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notes on methodology

Microdetermination of cholesterol in serum lipoproteins

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Summary A two-step procedure for the microdetermination of cholesterol in serum lipoproteins is compared with cholesterol quantitation after density gradient ultracentrifugation. Serum lipoproteins from 10 μl of serum are separated by electrophoresis on agarose and visualized by precipitation with dextran sulfate–$CaCl_2$. The lipoprotein bands are cut off from the plates, the agarose slices are hydrolyzed, and cholesterol is extracted with heptane and quantitated by gas–liquid chromatography. The comparison between the two procedures reveals satisfactory correlations for β- and pre-β-lipoproteins and total serum. There is excellent recovery of cholesterol in fractionated lipoproteins.

Supplementary key words lipoprotein electrophoresis · gas–liquid chromatography

The "standard" procedure of cholesterol determination in serum lipoproteins uses the classical method of preparative ultracentrifugation (1). It has gained clinical importance for the differential diagnosis of the hyperlipoproteinemias. Since the method is expensive and time-consuming, alternate procedures have been developed. They are based on selective precipitation of lipoproteins by polyanions (2) or by detergents (3), which permit only an indirect determination of LDL-cholesterol, important for the diagnosis of type II hyperlipoproteinemia. With all procedures, considerable quantities of serum are needed for lipoprotein fractionation. The procedure described here eliminates the need for large serum samples. Direct cholesterol determination is achieved in all lipoprotein fractions. It is equally suitable for serial lipoprotein measurements and for studies in infants and small laboratory animals.

Materials and methods

Serum samples were obtained from unselected patients with or without hyperlipoproteinemias. Routine analyses of serum lipoproteins were performed by discontinuous gradient ultracentrifugation from 3 ml of serum with a Beckmann 40-3 rotor (105,000 *g*, 20 hr) (Beckman Instruments, Fullerton, CA) followed by polyanion precipitation with heparin–$MnCl_2$ (4). Cholesterol was extracted from all fractions (VLDL, LDL + HDL, HDL) with isopropanol in the presence of zeolite and was determined by the Liebermann-Burchard reaction on an AA II Technicon AutoAnalyzer (5) (Technicon Instruments, Tarrytown, NY). The quantification of cholesterol was performed according to the procedure of the Lipid Research Clinic laboratories. For calibration, "Sercal" serum was obtained from the Center for Disease Control (Atlanta). LDL-cholesterol concentrations were calculated by subtracting HDL-cholesterol from cholesterol in the (LDL + HDL) fraction. The sums of cholesterol concentrations in lipoproteins yielded 90% or more of the total serum cholesterol in all investigated serum samples.

Lipoprotein electrophoreses were performed on the same serums. A 50 μl portion of serum was mixed with 50 μl of agarose suspension (2%) at 37°C. Twenty μl of the mixture was transferred into reservoirs of freshly prepared agarose gel beds on glass plates. The electrophoreses were performed in barbital buffer at pH 8.6.

Subsequent to electrophoretic separation the lipoproteins were precipitated with dextran sulfate 2000 (0.6%, Parmacia, Frankfurt)–$CaCl_2$ (0.2 M) (6). The areas with the precipitates were cut off with a scalpel and transferred into glass tubes with Teflon stoppers. One ml of 50% KOH (w/v) was added. The mixtures were heated to the boiling point for 40 sec under vigorous shaking over a gas flame to achieve hydrolysis of the agarose and cholesteryl esters. The solution was cooled to 15°C and 20 μl of 0.1% (w/v) α-cholestane solution in ethanol and 2 ml of 99.6% ethanol were added. The solution was heated at 60°C for 10 min (alternatively the solution can be shaken for 10 min) and 1 ml of heptane and 7 ml of water were added. The mixture was then shaken for an additional 15 min, centrifuged, and the heptane phase containing cholesterol and α-cholestane was used for gas–liquid chromatography. The precision

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; GLC, gas–liquid chromatography.

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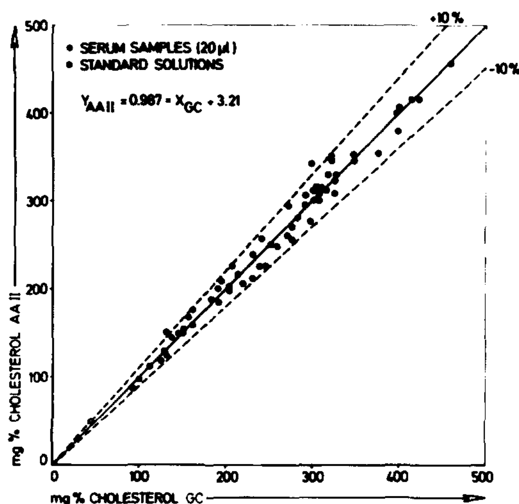

Fig. 1. Correlation between GLC determinations of total serum cholesterol and photometric estimation on an AA II Technicon AutoAnalyzer with the Liebermann-Burchard reaction (n = 58).

of the procedure was determined from 18 replicate electrophoretic separations of the same serum sample.

The quantitation of cholesterol from standard solutions in isopropanol or of total serum cholesterol in serums from patients in the department of internal medicine was performed as follows. Twenty µl of serum or standard solution was shaken with 100 µl of 50% KOH, 20 µl of α-cholestane standard, and 350 µl of ethanol. The solution was heated to 60°C for 15 min. After addition of 1 ml of heptane and 850 µl of water, the mixture was shaken for 15 min and then centrifuged. One µl of the heptane phase was used for GLC on a Hewlett Packard (Palo Alto, CA) H-P 5830 A gas chromatograph equipped with auto-sampler, Hewlett-Packard terminal, and a glass flame ionization detector. Six-foot glass columns (ID 2 mm) containing 3% SE 30 on Gaschrome Q 125–150 µm [Serva, Heidelberg, Germany] were used; injection temperature, 260°C; column temperature, 245°C; detector temperature 280°C; and N_2 flow 50 cc/min. The retention times were 4.8 min for α-cholestane and 8.6 min for cholesterol. The precision of the procedure was estimated from 18 replicate determinations of one serum sample. The sensitivity of the method was measured with a dilution series of cholesterol dissolved in isopropanol.

Results

The comparison between the microdetermination of cholesterol by GLC and by the commonly used Liebermann-Burchard reaction from standard solutions in isopropanol and from serum samples is shown in **Fig. 1**. Analyses of standard solutions gave nearly identical results. The differences in determinations from serum samples are less than 10% ($r = 0.99$) provided that the extraction with isopropanol and zeolite is performed prior to the photometric determinations and that the AutoAnalyzer procedure is standardized and corrected corresponding to the quantitation with the procedure of Abell et al. (7). Otherwise the Liebermann-Burchard reaction results in values that are about 12% higher than the determination by GLC. The precision for total serum cholesterol estimated from 18 aliquots of the same serum sample by GLC had a coefficient of variation of 3.3 (**Table 1**).

The sensitivity of the procedure has been investigated with cholesterol in isopropanol at decreasing concentrations. As shown in **Fig. 2,** quantitation is possible from 200 pg/µl of heptane solution. Thus, less than 1 mg/100 ml cholesterol can be estimated from 10 µl of serum.

To evaluate the precision of the cholesterol determination in lipoproteins separated from 10 µl of serum by lipoprotein electrophoresis, 18 replicate fractionations were carried out by agarose gel electrophoresis. Cholesterol was determined in each fraction (Table 1). The coefficient of variation decreases with increasing cholesterol concentration (β-lipoprotein cholesterol, *CV* 3.7; pre-β-lipoprotein cholesterol *CV*, 13.4; α-lipoprotein cholesterol, *CV* 6.8). In our experience the variance of the pre-β-lipoprotein cholesterol quantitation is comparable with the precision of the ultracentrifugation procedure

TABLE 1. GLC determination of cholesterol after fractionation of lipoproteins by electrophoresis on agarose gel

	Cholesterol			
	β-Lipoprotein	Pre-β-lipoprotein	α-Lipoprotein	Total Serum
	mg/ml			
Mean (n:18)	322 ± 11.8	21.5 ± 2.8	37.7 ± 2.6	395.0 ± 13.0
CV	3.7	13.4	6.8	3.3
Ultracentrifugation (n = 4)	338 ± 10.1	14 ± 4	34 ± 4	

Eighteen replicate analyses from one serum sample. CV is coefficient of variation.

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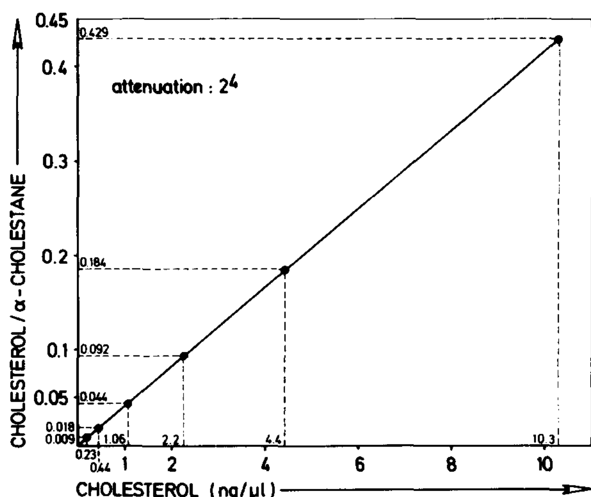

Fig. 2. Evaluation of the sensitivity of the GLC quantitation of cholesterol dissolved in isopropanol. (Internal standard for GLC, α-cholestane.)

(*CV* 15.0). The sum of cholesterol concentrations in all lipoprotein fractions was 96% of the total serum cholesterol (*CV* 3.6).

Serums from 37 patients who were normolipidemic or hyperlipoproteinemic (type IIa, IIb, IV) were analyzed both by ultracentrifugation and by the combined procedure of lipoprotein electrophoresis and GLC. Cholesterol recovery in the former procedure was 90% or better of total serum cholesterol. The correlations of the determinations in each lipoprotein fraction are presented in Figs. 3, 4 and 5. A high correlation was obtained for the β-lipoprotein fraction ($r = 0.954$). The correlation was linear in the range between 150 and 350 mg/dl of β-lipoprotein cholesterol (**Fig. 3**). The correlation for pre-β-lipoprotein cholesterol determinations was also satisfactory ($r = 0.926$) (**Fig. 4**). At low concentrations, generally higher values were obtained by the microprocedure, whereas at higher pre-β-lipoprotein concentrations, higher values were obtained by the ultracentrifugation. For α-lipoprotein cholesterol, similarly slightly higher values were measured by the GLC procedure ($r = 0.720$) (**Fig. 5**). A comparison between the sums of cholesterol concentrations from all lipoprotein fractions and total serum cholesterol estimated by the Liebermann-Burchard reaction is presented in **Fig. 6**. The recovery was usually better than 95% for the micromethod.

To demonstrate one possibility of the microprocedure, one rat (250 g wt) was fasted for 12 hr. During this time 200 µl of blood was taken at each hour. The serums were fractionated by agarose gel electrophoresis. The precipitation of lipoproteins revealed one band in the β-position and two bands in the α-position (α_1 and α_2).

The results of the cholesterol quantitation by GLC are shown in **Fig. 7**. They clearly demonstrate the usefulness of the method for measurements that cannot be carried out by common procedures.

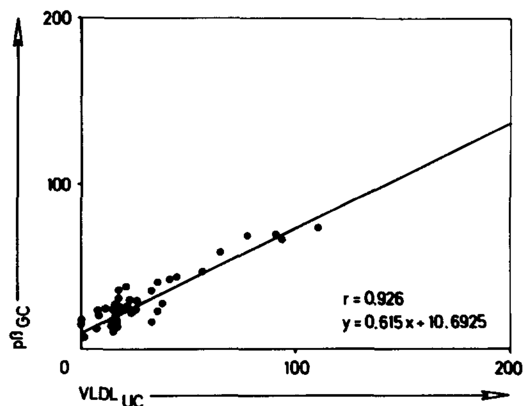

Fig. 4. Correlation between GLC-determined pre-β-lipoprotein cholesterol and VLDL-cholesterol from ultracentrifugation (n:37).

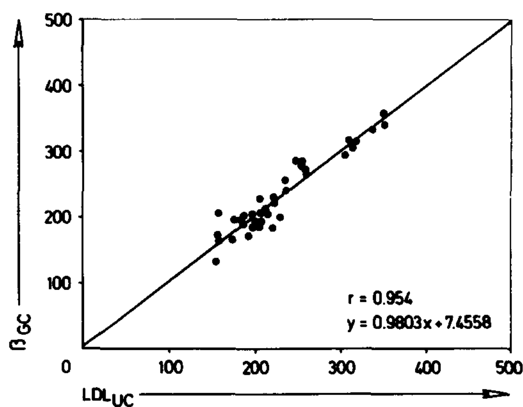

Fig. 3. Correlation between GLC-determined β-lipoprotein cholesterol and LDL-cholesterol from ultracentrifugation (n:37).

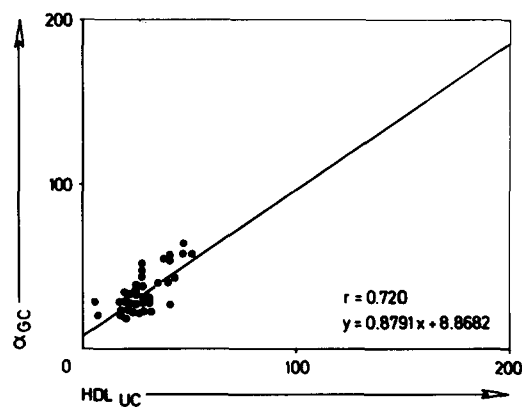

Fig. 5. Correlation between GLC-determined α-lipoprotein cholesterol and HDL-cholesterol from ultracentrifugation (n:37).

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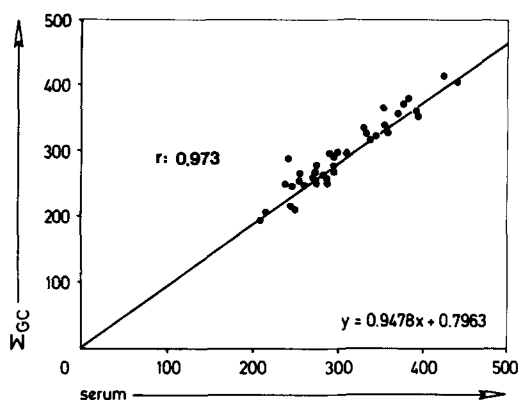

Fig. 6. Correlation between the sums of GLC-determined cholesterol from electrophoretically separated lipoprotein and total serum cholesterol (n:37).

Discussion

Ishikawa et al. (8) have recently developed a microprocedure for cholesterol quantitation by GLC. Their reagent tetramethylammonium hydroxide does not hydrolyze agarose within a short time period. This can be accomplished with 50% KOH, which leads to a hydrolysis of the gel at the boiling point of the solution within 30–40 sec. At this step it is necessary to cool the hydrolyzate to 15°C to prevent an accelerated oxidation of cholesterol; otherwise, lower recoveries will be obtained. The determination of cholesterol in lipoproteins fractionated from 10 μl of serum by agarose gel electrophoresis is as precise as the ultracentrifugation, provided that the lipoprotein precipitates can be visualized as distinct bands.

Several factors have to be considered when comparing the quantification of cholesterol by lipoprotein electrophoresis and GLC or by ultracentrifugation and the Liebermann-Burchard reaction. In gas–liquid chromatography cholesterol is determined as free cholesterol, whereas in the Liebermann-Burchard reaction it is measured both in its free and esterified forms. In the automated method, cholesteryl esters reveal a higher absorption than free cholesterol. This accounts for the difference of about 12% between values measured by the AA II-AutoAnalyzer method and by the procedure of Abell et al. (7). In normal human sera the concentration of free cholesterol accounts for about 30% of total serum cholesterol. Yet this ratio is not the same in isolated lipoproteins. The highest divergence from this value is found in HDL (free cholesterol/esterified cholesterol, 15/85). It should result in relatively lower determinations of cholesterol by GLC in this fraction.

On the other hand, the lipoproteins termed "HDL" are not necessarily identical with the α-fraction in lipoprotein electrophoresis. The routine determination of "HDL"-cholesterol by ultracentrifugation and polyanion precipitation does not distinguish between HDL (d 1.063–1.21) and very high density lipoproteins (d > 1.21). In addition, a small portion of HDL may have an electrophoretic mobility like that of β-lipoproteins (9). Similarly the VLDL (d < 1.006) does not behave homogenously with regard to electrophoretic mobility. A small portion may show β-mobility (6). Taking all into consideration, the microprocedure should result in a satisfactory correlation with slightly higher values for LDL/β-lipoprotein cholesterol. For VLDL/pre-β-lipoprotein cholesterol and HDL/α-lipoprotein cholesterol, the correlation cannot be expected to be as precise, because cholesterol concentrations in these fractions are considerably lower. In our experience the recovery of cholesterol in VLDL and HDL after ultracentrifugation fluctuates (*CV* 10–20); it is not more than 80% due to losses in the ultracentrifugation step (10). This may explain why the microprocedure yields generally higher values for HDL/α-lipoprotein cholesterol and VLDL/pre-β-lipoprotein cholesterol in serums with low VLDL concentrations.

The high correlation of β-lipoprotein concentrations demonstrates the validity of the micromethod. With adequate equipment, β-lipoprotein determinations from more than 35 serums can be accomplished easily within 24 hr. As minimal serum volumes are needed for complete quantitation, the procedure

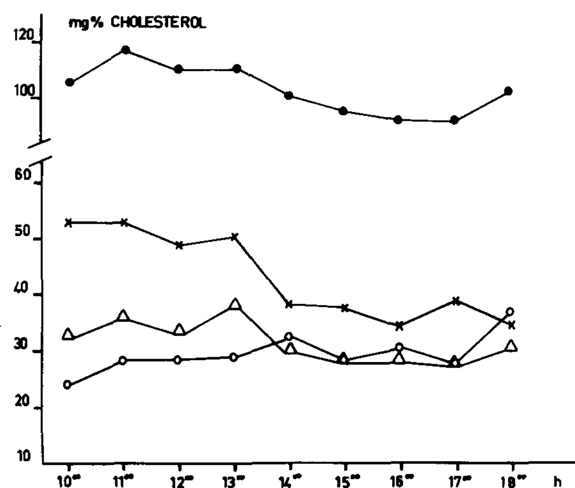

Fig. 7. Cholesterol concentrations of electrophoretically separated lipoproteins in blood samples from one rat taken at 1-hr time intervals within a period of 8 hr. The rat was fasted 4 hr before and during the time of the study. Total cholesterol, ●; β-lipoprotein cholesterol, △; α_1-lipoprotein cholesterol, ×; α_2-lipoprotein cholesterol, ○.

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not only facilitates routine analyses by avoiding ultracentrifugation, but it is also valid for investigations when only small plasma volumes are available.

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