notes on methodology

Analysis of α-lipoprotein cholesterol in 50 μl of plasma

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Summary A micromethod for quantitation of α-lipoprotein cholesterol was devised for gas–liquid chromatography to minimize plasma sample size and facilitate lipid studies using capillary blood from children or small animals. α-Lipoprotein cholesterol was measured by gas–liquid chromatography in 20 μl of the centrifuged supernate obtained after addition of 5 μl of mixed heparin–manganese chloride solution 1:1 (v/v) to 50 μl of plasma. Comparison of venous α-lipoprotein cholesterol measured by gas–liquid chromatography with venous α-lipoprotein cholesterol measured by conventional heparin–manganese precipitation and ferric chloride (colorimetric) cholesterol determination gave a correlation coefficient of 0.98 for 80 plasma samples. Capillary α-lipoprotein cholesterol and venous α-lipoprotein cholesterol were closely correlated in 31 patients (r = 0.97).

Supplementary key words gas–liquid chromatography

Plasma cholesterol can be quantitated by GLC–microtechniques in 20 μl of plasma obtained by capillary sampling (1, 2), facilitating studies in children and small animals. Particularly in infancy, determination of α-lipoprotein cholesterol (C-HDL) is important because it constitutes fully half of all plasma cholesterol at birth (3, 4). Measurement of C-HDL is also important in conjunction with determination of β-lipoprotein cholesterol (C-LDL) by ultracentrifugation (4–6) or double-precipitation (7) methods.

In the present study we developed a microextraction, GLC method for analysis of C-HDL (in 50 μl of plasma) to complement the GLC micromethod for total cholesterol (1). This microextraction GLC method correlated highly (r = 0.98) with an established heparin–manganese precipitation (8) and colorimetric quantitation (9, 10) of C-HDL currently in use.

Equipment, columns and column packings, and cholesterol determination by GLC were the same as recently reported (1, 2), with two slight modifications: (*1*) 5 mg of 5α-cholestane was added to the tetramethylammonium hydroxide–isopropanol solution, and (*2*) the GLC electrometer attenuator was set at 4.

Capillary and venous blood samples. Two or three 75-mm capillary hematocrit tubes (Matheson Scientific, no. 8484-20) were taken by fingerstick in parallel with venous samples (2) from 31 consecutive unselected patients for comparison of HDL in capillary blood (microextraction and GLC) and venous blood (macroextraction and AA-1 determination). To compare microextraction–GLC and macroextraction methods for analysis of C-HDL in venous blood, an additional 36 adult venous and 44 umbilical cord samples were utilized.

Macroprecipitation of C-LDL and C-VLDL by heparin–manganese and determination of C-HDL by AA-1 (macro-AA). Each venous blood sample was routinely divided into two (plasma) aliquots. On the first aliquot, heparin–manganese precipitation of C-LDL and C-VLDL was carried out using the procedure of Burstein and Samaille (8). This macromethod involved addition of 0.05 ml of heparin (5000 USP units/ml) to 1 ml of plasma in a 10 × 75 mm test tube in an ice bath. After thorough mixing on a Vortex mixer, 0.05 ml of 1 M manganese chloride was added and the solution was mixed a second time. The precipitation reaction mixture was allowed to stand for 15 min in the ice bath, and it was then centrifuged for 15 min at 4°C at 8 × 10^2 *g*. C-HDL concentrations were quantitated by AA-1 methods after macroprecipitation and extraction of 0.5 ml of the supernate with isopropanol and zeolite (10). In this assay the final concentration of $MnCl_2$ was 0.046 M, and the final concentration of heparin was 227 units/ml. C-HDL measurement was accurate at final $MnCl_2$ concentrations of from 0.046 M to 0.14 M. C-HDL determination was inaccurate at final $MnCl_2$ concentrations of 0.041 M or less because of incomplete precipitation of C-LDL and C-VLDL. C-HDL measurement was accurate at final heparin concentrations of from 113 to 800 units/ml.

Macroprecipitation of C-LDL and C-VLDL by heparin–manganese and determination of C-HDL by GLC (macro-GLC). 20 μl of the macrosupernate (as prepared above) was retained for determination of C-HDL by GLC (1).

Microprecipitation of C-LDL and C-VLDL by heparin–manganese and determination of C-HDL by GLC (micro-GLC). The second aliquot (50 μl) of each venous plasma sample and a 50-μl aliquot of plasma separated from the centrifuged capillary hematocrit tubes (2) were precipitated with heparin and manganese using the fol-

Abbreviations: GLC, gas–liquid chromatography; C-HDL, α-lipoprotein cholesterol; C-LDL, β-lipoprotein cholesterol; C-VLDL, pre-β-lipoprotein cholesterol; AA-1, ferric chloride (colorimetric) cholesterol determination.

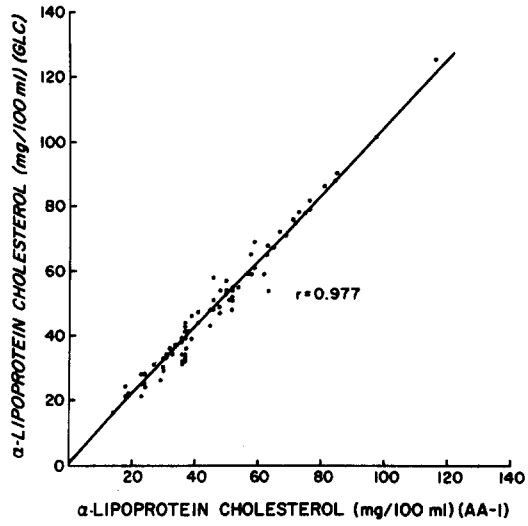

Fig. 1. Comparison of α-lipoprotein cholesterol levels in venous blood as measured by the micro-GLC method and AA-1 colorimetric method in 80 samples. Solid line, best fit regression line.

lowing micromethod. (*1*) Using a disposable micropipet, 50 μl of plasma was placed into a 5.5 × 50 mm polyethylene microsample tube (Bel-Art Products, Pequannock, N.J.) standing in an ice bath. (*2*) 5 μl of a freshly prepared 1:1 mixture of heparin (5000 units/ml) and 1 M manganese chloride solution was added to the plasma from a 10-μl glass microsyringe. (*3*) The contents of the tube were mixed, allowed to stand in the ice bath for 15 min, and then centrifuged at 8×10^2 *g* for 15 min at 4°C. Using a 20-μl disposable capillary pipet, 20 μl of the supernate was removed, taking care not to aspirate any of the packed precipitate at the bottom of the tube. This 20-μl aliquot of clear supernate was then used directly for the micro-GLC cholesterol determination (1). If necessary, as little as 5 μl of the supernate could be taken (1). (*4*) A brown precipitate (probably manganese dioxide) is formed during the saponification (1) of the plasma extract. Although we have not observed any major effects of this precipitate on the column performance or the analysis of cholesterol, we avoid injecting any of it into the column and carefully wipe away any precipitate adhering to the injection needle.

Comparison of C-HDL determination in venous blood by the macro-AA, macro-GLC, and micro-GLC analytical techniques. 80 venous plasma samples from 36 adults and 44 neonates were divided into two aliquots. C-HDL was measured in the supernate of the first aliquot by AA-1 (macro-AA), and also by GLC (macro-GLC) (as described above). In the second aliquot of plasma, a microprecipitation with heparin–manganese was carried out, and C-HDL was determined in the supernate by GLC (micro-GLC) (as described above). The three analytical techniques were compared as follows. (*1*) Macro-AA was compared with macro-GLC to determine if the two different analytical methods differed in regard to quantitation of C-HDL. (*2*) Macro-GLC was compared with micro-GLC to determine if miniaturization of the precipitation procedure affected quantitation of C-HDL, using identical analytical methods. (*3*) Macro-AA was then compared with micro-GLC to determine if *both* miniaturization of the precipitation process and differing analytical techniques affected the overall quantitation of C-HDL.

Comparisons of these three approaches to the plasma samples were not made until the final data had been recorded separately in discrete laboratory data books, by three separate technicians, following a strict double-blind protocol.

Comparison of C-HDL in capillary blood by micro-GLC techniques and C-HDL in venous blood by macro-AA analysis. In 31 subjects C-HDL was determined after capillary blood sampling by GLC (capillary micro-GLC) and was compared with C-HDL in venous blood samples quantitated by macro-AA analysis.

Same-day and inter-day studies of precision. A large pool of plasma was divided into 30 aliquots. C-HDL was measured by macro-AA, macro-GLC, and micro-GLC on each of 10 triplicate aliquots on the same day. This allowed for estimation of "same-day" precision.

A large plasma pool was used over a 21-day period with aliquots taken for sampling at 2-day intervals. C-HDL was determined in duplicate aliquots by macro-AA and micro-GLC to estimate "inter-day" precision.

Statistical analysis. Correlations between the three analytical methods for quantitation of C-HDL were obtained (11) (Table 1), and comparisons of these three approaches were made with two-way analyses of variance (12). Additional analyses of quantitative differences between C-HDL measurements by macro-AA and micro-GLC were performed according to the methods of Friedewald, Levy, and Fredrickson (6) and are summarized in Table 2.

Accuracy and comparability of macro- and micro-measurement of C-HDL in venous blood. Overall, 80 venous plasma samples were evaluated using macro-AA, macro-GLC, and micro-GLC methods. C-HDL levels in the 80 plasma samples measured with the three different techniques were quite similar (**Table 1**). There was a high degree of correlation between the macro-AA and micro-GLC methods, between the macro-AA and macro-GLC methods, and between the macro-GLC and micro-GLC methods (Table 1, $P < 0.001$) (**Fig. 1**). The simple "scaling-down" of the reaction mixture size for the precipitation of VLDL and LDL did not affect determination of C-HDL, as seen by the close concordance of C-HDL measured by macro-GLC and by micro-GLC (Table 1). The close relationship of C-HDL measured by macro-GLC and macro-AA techniques demonstrated that very different methodologies did not affect C-HDL quantitation (Table 1). The combination of "scaling down" and differ-

TABLE 1. Comparison of three analytical methods for quantitation of HDL cholesterol in 80 plasma samples

Method	Mean	SD	SE
	mg/100 ml		
Macro-AA[a]	41.2	14.5	1.6
Macro-GLC[b]	43.5	15.1	1.7
Micro-GLC[c]	43.1	15.4	1.7

The *correlation coefficients* were: macro-AA:macro-GLC, $r = 0.972$ ($P < 0.001$); macro-GLC:micro-GLC, $r = 0.990$ ($P < 0.001$); micro-GLC:macro-AA, $r = 0.977$ ($P < 0.001$).

[a] HDL cholesterol measured by macroprecipitation and Auto-Analyzer (AA-1).

[b] HDL cholesterol measured by macroprecipitation and GLC.

[c] HDL cholesterol measured by microprecipitation and GLC.

TABLE 2. Comparison of HDL cholesterol concentration (mg/100 ml) measured by macroprecipitation and Auto-Analyzer determination and HDL cholesterol measured by microprecipitation and GLC

Number of Values	(C-HDL AA-1) − (C-HDL GLC)[a]			[HDL AA-1 − HDL GLC]	
	Mean ($\bar{x}$)	SD	95% Confidence Interval[b]	Mean ($\bar{x}$)[c]	% Error[d]
80	−1.9	3.1	−1.1, −2.7	3.2	7.9

[a] HDL cholesterol, macroprecipitation, analyzed on AA-1 Auto-Analyzer, minus HDL cholesterol, microprecipitation, analyzed by GLC.

[b] Calculated as $\bar{x} \pm [t \text{ value } (0.025, n - 1)\ SD/\sqrt{n}]$.

[c] Absolute value of (HDL AA-1 − HDL GLC), irrespective of sign.

[d] % error = (mean of [HDL AA-1 − HDL GLC]) × 100/(mean of HDL AA-1)

ing techniques did not alter quantitation of C-HDL, as shown by the close relationship of C-HDL by micro-GLC and by macro-AA (Table 1).

Additional analyses of quantitative differences between determination of C-HDL by macro-AA and micro-GLC methods were performed by the methods of Friedewald et al. (6) (**Table 2**). Mean C-HDL by micro-GLC was slightly higher than that by macro-AA. When C-HDL by micro-GLC was subtracted from C-HDL by macro-AA and the 95% confidence interval for the differences was calculated, the great majority of the differences fell between the tight range of 1.1 and 2.7 mg/100 ml.

In further comparison of the three methods, C-HDL determined by macro-GLC did not differ from C-HDL determined by micro-GLC by two-way analysis of variance ($F = 0.19$; df = 2, 237; $P > 0.2$).

C-HDL determined by macro-AA in venous blood was slightly lower than C-HDL in venous blood by micro-GLC. This slight difference (mean difference −1.9 mg/100 ml, Table 2) was significant by two-way analysis of variance ($F = 27.9$; df = 1, 158; $P < 0.01$). This small difference, though statistically significant, is probably immaterial on a clinical or chemical basis within the range of usual measurement of C-HDL.

Same-day and inter-day precision. The same-day precision of the three methods was evaluated by determination of C-HDL in 30 aliquots from a single pool of plasma. The coefficient of variation was 1.4% for macro-AA, 2.6% for macro-GLC, and 3.5% for micro-GLC, with mean C-HDL of the pool being 35 mg/100 ml.

To assess inter-day variance, a second assayed reference pool of plasma was studied repetitively (every other day) over a 3-wk period. The "inter-day" coefficient of variation for C-HDL by macro-AA was 10.3% and by micro-GLC was 3.0%.

Comparison of C-HDL in capillary and venous blood samples. In 31 subjects, mean ± SD capillary blood C-HDL was 47 ± 17 mg/100 ml, slightly lower than paired measurement of C-HDL in venous blood, 51 ± 18. Capillary and venous C-HDL were highly correlated: $r = 0.97$, $P < 0.001$. C-HDL in capillary blood differed from C-HDL in venous blood by a mean of 4.3 mg/100 ml, a significant difference by two-way analysis of variance ($F = 31.2$; df = 1, 60; $P < 0.01$). Using the AA-1 method as a reference, or independent variable, a linear regression line was calculated (1, 2): the regression coefficient was 0.96 and the y axis intercept was −2.2. The 95% confidence limits about the regression line were parallel through the range tested and allowed prediction of capillary C-HDL (GLC) from venous C-HDL (AA) with 95% confidence limits of ±9 mg/100 ml.

Utility of the micro C-HDL method. The heparin–manganese method (8) of precipitation of C-LDL and C-VLDL has been used to determine "normal" levels of both C-HDL and C-LDL (10, 13). Recent comparisons of the heparin–manganese precipitation and dextran sulfate–calcium chloride methods (14–17) have suggested that C-HDL (heparin–manganese) correlated much better with C-HDL (preparative ultracentrifugation) than with C-HDL derived from dextran precipitation (7).

Highly correlated with C-HDL measured by the established method of Burstein and Samaille (8), C-HDL can be quantitated in 50 µl of plasma by GLC 45 min after receipt of the sample. In a normal working day, without an automatic sample injection apparatus, 25 unknown samples can easily be run. Coupled with the micromethod for measurement of total cholesterol and capillary blood sampling (1, 2) the micro-HDL method allows quantitation of total and HDL cholesterol with a total plasma requirement of only 70 µl.

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