, H.R. Scholnick, and R. Morfin, *J. Lipid Res.*, **11**, 583 (1970).
17) Manual of Lipid Operations. Lipid Research Clinics Program, Vol. I: Lipid and Lipoprotein Analysis, U.S. Department of Health, Education and Welfare, Pub. No. (NIH) 75-628 (1974).

Comparison with colorimetric methods based on the enzymatic reaction (CEH–COD–POD system) with chromogenic substrates (4-aminoantipyrine-phenol[2,11]) for serum total cholesterol and HDL cholesterol showed correlation coefficients of 0.933 ($n=48$) and 0.975 ($n=30$), respectively, and the regression equations for the present method (x) against the colorimetric methods were $y=1.03x+2.2$ and $y=0.98x+2.2$, respectively. This indicates that the present method gives values virtually identical to those obtained by the colorimetric methods.

The fluorimetric method is precise and simple, and should be useful in cases where only an extremely small amount of serum is obtainable.

Acknowledgement The supply of sera from the Central Clinical Laboratory of Kyushu University Hospital, and from the Japan Medical Laboratory-Kyushu is acknowledged.

[Chem. Pharm. Bull. 28(10)3134—3137(1980)]

4,4-Dimethyl Effect. (1). Stereochemistries of the Hydrogenation Products of α-Onocerin[1,2]

YOSHISUKE TSUDA[3a] and TAKEHIRO SANO[3b]

Faculty of Pharmaceutical Sciences, Kanazawa University[3a] *and Showa College of Pharmaceutical Sciences*[3b]

(Received May 19, 1980)

Three isomeric hydrogenation products of α-onocerin diacetate, onoceranediol diacetates-I, -II, and -III, were converted to the corresponding diones-I, -II, and -III, which exhibited a positive, a double humped, and a negative CD spectrum, respectively. The configurations of onocerane-I, -II, and -III were therefore elucidated as 8αH-14βH, 8αH-14αH, and 8βH-14αH, respectively.

Keywords——α-onocerin; onoceranes; onocerane-3,21-diones; CD spectra; 4,4-dimethyl effect; 4,4,10-trimethyl-*trans*-decalin-3-ones

The structure and stereochemistry of α-onocerin (**1**), a tetracyclic triterpenoid of *Ononis spinosa* (Leguminosae) were determined more than two decades ago.[4,5] Later, this triterpenoid was found in various plants of *Lycopodium* (Lycopodiaceae) as a common constituent and it is regarded as a probable biogenetic precursor of the triterpenoids of the serratane group.[6] During its structure investigation, Barton and Overton[4] prepared three isomeric hydrogenation products, onoceranediol-I, -II, and -III. The stereochemistries of these compounds, however, were not determined until we correctly assigned their configurations as **3**, **4**, and **5**, respectively, in 1975.[2] This paper presents details of the experiments.

Hydrogenation of α-onocerin diacetate (**2**) in EtOAc over PtO_2 produced, in accord with the previous result,[4] three isomeric diacetates which were separated by column chromatography on acid-washed alumina. Alkaline hydrolysis of the diacetates-I, -II, and -III, and Jones oxidation of the resulting diols afforded the diketones-I, -II, and -III, respectively. The mp's of all these derivatives are in accord with the data reported previously.[4]

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1) Triterpenoid Chemistry. XIV. Part XIII: T. Sano, T. Fujimoto, and Y. Tsuda, *Chem. Pharm. Bull.*, **23**, 1784 (1975).
2) A preliminary report of this work was presented at the 95th Annual Meeting of the Pharmaceutical Society of Japan. Y. Tsuda, T. Sano, and T. Fujimoto, Abstract-II, p. 253, Nishinomiya, April (1975).
3) Location: *a) 13-1 Takara-machi, Kanazawa 920, Japan*; *b) 5-1-8 Tsurumaki, Setagaya-ku, Tokyo 154, Japan.*
4) D.H.R. Barton and K.H. Overton, *J. Chem. Soc.*, **1955**, 2639.