Acta Chemica Scandinavica, 1993: 47: 846–848
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ACTA
CHEMICA SCANDINAVICA
ISSN 0904–213X

Short Communication

Adaptation of an Enzymatic Kit for the Assay of Cholesterol in Tissue Lipid Extracts

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Wang, W. and Gustafson, A., 1993. Adaptation of an Enzymatic Kit for the Assay of Cholesterol in Tissue Lipid Extracts. – Acta Chem. Scand. 47: 846–848.

Enzymatic methods have been frequently used in the assay for cholesterol in plasma or serum, owing to their higher sensitivity and simplicity compared with chemical methods. Their use in the essay of lipid extracts has been attempted by only a few investigators.[1–3] The problem with this application is the additives needed to dissolve the cholesterol before the colorimetric reaction. The additives that have been tested include ethanol and Triton X-100. We have adapted the Monotest Cholesterol, high performance CHOD-PAP reagent (236 691)[4] for cholesterol determination in animal tissue extracts. An unstable and lower cholesterol absorbance was frequently experienced when the kit was applied to dried lipid extracts in accordance with the manufactures instructions. After testing several other additives, we were able to improve the enzymatic cholesterol determination by the use of isopropyl alcohol. A special problem in the assay for cholesterol in red blood cell (RBC) lipid extract, the co-extracted pigment, was solved by use of a correction formula.

Materials and experiments

Analytical reagent-grade chemicals and solvents were used. The following materials were purchased from Merck D-6100 Darmstadt (Germany): chloroform, methanol, ethanol, isopropyl alcohol, Triton X-100; and from Sigma Chemical Co. (St. Louis, USA): 5(6)-cholesten-3β-ol and cholesteryl oleate. CHOD-PAP reagent kits (Monotest® Cholesterol high performance CHOD-PAP 236 691) and (Test-Combination Free Cholesterol CHOD-PAP method 310 328) were from Boehringer Mannheim GmbH. The spectrophotometer used was a Shimadzu UV-260.

Standard total cholesterol determination procedure. To dried lipid extracts were added 0.2 ml isopropyl alcohol and 0.9 ml reagent (CHOD-PAP 236 691). The mixture was incubated at 22–25°C for 15 min. The absorbance was measured at 500 nm against the isopropyl alcohol–reagent blank.

The addition to dried aliquots of standard cholesterol of different alcohols prior to the reagent promoted the colorimetric reaction; the corresponding addition of Triton X-100 (0.5% w/v) decreased it (Fig. 1). Compared with the constant cholesterol absorbance in pure reagent, the addition of methanol, ethanol and isopropyl alcohol enhanced the calorimetric reaction by 12, 16 and 33%, respectively ($p < 0.001$), indicating improved sensitivity of the kit method. By adding pure reagent to cholesterol, a constant absorbance was obtained after incubation for 2 h, while using additional isopropyl alcohol, it occurred after only 15 min (Fig. 1). The best ratio of isopropyl

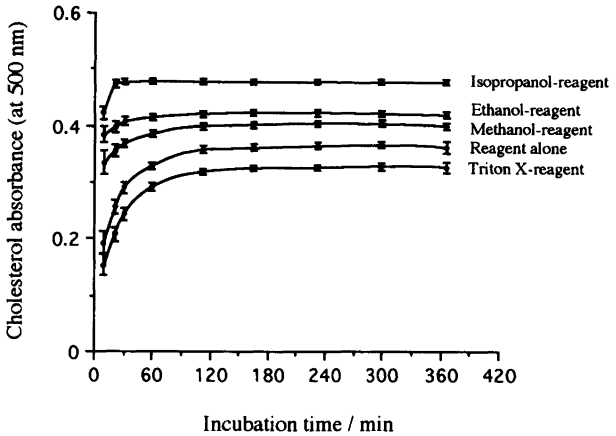

Fig. 1. Development of cholesterol absorbance in alcohol-, and Triton X-reagent mixture in relation to incubation time. Dried triplicate aliquots of free cholesterol (26.6 μg) were mixed with 1.1 ml CHOD-PAP (236 691) reagent or 0.2 ml each of the following solvents: methanol, ethanol, isopropyl alcohol, and Triton X-100 (0.5%) respectively, and 0.9 ml reagent. Absorbance was measured at 500 nm against the reagent, or solvent–reagent, respectively, after incubation at room temperature for 0 to 400 min. Values are expressed as mean ± sd.

alcohol to reagent was found to be 0.22 : 1 (v/v) (Fig. 2) and the addition of isopropyl alcohol before the reagent (with a 2 min interval) gave better results than addition after, or simultaneously with the reagent.

Cholesteryl oleate or a mixture of free and esterified cholesterol gave similar results. A standard curve for free and esterified cholesterol was established between 1.45 and 160 μg and 1.8 and 130 μg, respectively. The hydrolysis of cholesteryl oleate by the kit enzyme was not affected by the addition of isopropyl alcohol, as evidenced by thin-layer chromatography of the extracted reaction mixture (Silica gel H plate, coupled with the developing solvent: petroleum ether–ether–methanol–acetic acid 80 : 20 : 3 : 1 v/v). The isopropyl alcohol–reagent method (X) and the Zlatkis method[5] (Y) showed a good correlation: $Y = 0.95\,X + 0.09$ ($n = 24$, $r = 0.997$). Our method was successfully applied to lipid extracts of rat liver, brain, aorta and intestinal tissue. The coefficient of variation within the assays was 1.7% (in 16 determinations), while the day-to-day precision was 2.6% in triplicate determinations over 6 days.

The kit reagent (310 328)[6] for the determination of free cholesterol in plasma was also tested for the isopropyl alcohol modification. When used on dried lipid extracts, a continuous absorbance development was experienced. It was found that the change in absorbance of the reagent blank *per se* was a function of the incubation time. Addition of isopropyl alcohol prior to the reagent did not completely abolish this phenomenon.

Correction of pigment absorbance from RBC lipid extract. Pigment from haemoglobin interferes with cholesterol determination by both chemical and enzymatic reagents. The co-extraction of pigment with cholesterol from RBC is unavoidable in methanol-containing solvents and the pigment was believed to be heme.[7] We found that standard heme shows, identical with pigment, its highest absorbance peak at 400 nm as well as at 500 nm. Theoretically, the pigment absorbance can be corrected by using a reagent without cholesterol oxidase, but such a reagent is not available. At 500 nm, heme absorbance is too low to be relied upon so it was measured at 400 nm and corrected to 500 nm (Fig. 3). The net cholesterol absorbance in isopropyl alcohol–reagent at 500 nm was the total absorbance of cholesterol and heme, minus the corrected heme absorbance (at 500 nm).

Such a correction was acceptable since the total absorbance of the heme and cholesterol was equal to the sum of each of them in the isopropyl alcohol–reagent (Fig. 2). The amount of heme chosen here gave an absorbance of 0.5 at 400 nm. At the most, the absorbance of heme extracted by methanol/chloroform mixtures from RBC was less than 0.6 (RBC was extracted by 19 volumes of methanol–chloroform 1 : 2 to 2 : 1). The accuracy of such a subtraction for RBC lipid extracts was verified by the Zlatkis method[5] in two ways: by quantifying the free cholesterol content after isolation of heme by thin-layer chromatography, and secondly by correcting the heme absorbance using a non-ferric chloride reagent. In practice, whenever the pigment absorbance at 400 nm was less than 0.1, the correction could be omitted.

An alternative way of avoiding the heme extraction from RBC is to use a less polar alcohol in the extraction. We found that the method of Rose and Oklander,[7] using isopropyl alcohol–chloroform (11 : 7, 19 v), extracted much less pigment from RBC than methanol-containing solvents so that the correction of pigment absorbance could perhaps be omitted. However, at the same time, this

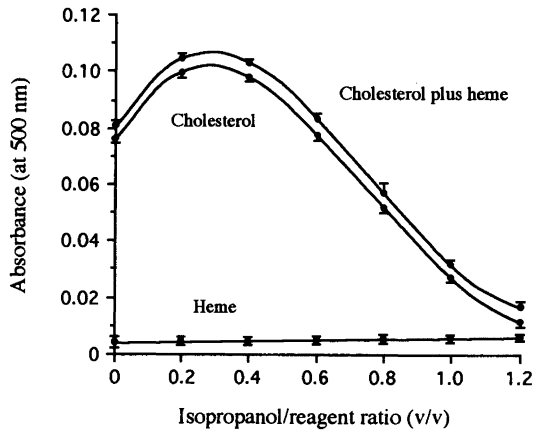

Fig. 2. Absorbance of cholesterol and heme in isopropyl alcohol–reagent. Dried aliquots of heme and cholesterol (5.8 μg) were added to tubes together or separately, mixed in quadruplicate with 1.1 ml of isopropyl alcohol–reagent (CHOD-PAP 236 691) in ratios from 0 : 1 to 2.67 : 1 (isopropyl alcohol added prior to the reagent). Absorbance was measured 30 min later at 500 nm against the corresponding isopropyl alcohol–reagent. Values are expressed as mean ± sd.

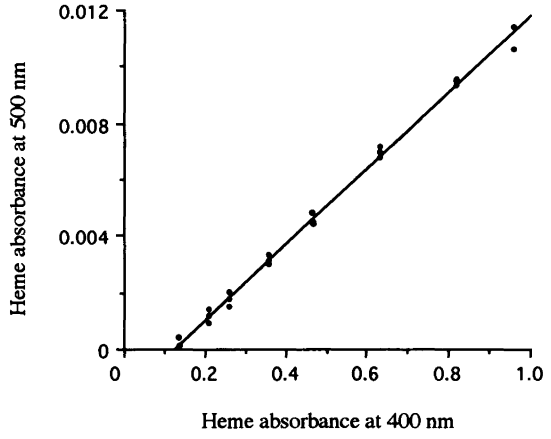

Fig. 3. Relationship between heme absorbance at 400 nm and that at 500 nm. Triplicate aliquots of 0.1 ml heme (in methanol–chloroform 1 : 1 and corresponding to absorbance between 0.136 and 0.960 read at 400 nm against chloroform blank) (X), were evaporated, mixed with 0.2 ml isopropyl alcohol and 0.9 ml reagent (CHOD-PAP 236 691). Absorbance was measured 15 min later at 500 nm against the isopropyl alcohol–reagent blank (Y). $Y = 0.0135\,X - 0.0016$, $r = 0.996$.

solvent extracted less cholesterol and total phospholipids. A reliable and complete extraction of such lipids can be fulfilled only by more than 28 volumes of isopropyl alcohol–chloroform solvent (paper in preparation).

Discussion

Our results indicate that the isopropyl alcohol–CHOD-PAP (236 691) reagent complements existing methods for the determination of total cholesterol from dried tissue lipid extracts. The reason for the promoted colorimetric reaction of the cholesterol in such mixtures is not clear. It may not simply be due to the enhanced solubility of cholesterol in the isopropyl alcohol, since it is higher in methanol. For the determination of free cholesterol, the CHOD-PAP (310 328) reagent is suggested. Unfortunately, the reagent *per se* was unstable. The reason might be the involvement of large amounts of methanol ($1.85\ mol\ l^{-1}$). Therefore, we would suggest that in the assay for free cholesterol, either cholesterol esterase in reagent (236 691) should be removed, or the methanol in reagent (310 328) should be replaced by isopropyl alcohol. However, total cholesterol can be determined from RBC lipid extracts by the isopropyl alcohol–reagent (236 691) mixture, since RBC contains solely free cholesterol. A formula has been developed to correct for pigment absorbance in cholesterol determination from RBC lipid extract.

Summary

In the assay for total cholesterol in lipid extracts by enzymatic methods, it was found necessary to redissolve the lipid prior to the reagent addition. Isopropyl alcohol was found to be best at promoting the colorimetric reaction. As little as 1.45 µg free cholesterol and 1.8 µg esterified cholesterol could be determined after 15 min incubation with the isopropyl alcohol–reagent. Using a formula for the correction of pigment absorbance, it was also possible to apply the enzymatic kit method to red blood cell lipid extracts.

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Received March 3, 1993.