Quantitative analysis of cholesterol in 5 to 20 μl of plasma

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Summary A gas–liquid chromatographic micromethod for quantitation of cholesterol in 20 μl of plasma was developed using 5α-cholestane as an internal standard, saponification with tetramethylammonium hydroxide–isopropanol, and extraction with tetrachloroethylene–methyl butyrate. Cholesterol levels in plasma samples were calculated by comparing cholesterol–cholestane peak height ratios with those of preassayed reference plasma. Over a plasma cholesterol range of 44 to 468 mg/100 ml, the gas–liquid chromatographic micromethod and the automated ferric chloride colorimetric method gave nearly identical results ($r = 0.99$) in duplicate aliquots of 131 plasma samples.

Supplementary key words gas–liquid chromatography · cholesterol–cholestane peak height ratios

Recently we reported a practical micromethod (using 50 μl of plasma) for the determination of total cholesterol by GLC (1). This method is simple, precise, accurate, and gives results that correlate highly ($r = 0.99$) with the ferric chloride automated colorimetric method (2). This method is limited to a minimum sample size of 50 μl because further reduction in sample size can be accomplished only by sacrificing the resolution of the GLC chromatograms. We have revised our original method to utilize from 5 to 20 μl of plasma (3) with the same accuracy, precision, and sensitivity as reported for 50 μl. This revised micromethod should have particular usefulness in pediatric or small animal lipid studies by reducing blood sample size and utilizing capillary blood sampling.

Methods

Patients. The samples were obtained from unselected children and adults in the family lipid clinic of the General Clinical Research Center and from our ongoing cord blood screening program of unselected live births. 131 plasma samples were evaluated in parallel by the GLC and colorimetric methods. 49 samples came from pediatric follow-up patients (ages 7 months to 9 yr); 45 samples were obtained from adults, and 37 from cord bloods.

Gas–liquid chromatography: sample preparation. Using a 100-μl gas-tight microsyringe (Hamilton Co. no. 1910), 100 μl of a tetramethylammonium hydroxide–isopropanol (TMH-i) solution, containing 20 μg of 5α-cholestane (Applied Science Laboratories, no. 19505) as an internal standard, was delivered into a 3-ml glass-stoppered conical centrifuge tube. The TMH-i solution was made by diluting 20 mg of 5α-cholestane (dissolved in 2 ml of diethyl ether) and 25 ml of tetramethylammonium hydroxide (24% in methanol, Matheson Coleman & Bell [MCB], no. 9002) to 100 ml with isopropanol (MCB, no. SG2857). The TMH-i solution was placed in each of the centrifuge tubes for a set of 10–18 unknown plasma samples. 20 μl of plasma sample was then added with a disposable capillary pipet to the TMH-i solution. After the addition of 20 μl of plasma, the centrifuge tube was stoppered and mixed on a Vortex mixer for 10 sec. The tube was placed in a heating block for 15 min at 80°C. The tube was removed from the heating block and allowed to cool for 30 sec. Then 50 μl of a solution containing 1 part tetrachloroethylene (MCB, no. 5717) and 3 parts methyl butyrate (MCB, no. 7163) was added; the contents were thoroughly mixed for 15 sec. 200 μl of distilled water was then added to the tube, followed by vigorous hand shaking for 5 sec while holding the stopper firmly in place. The tube was then centrifuged at room temperature for 10 min at 2000–2500 rpm. 0.5–1 μl of the clear lower phase

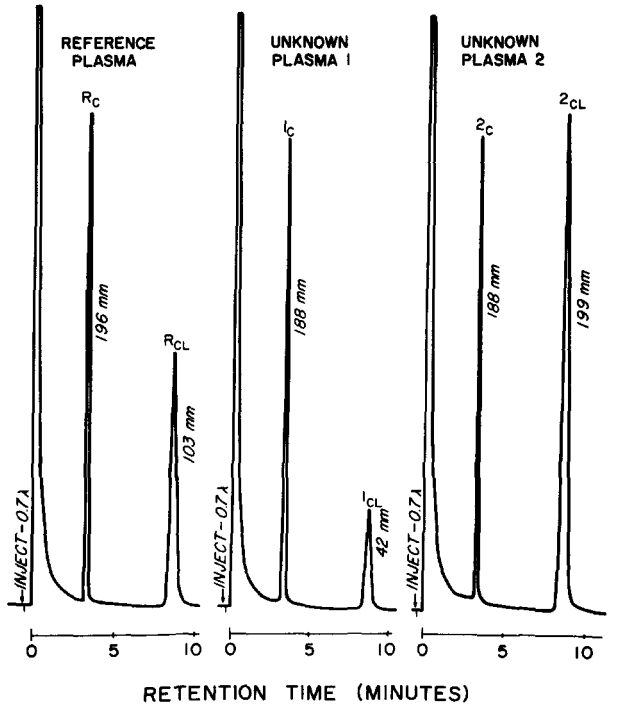

Fig. 1. Gas–liquid chromatogram of reference and unknown plasmas. R_c, peak height of cholestane of reference plasma; R_{cl}, peak height of cholesterol of reference plasma; 1_c, peak height of cholestane of unknown plasma 1; 1_{cl}, peak height of cholesterol of unknown plasma 1; 2_c, peak height of cholestane of unknown plasma 2; 2_{cl}, peak height of cholesterol of unknown plasma 2.

Abbreviations: GLC, gas–liquid chromatography; TMH, tetramethylammonium hydroxide.

(tetrachloroethylene) was injected into the gas chromatograph (Hewlett-Packard, model 402B) equipped with flame ionization detectors. The electrometer settings with a 1 mV (Hewlett-Packard, model 712A) recorder were range, 100, attenuator, 8. These settings conform to the signal-to-noise ratio sensitivity we have reported previously (1). The cholestane is completely eluted in 3.3 min and cholesterol in 8.7 min (Fig. 1). The inadvertent injection of the TMH upper phase has no deleterious effect on the column packing (1), in contrast to other saponifying reagents (4–6).

Extracts from 20-μl aliquots of two reference sera were injected before each run and after each 12 samples. Serachol, a weighed-in cholesterol reference serum (77% esterified cholesterol, Warner-Chilcott Laboratories), and Monitrol-I (Dade Div., American Hospital Supply Corp.) were used.

Columns and column packings. Two different columns (glass) were used with equivalent efficiency. Both were silanized with Sylon-CT (Supelco, Inc., no. 03-3065) before packing. The first column, 1.8 m × 2 mm (ID), was packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Laboratories, no. 12727). The second column, 1.2 m × 2 mm (ID) was packed with 3% SP-2250 on 100–120 mesh Supelcoport, (Supelco, no. 01-1985). In order to keep the packing material below the aluminum heating collar in the gas chromatograph, the columns were not packed within 5 cm of the end of the column on the injection port side; silanized glass wool (Alltech Associates, Inc., no. 4037) was used to contain the packing material. Column temperature was maintained at 260°C, the injection port was set at 290°C, and the detector was at 300°C. The nitrogen carrier gas was maintained at a flow rate of 50 ml/min, hydrogen was set at 30 ml/min, and nitrogen–oxygen (60%–40%) was set at 300 ml/min. Under these conditions the columns generally have an effective life of at least 9 months. The column life we have obtained, as opposed to those reported by others (4–7), is due in part to our use of a quaternary ammonium hydroxide saponifying agent, tetramethylammonium hydroxide, rather than an alkali metal hydroxide (1), and periodic treatment in situ with Silyl-8 (Pierce, no. 38014).

To minimize the effects of adsorption of cholesterol on the coated solid support, which could result in a low cholesterol–cholestane ratio (4–6), 2 μl of a solution of cholesterol (Supelco, no. 04-5000, 300 mg/100 ml of heptane) was used to prime the columns prior to the injection of a set of samples. 1 μl of the extracting solvent, tetrachloroethylene, was injected after priming to check for elution of the primer by solvent alone. Less than 1 mg/100 ml of the primer cholesterol was recovered by solvent injection alone.

Colorimetric analysis of cholesterol. The plasma samples analyzed by GLC were run in parallel on the AutoAnalyzer (AA-1 model) using the ferric chloride method (2). The extracts used in the colorimetric determinations were made according to the procedure described by Kessler and Lederer (8). A strict double-blind protocol was followed by five analysts in preparing, analyzing, calculating, and recording the results obtained by the two methods.

Calculations. The cholesterol concentration of the unknown samples was determined by comparing the cholesterol–cholestane peak height ratios of the sample with that of the Monitrol-I and Serachol reference sera. The serum cholesterol determination was based on resolution of a two-step formula, summarized in the Appendix, with a representative gas chromatogram (Fig. 1) used in the calculations.

Recovery of a weighed amount of pure material. Weighed amounts of pure cholesterol (National Bureau of Standards, no. 911) were solubilized in isopropanol and subjected to the GLC analytical procedures as described above as an additional approach in estimating the accuracy of the method. From peak height calculations, using the reference sera of the working analytical method, the amount of NBS cholesterol recovered was compared with its known initial weight, and percentage recovery was calculated. As a second recovery, a weighed sample of an aqueous cholesterol standard (Tekit-Searle Diagnostics Inc.) was carried through the working procedure in a manner similar to the NBS weighed sample. A weighed amount of cholesteryl palmitate (Supelco, no. 04-5001)

TABLE 1. Comparison between AutoAnalyzer (AA) and gas–liquid chromatographic methods for measurement of total plasma cholesterol

Patient Group	Mean	SD	SE	n	Correlation Coefficients[a] Pearson r	Correlation Coefficients[a] Spearman ρ
	mg/100 ml					
Adult						
AA	212.3	45.0	6.7			
				45	0.99	0.98
GLC	213.1	49.9	7.4			
Children						
AA	168.3	63.3	9.0			
				49	0.99	0.98
GLC	164.6	64.2	9.1			
Cord blood						
AA	68.8	14.7	2.4			
				37	0.95	0.94
GLC	69.1	15.0	2.5			
Above data combined						
AA	155.3	74.5	6.5			
				131	0.99	0.99
GLC	154.3	75.7	6.6			

[a] $P < 0.001$ in all cases.

was similarly analyzed to determine recovery of a cholesteryl ester. In addition, the efficiency of saponification of this cholesteryl ester was studied by subjecting replicate samples to saponification varying from 5 to 45 min.

Statistical analysis. The concordance of plasma cholesterol levels obtained by GLC and the AutoAnalyzer was calculated by the Pearson product moment correlation coefficient (9, 10) and by Spearman's rank-order method (9, 10). Differences between the colorimetric and GLC methods were also analyzed by two-way analysis of variance (11).

Results

Accuracy. Cholesterol values obtained by the ferric chloride and GLC methods in 131 plasma samples were very nearly identical (Table 1). These samples reflected population groups currently under study in our lipoprotein research screening clinics and included 37 cord blood

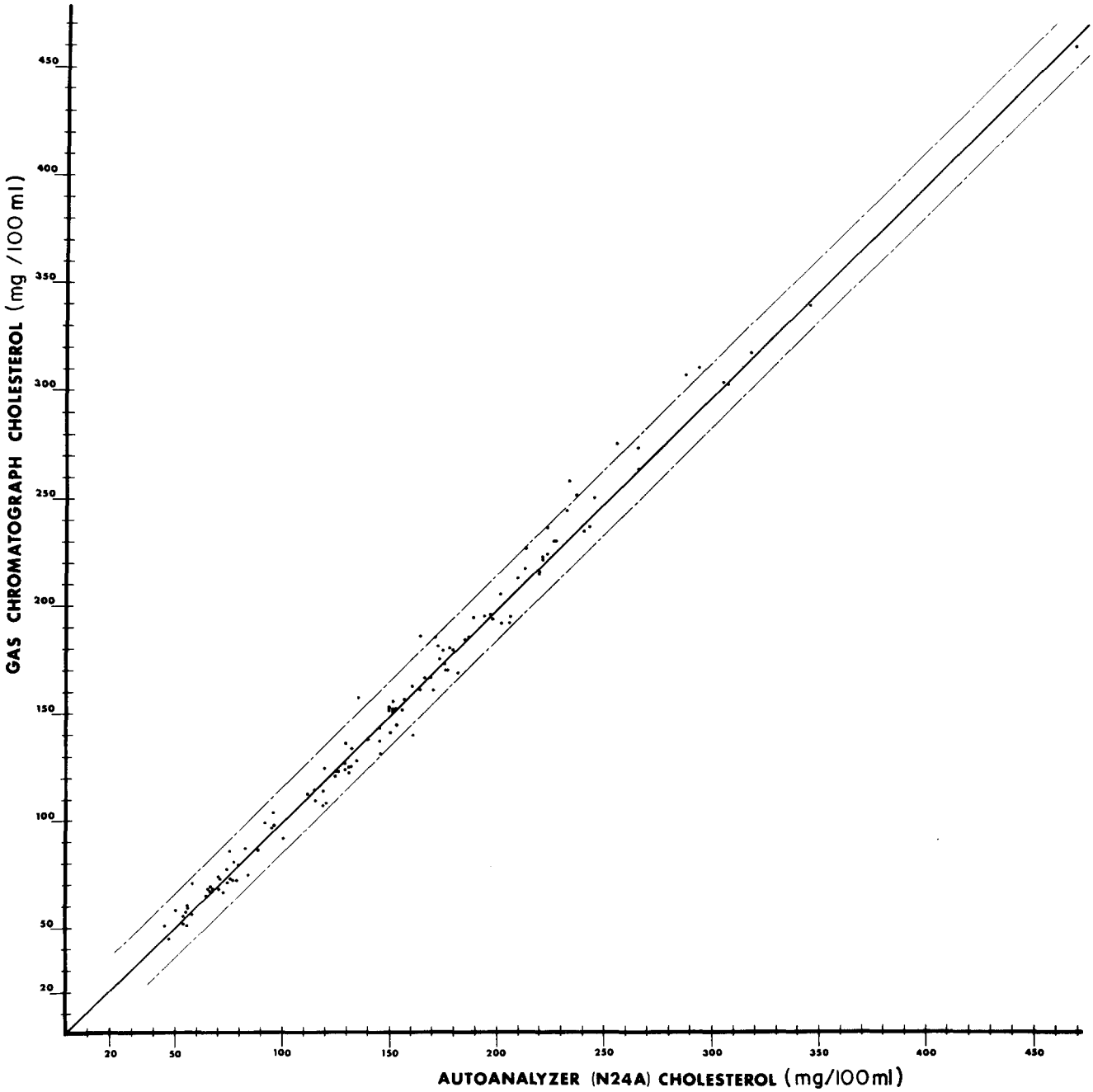

Fig. 2. Comparison of cholesterol levels measured by the GLC method with those measured by the automated colorimetric method (131 plasma samples). Solid line, best fit regression line; broken lines, 95% confidence limits for the regression line.

samples, 49 samples taken in follow-up of pediatric patients, and 45 samples from adults. Highly significant correlations were found between the cholesterol values obtained by the ferric chloride and the GLC methods for the samples from adults (mean = 212.3 mg/100 ml, r = 0.99), for the samples from the pediatric population (mean = 168.3 mg/100 ml, r = 0.99), and for the samples from cord blood (mean = 68.8 mg/100 ml, r = 0.95). A significant agreement (r = 0.99, $P < 0.001$) between the two methods was obtained for all 131 samples over a range in cholesterol values from 44 to 468 mg/100 ml (Table 1 and Fig. 2). Nonparametric analysis (Spearman rank-order correlation coefficient) revealed the same high degree of concordance between the ferric chloride and GLC methods (Table 1).

Using the ferric chloride determination as a standard, or independent variable, a linear regression line (Fig. 2), $y = a + bx$, was calculated using the least squares method. The regression coefficient was 1.01 with a standard error of 0.0089, and the y-axis intercept was −2.81. The 95% confidence limits about the regression line were essentially parallel (Fig. 2) through the range tested and permit prediction with 95% confidence of ± 16 mg/100 ml.

Using two-way analysis of variance (patients by methods), the differences in methods were not found to be significantly different (F = 2.411, df 1/130, $P > 0.12$) in spite of a sensitivity in this study that would have declared a difference between methods as small as 1.6 mg/100 ml significant at the 5% level.

Serial dilution of a pure cholesterol sample produced a linear response for the cholesterol–cholestane ratio over the range of 30–380 mg/100 ml (Fig. 3). This relationship was obtained when the column had approximately 6 months of use.

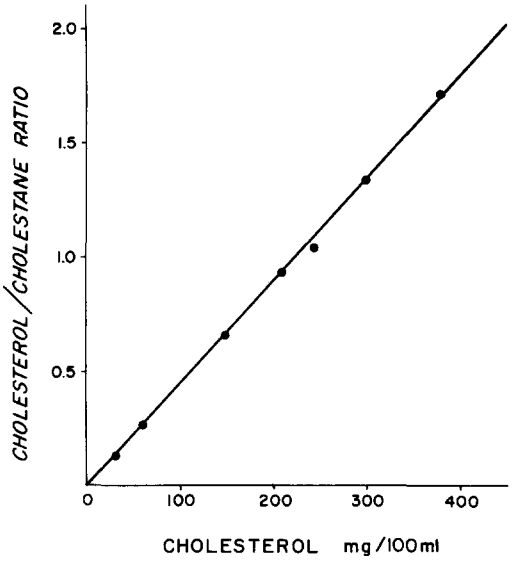

Fig. 3. Linear relationship between cholesterol–cholestane peak height ratios and concentration of a pure cholesterol sample after serial dilution.

A further indication of the accuracy of the GLC micromethod was seen in serial studies using a weighed amount of cholesterol and an aqueous cholesterol standard. Mean (± 1 SD) recoveries were, respectively, 99.9 ± 1.6% and 98.8 ± 2.0%. In studies using a weighed amount of cholesteryl palmitate the recovery was 95.4 ± 1.5%. Analysis of duration of saponification of cholesteryl palmitate from 5 to 45 min revealed that release of cholesterol was complete in 10 min at 80°C. The 15-min saponification in the working method (at 80°C) was more than adequate for complete saponification.

Precision. 36 aliquots of a single plasma sample were injected on a single day and were analyzed for "same-day" precision. The "same-day" coefficient of variation for the ferric chloride method was 1.0% and was 1.2% for the GLC method. "Inter-day" precision was estimated by analyzing a single reference sample of plasma for a period of 9 wk with repetitive parallel determinations by GLC and colorimetric methods. The coefficient of variation obtained with 25 samples was 2.4% for the colorimetric method and 1.7% for the GLC method.

Sensitivity. A definition of sensitivity summarized by Walker, Jackson, and Maynard (12) was applied to the GLC micromethod, i.e., sensitivity equals the ratio of signal response to background noise. At the maximum sensitivity of the instrument (attenuator setting 1, range 1), noise was 2.5 cm. An extrapolated level for noise under our standard operating conditions, attenuator 8, range 100, was 0.0031 cm. For the lowest range cholesterol sample measured (44 mg/100 ml), the signal-to-noise ratio was 1385/1, evidence of excellent sensitivity of this method.

Specificity of the micromethod was evaluated by comparison of retention times for cholestane and cholesterol (3.3 and 8.7 min, respectively, Fig. 1) with retention times of other serum sterols closely related to cholesterol (13, 14). Desmosterol, β-sitosterol, campesterol, stigmasterol, and α-tocopherol had clearly different retention times

TABLE 2. Retention times of sterols and α-tocopherol

	Stationary Phase	
	SP-2250	OV-17
	min	
Cholestane	3.3	3.3
Cholesterol	8.7	8.7
Cholestanol	8.7	8.7
Desmosterol	10.4	10.4
Campesterol	11.7	11.6
Stigmasterol	12.8	12.6
β-Sitosterol	14.3	14.2
α-Tocopherol	9.4	9.3

in this system compared with cholesterol. Only cholestanol had a similar retention time (Table 2).

Discussion

Availability of a micromethod for analysis of plasma cholesterol would simplify studies in pediatric and adult populations by making venesections unnecessary and by reducing sample size to 20 μl or less, well within the range of capillary blood screening. Studies in small animals would be facilitated.

An established, widely used colorimetric method for cholesterol measurement and our GLC micromethod gave nearly identical results, with a comparable coefficient of variation for both methods.

The micromethod requires only two precise volumetric measurements, 20 μl for the sample and 100 μl for the TMH-i solution. The method is quick, with final results available 45 min after receipt of the sample. Reagent costs, including internal standards and reference plasma are about one-tenth the cost for comparable studies using the colorimetric method. In a normal working day, without use of an automatic sample injector, 50 samples can easily be run in addition to a full battery of reference plasmas and standards.

This GLC method is accurate with as little as 5 or 10 μl of plasma. The coefficient of variation for the 5-μl plasma sample was 3.04%. In most clinical research laboratories, it may be more expedient to use the somewhat larger plasma sample size (20 μl).

The miniturization we have achieved over our previously reported procedure (1) was accomplished by using a mixture of tetrachloroethylene and methyl butyrate in place of ethyl acetate as the neutralizing and extracting solvent. This mixture was chosen in order to obtain the cholesterol in the lower phase. This allows for recovery of an aliquot from a much smaller volume of extractant than is possible with the upper phase extractant. Additional refinements of this micromethod might include shortening the GLC column to obtain shorter retention times and even higher recoveries of cholesterol (15), and quantitation of both free and esterified cholesterol by running duplicate aliquots with and without saponification.

The micromethod is simple, quick, inexpensive, and practical with conventional GLC instrumentation and easily available reagents. The method appears to be accurate, precise, sensitive, and specific for quantitation of cholesterol in plasma or serum.

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APPENDIX

Two-step formula for the determination of total plasma cholesterol

Step 1:

$$F = \frac{\text{concentration of cholesterol in reference serum (mg/100 ml)}}{\left(\frac{\text{peak height of cholesterol in reference serum}}{\text{peak height of cholestane in reference serum}}\right)}$$

Step 2: $\left(\frac{\text{peak height of cholesterol in unknown}}{\text{peak height of cholestane in unknown}}\right) \times F =$ cholesterol in unknown (mg/100 ml)

A representative calculation is given below for determination of cholesterol in unknown plasma, Fig. 1, using the following abbreviations:

$CRcl$ = concentration of cholesterol in reference serum (mg/100 ml)
Rcl = peak height of cholesterol of reference serum
Rc = peak height of cholestane of reference serum
$1cl$ = peak height of cholesterol of unknown 1
$1c$ = peak height of cholestane of unknown 1
$2cl$ = peak height of cholesterol of unknown 2
$2c$ = peak height of cholestane of unknown 2

Step 1: $F = \frac{CRcl}{\left(\frac{Rcl}{Rc}\right)} = \frac{142 \text{ mg/100 ml}}{\left(\frac{103 \text{ mm}}{196 \text{ mm}}\right)} = 270.48 \text{ mg/100 ml}$

Step 2: $\frac{1cl}{1c} \times F = \frac{42 \text{ mm}}{188 \text{ mm}} \times 270.48 \text{ mg/100 ml} =$ 60.32 mg/100 ml cholesterol in unknown 1

$\frac{2cl}{2c} \times F = \frac{199 \text{ mm}}{188 \text{ mm}} \times 270.48 \text{ mg/100 ml} =$ 286.16 mg/100 ml cholesterol in unknown 2

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