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COMPARATIVE DETERMINATION OF PLASMA CHOLESTEROL AND TRIACYLGLYCEROL LEVELS BY AUTOMATED GAS—LIQUID CHROMATOGRAPHIC AND AUTOANALYZER METHODS

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SUMMARY

Plasma samples obtained during a prevalence study of hyperlipemia in a free living urban population were analyzed for total cholesterol and triacylglycerol content by automated high-temperature gas—liquid chromatographic (GLC) and automated colorimetric (Auto-Analyzer, AA11) methods. The analyses were done over a three-year period. The methods gave excellent overall correlation for both total cholesterol (r = 0.9811) and total triacylglycerols (r = 0.9739). Detailed comparisons of the results obtained by the two methods with natural samples over the entire concentration range, indicated that the GLC method gave cholesterol values 5—10 mg% lower and triacylglycerol values 10—20 mg% lower than the corresponding AA11 determinations. The differences between the two methods are attributed to an overestimation of the cholesterol and triacylglycerol levels by the AA11 method due to presence of variable amounts of interfering chromogens in the plasma extracts. The between-method relative error ranged from 3 to 5% for cholesterol and from 5 to 10% for triacylglycerols. The within-day standard deviation of GLC averaged 2.3 mg% for cholesterol and 3.5 mg% for triacylglycerols. The between-day standard deviation of the GLC method averaged about 6 mg% for both cholesterol and triacylglycerols. The within-day, within GLC, relative error averaged 1.12% for cholesterol and 2.66% for triacylglycerols. The apparent high precision and high accuracy of the GLC method recommend it as an alternative to the indirect methods of plasma cholesterol and triacylglycerol analysis, especially where a smaller throughput of samples is not a limitation and where both total amount and composition of the lipids is of interest.

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INTRODUCTION

High-temperature gas—liquid chromatography (GLC) is a sensitive and rapid technique by which neutral plasma lipids can be separated into individual lipid classes or molecular species [1—5] and accurate identification and quantitation obtained for each component. We have recently demonstrated that this method of total plasma lipid analysis can be automated and that the results obtained for total cholesterol and total triacylglycerols compare favourably to those realized with colorimetric methods, when tested with reference standards [4]. In the present study we have compared the results of the GLC and the AutoAnalyzer methods for analysis of total cholesterol and triacylglycerols in over 1000 samples of plasma from a free living urban population. In general the GLC method gave values which were 5—20 mg% below those of the AutoAnalyzer method [6]. The discrepancies are traced to various inherent errors and biases in the GLC and AutoAnalyzer methods.

MATERIALS AND METHODS

The standard acylglycerol, free cholesterol and cholesteryl ester mixtures employed in the study were prepared in the laboratory from chromatographically pure (99% + single components) materials supplied by Serdary Research Labs. (London, Canada) and the Applied Science Labs. (State College, Pa., U.S.A.). BDH Control Sera were obtained from BDH (Toronto, Canada). To each vial of the latter containing the freeze-dried solids from 10 ml plasma, 10 ml of distilled water were added to obtain complete solution. Other control plasma samples of known content of total cholesterol and total triacylglycerol (samples LRC 1, LRC 2, and LRC 3), and the unknown samples from a population survey were supplied by the Toronto-McMaster Lipid Research Clinic (University of Toronto, Toronto, Canada). The unknown plasma samples had been prepared from fresh blood collected in ethylene diaminetetraacetate-containing vials, and were stored in a frozen state at —20° for a maximum of 3 months before analysis. Prior to withdrawal of any aliquots the thawed samples were thoroughly shaken to avoid concentration gradients [7]. Phospholipase C (α-toxin of *Clostridium welchii*) was purchased from Sigma (St. Louis, Mo., U.S.A.). Trisil—BSA reagent was supplied by Pierce (Rockford, Ill., U.S.A.). Other reagents and solvents were of Fisher certified-reagent grade and were tested for lipid contaminants prior to use.

Preparation of samples for analysis

EDTA (0.01%)—plasma (0.2—0.5 ml) was added to a PTFE-lined screw-cap centrifuge tube (18-ml capacity) containing 0.2—0.4 mg phospholipase C in 4 ml of 17.5 m*M* Tris buffer, pH 7.3, along with 1.3 ml of 1% $CaCl_2$ and 1 ml of diethyl ether, and the mixture incubated with shaking for 2 h at 30°. The reaction mixture was then treated with 5 drops of 0.1 *N* HCl and extracted once by vigorous shaking with 10 ml of chloroform—methanol (2:1) containing 150—250 μg tridecanoylglycerol as internal standard. The solvent phases were separated by centrifuging for 10 min at 200 *g*. The clear chloroform phase was removed from the bottom of the tube and was dried by passing

through a Pasteur pipet containing 2 g of anhydrous sodium sulphate. The effluent was evaporated under nitrogen and the residue dissolved in Trisil—BSA (150—250 μl) and transferred to a sampling vial, and the vial sealed.

Gas chromatographic methods

The automated high-temperature GLC analysis was performed on a Hewlett-Packard Model 5700 A automatic gas chromatograph equipped with dual stainless-steel columns (50 cm × 2 mm I.D.) containing 3% OV-1 on 100—120 mesh Gas-Chrom Q (Applied Science Labs.) and an automatic liquid sample injector (Model HP 7671 A), as previously described [4]. The GLC separations were routinely made by temperature programming from 175 to 350° at either 4 or 8°/min with the columns in the dual compensating mode and dry nitrogen as the carrier gas (40 ml/min).

After every 200 analyses the first 1—2 in. of the column packing were replaced with fresh packing and the columns reconditioned at 350° with the normal carrier gas flow. Silicone oxide deposits, which accumulated on the detector due to injection of the silylation mixture, were routinely removed by scrubbing with chloroform every two weeks.

The integrator output was simultaneously recorded on a paper chart and on a punched paper tape indicating the tube number, the peak retention time and area in a computer compatible ASC 11 code, which is a basic language program for off-line data processing. The punched tape record was processed using modifications of the computer programs provided by Hewlett-Packard (CALIST, CALIB and HP7600), as previously described [4]. The peak areas for the free cholesterol and cholesteryl esters, and the triacylglycerols, respectively, were summed using appropriate calibration and conversion factors to provide estimates for total-plasma cholesterol and triacylglycerols. It was noted (see Results) that the integrator record could be quite erratic for certain slope sensitivity settings and that it required a systematic examination for errors in baseline resetting if precise results were to be obtained. The absolute amounts of plasma lipids were quantitated by means of an internal standard (tridecanoylglycerol) added to the plasma at the time of lipid extraction at a relative proportion of 10—20% of total. The quality of the analytical results was controlled by systematic monitoring of a synthetic and a natural plasma external reference standard, which were analyzed simultaneously with any unknown samples.

AutoAnalyzer methods

The colorimetric analyses were performed with an AutoAnalyzer AA11 (Technicon, Tarrytown, N.Y., U.S.A.) instrument. The estimates for total cholesterol and total triacylglycerols were obtained on Zeolite-treated isopropanol extracts as outlined in the Manual of Laboratory Operations, Lipid Research Clinics Program [8]. Pure cholesterol and trioleoylglycerol (triolein) standards and a serum calibrator were supplied by the Lipid Standardization Laboratory (Center for Disease Control, Atlanta, Ga., U.S.A.). Each AA11 run was initially set up and checked with free cholesterol standards. Subsequently the output was adjusted downward on the basis of a daily analysis of a cholesterol serum calibrator with a cholesterol value determined by the

method of Abell and Kendall as modified by the Lipid Standardization Laboratory [8]. When a step-up series of cholesterol standards was analyzed, the AA11 generally gave a cholesterol value of 325 ± 5 mg% for a serum calibrator with a target value of 296. The triacylglycerols were determined by color development with the glycerol liberated from the neutral lipid extract of plasma upon saponification. The glycerol yield was expressed as mg% of trioleoylglycerol.

Statistical analysis

The evaluation of the GLC procedure for cholesterol and triacylglycerol determination was modeled on a comparable study of methodology reported by Lippel et al. [9]. Systematic errors were measured by the difference between average GLC values and the AA11 or target values by a bias statistic. The magnitude of random errors was measured by the variance or standard deviation. The relative error values are averages of percent deviations defined as:

$$\text{Relative error} = \frac{\text{GLC value} - \text{AA11 Value}}{\text{AA11 value}} \times 100$$

A coefficient of variation between duplicates was calculated using the formula

$$\text{C.V. \%} = \frac{100\sqrt{d^2/2}}{\bar{x}}$$

where d is the difference between duplicates and $\bar{x}$ is the mean.

The within-day standard deviation was used as the measure of within-day variability. The overall standard deviation was used as the measure of the variability of a single determination of a quality control sample by the GLC method. A correlation coefficient and a regression coefficient for the data were calculated according to established statistical procedure [10]. A Hewlett-Packard HP-9821A programmable calculator was used to assist in these determinations.

RESULTS

The overall analytical routine was tested with standard mixtures of neutral lipids prepared in the laboratory and with standard plasma lipid samples purchased commercially or acquired from the Lipid Research Clinics Program and satisfactory results were obtained as previously described [4]. It remained to be demonstrated that such analyses could be performed routinely on a large number of unknown samples and to establish how the GLC values compared to those obtained by the AutoAnalyzer method generally employed for the determination of plasma total cholesterol and triacylglycerols in clinical laboratories.

Acquisition of data

Following the preliminary enzymic and chemical modification of the plasma lipids, the quantitative estimates of plasma total cholesterol and triacylglycerols

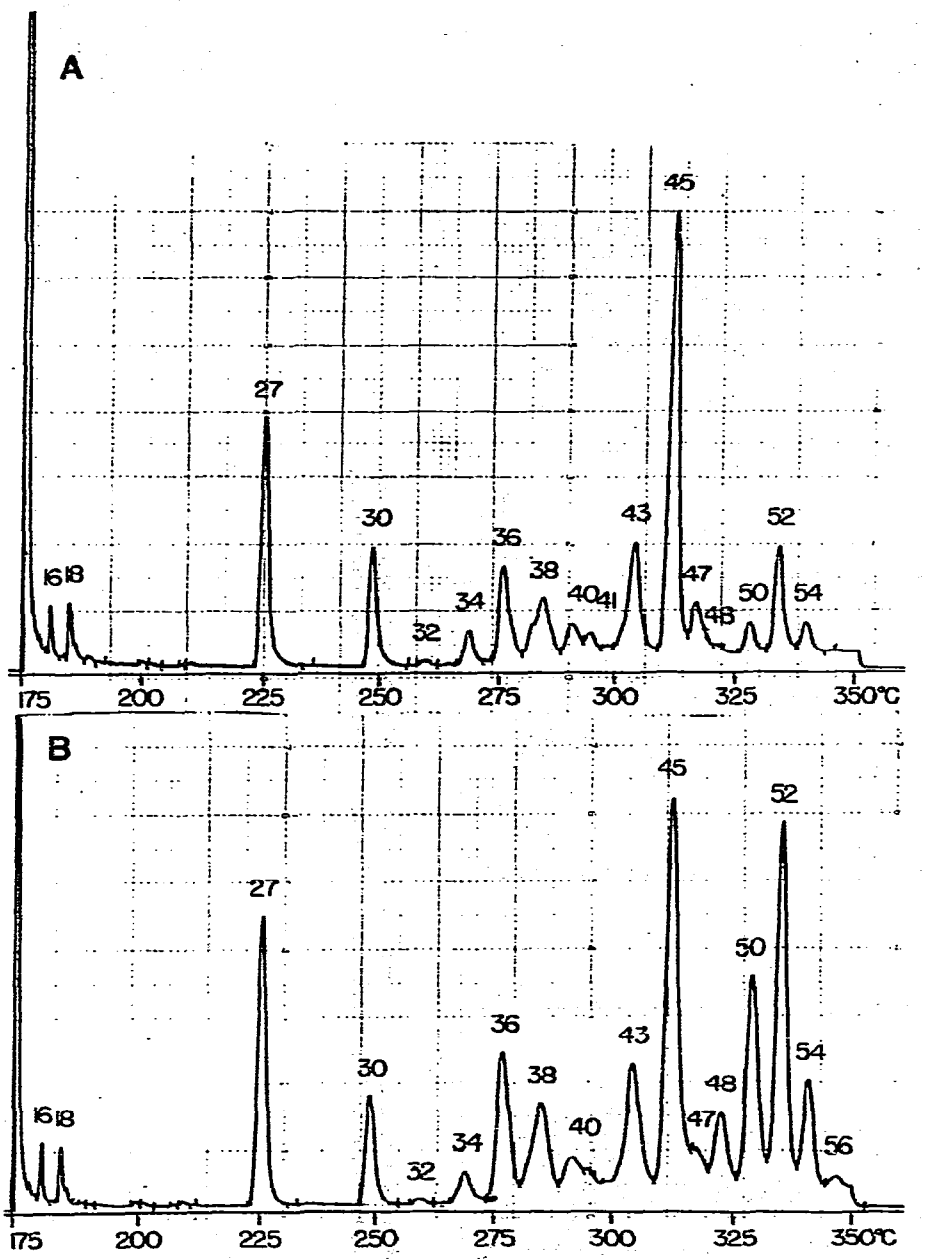

Fig. 1. Total lipid profiles (A) a normolipemic and (B) a hyperlipemic plasma as obtained using a low-temperature gradient GLC. Conditions of high-temperature GLC as given in text. Peaks 16 and 18, trimethylsilylesters of free fatty acids with 16 and 18 acyl carbons; peak 27, trimethylsilylether of cholesterol; peak 30, tridecanoylglycerol internal standard; peak 34, trimethylsilylether of palmitoylsphingosine; peaks 36—42, trimethylsilylethers of diacylglycerols of a total number of 34—40 acyl carbons; peaks 43—47, cholesteryl esters of fatty acids with a total number of 16—20 acyl carbons; peaks 48—56, triacylglycerols with a total number of 48—56 acyl carbons. Sample size: 1 μl of an approximately 1% solution in silylation mixture. Attenuation: 100 times full sensitivity.

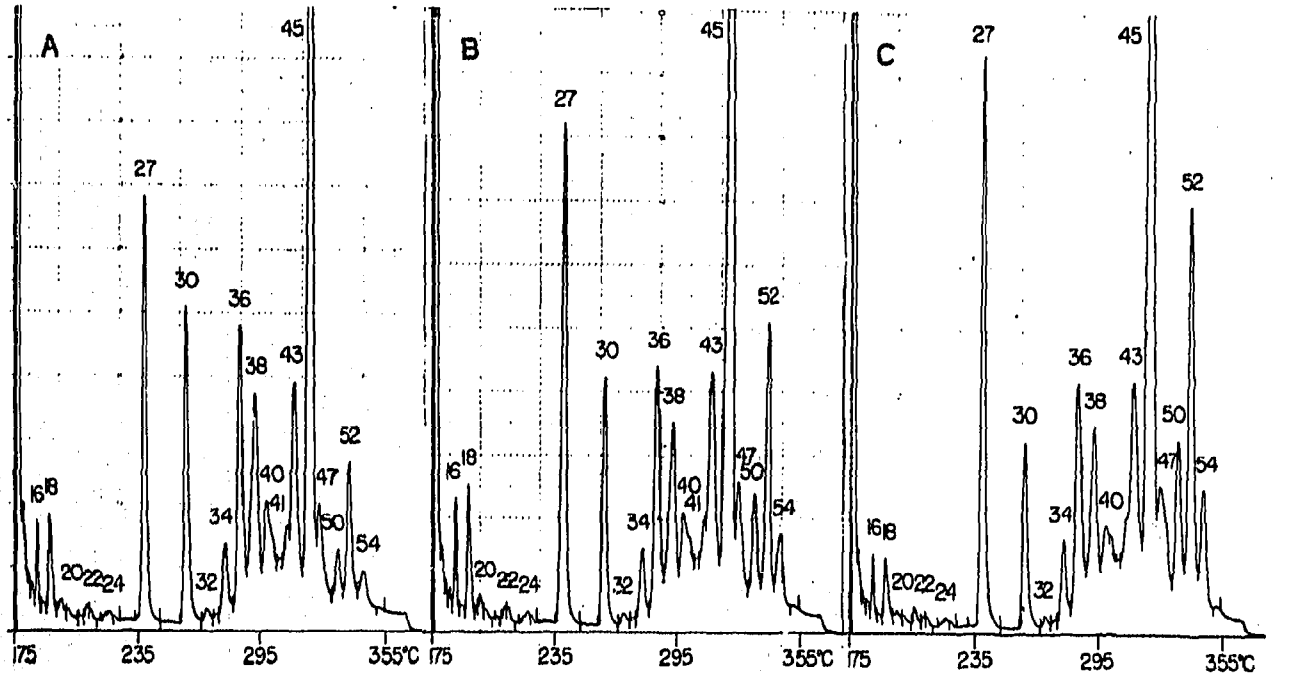

Fig. 2. Total-lipid profiles of plasma of varying lipid content as obtained by a high-temperature gradient GLC. Conditions of high-temperature GLC as given in text. Peak identification as in Fig. 1.

were obtained by GLC using two different rates of temperature programming. For optimum resolution of peaks the column temperature was programmed in the range 175—350° at a rate of 4°/min. Fig. 1 shows representative total plasma lipid profiles of a normal subject and a patient with hyperlipoproteinemia. It is seen that the peaks for free cholesterol, the tridecanoylglycerol internal standard, and the various molecular species of the cholesteryl esters and triacylglycerols are clearly resolved for both the normolipemic and the hyperlipemic plasma, although some overlap may occur for the cholesteryl esters and triacylglycerols in the hyperlipemic plasma. The partial overlap of the cholesteryl esters and triacylglycerols may be avoided by appropriate dilution of the sample and suitable adjustment in the amount of the added internal standard. The overlapping of the cholesteryl esters and triacylglycerols is much more serious and cannot be avoided by dilution of the sample when it is due to high levels of short-chain fatty acids in the triacylglycerol fraction. Under normal conditions, however, short-chain fatty acids are largely absent from plasma triacylglycerols and carbon numbers of C_{42}—C_{46} usually make up only a minor proportion of the total mass of plasma triacylglycerols. It should also be noted that in all instances the baseline elevation due to column bleed has been minimal, as indicated by the small difference between the final baseline elevation and the point of the last baseline reset (usually between the peaks for free cholesterol and the tridecanoylglycerol). Despite the excellent peak resolution, this program failed to give a reliable peak area quantitation by automated integration due to erratic baseline reset by the peak slope sensor (see below).

Fig. 2 gives the total lipid profiles of three plasma samples of varying total lipid content as obtained in the temperature range 175—350° using a heating rate of 8°/min. Under these conditions the slopes of the peaks are much steeper and the frequency of erratic baseline resetting much lower. However, there is also much more peak overlapping than when the lower rate of temperature programming is employed. Nevertheless, the major chemical classes and molecular species of cholesteryl esters and acylglycerols are reasonably well resolved so that precise and accurate quantitation of peak areas may be expected. Furthermore, a faster program rate improves the detection and quantitation of the peak areas due to minor components (monoacylglycerols). The faster program rate also shortens the overall time of analysis and thereby minimizes the decomposition of the more sensitive components and increases the overall efficiency of the operation of the analytical system. With proper peak area measurements both temperature programming rates gave comparable quantitative results, but the esthetic impression was more favourable with the slower heating rate.

In both instances the total cholesterol value was obtained by adding the peak areas for the free cholesterol and the various cholesteryl esters using appropriate calibration factors and molar conversion ratios. The total triacylglycerols were calculated by summing the peak areas in the range C_{48}—C_{56} using appropriate peak area correction factors. Alternatively the peak areas were summed over a range of preselected elution times (windows) and overall correction factors applied [4]. This method avoided the need for

TABLE I

FREQUENCY OF DIFFERENT ERRORS IN AN AUTOMATED GLC ANALYSIS OF TOTAL LIPIDS OF FROZEN PLASMA SAMPLES

Number of samples	Error		
	Faulty integration	Copying error	Non-representative sampling*
Slow temperature program (4°/min)			
Batch of 800	130	10	40
Batch of 250	14	2	4
Batch of 400	45	6	20
Total 1450	189	18	66
Fast temperature program (8°/min)			
Batch of 197	8	2	5
Batch of 400	23	2	12
Batch of 100	2	0	3
Total 697	32	4	20

*Non-representative sampling due to incomplete dissolution of precipitate in frozen plasma samples.

accurate peak identification and the use of specific correction factors, which was not always possible to obtain.

Table I gives the frequency of erratic resetting of baseline by the electronic peak area integrator. With the slower temperature program over 10% of the runs contained baseline resetting errors. This problem was corrected to large extent (less than 5% error) by substituting a faster temperature program, which produced steeper peak slopes. The faster program also appears to have decreased the other errors, but this was due to greater care taken in sample handling and in data recording in the more recent experiments.

Precision of analyses

A measure of the precision or reproducibility of the GLC method of determining total cholesterol and total triacylglycerols in unknown plasma was obtained by calculation of the standard deviation and the coefficient of variation on repeat analyses at several levels of concentration of plasma lipids. Table II gives the mean values, standard deviations and coefficients of variation for 4 repeat analyses for total cholesterol and total triacylglycerols from 17 random samples of plasma. The overall coefficients of variation are 1.12 and 2.66% for the total cholesterol and total triacylglycerols, respectively, The reproducibility of these values or the precision of the GLC analysis itself for the natural samples is therefore of the same order as that previously observed for standard free cholesterol and triacylglycerols by this method [4]. Table III gives the range of values, mean of values, standard deviations and the

TABLE II

PRECISION OF QUADRUPLICATE REPEAT GLC ANALYSES OF PLASMA TOTAL CHOLESTEROL AND TRIACYLGLYCEROLS

Each sample was injected four times into the gas chromatograph in four cycles over a period of two days. C.V. = coefficient of variation; S.D. = standard deviation.

Sample	Cholesterol		Triacylglycerol	
	Mean ± S.D.	C.V. (%)	Mean ± S.D.	C.V. (%)
1	187.8±0.64	0.34	31.7±1.0	3.2
2	182.5±4.5	2.5	32.8±1.3	4.1
3	168.0±1.2	0.70	18.2±0.30	1.6
4	151.3±0.75	0.50	38.5±1.15	3.0
5	140.5±1.4	1.00	48.1±2.5	5.2
6	143.9±0.55	0.38	58.1±0.85	1.5
7	132.4±1.6	1.2	35.7±2.3	6.3
8	130.6±3.0	2.3	50.9±0.83	1.6
9	129.1±0.67	0.52	41.1±0.89	2.2
10	156.3±1.9	1.2	42.9±0.65	1.5
11	132.1±0.3	0.23	52.9±0.48	0.90
12	154.0±2.6	1.7	57.4±1.7	3.0
13	158.4±0.13	0.08	53.7±0.59	0.93
14	143.9±0.48	0.34	36.3±0.93	2.5
15	140.5±4.3	3.0	65.5±1.1	1.7
16	138.5±3.5	2.5	50.4±1.8	3.6
17	130.9±0.64	0.49	36.2±0.82	2.3
Average	148.2±1.66	1.12	45.1±1.12	2.66

coefficients of variation for the within-day variation of the estimates of total cholesterol and triacylglycerols as obtained on three samples of standard plasma of markedly different total lipid content following decaplicate repetition of the entire analytical routine on each sample, but excluding major adjustments in instrumentation. The overall coefficients of variation obtained in this instance are 1.14 and 1.93% for total cholesterol and total triacylglycerols, respectively. These values again are of the order obtained on repeat injections of the same sample. Table IV gives the range of values, the means and the standard deviations of the within-day and between-day variation observed for the entire GLC method when major instrument adjustments are also included. These variations were recorded for an external quality control standard over a 60-day period. It is seen that the within-day standard deviations of 2.2 and 3.0 mg% for total cholesterol and total triacylglycerols, respectively, are somewhat higher than those observed for the within-day repeat injections of the same sample or for repeat processing and analysis of the same sample including appropriate correction factors (Tables II and III, respectively). However, the between-day variation was highly significant and emphasized the need for the external standard for quality control of the analyses. When the unknown values are corrected for the day-to-day variation of the external reference standard, the standard deviations of the day-to-day variation become of the order of those seen for the within-day varia-

TABLE III

WITHIN-DAY VARIATION OF THE ENTIRE GLC METHOD OF ANALYSIS OF TOTAL CHOLESTEROL AND TOTAL TRIACYLGLYCEROLS ON REFERENCE PLASMA SAMPLES

The individual samples were pools of plasma from normolipemic and hyperlipemic subjects by the Toronto-McMaster Lipid Research Clinic

Plasma samples	Range (mg%) of decaplicate analyses		Mean values (mg%) ± S.D. of decaplicates		Coefficient of variation of decaplicates (%)	
	Cholesterol	Triacylglycerols	Cholesterol	Triacylglycerols	Cholesterol	Triacylglycerols
LRC 1	177–188	72–78	180.30±3.09	75.181±1.83	1.71	2.43
LRC 2	258–264	150–157	260.90±2.46	153.50 ±2.76	0.94	1.80
LRC 3	374–382	270–281	378.56±2.96	276.40 ±4.27	0.78	1.55
Average					1.14	1.93

TABLE IV

WITHIN-DAY AND DAY-TO-DAY VARIATION OF THE ENTIRE GLC METHOD OF ANALYSIS OF TOTAL CHOLESTEROL AND TOTAL TRIACYLGLYCEROLS IN A QUALITY CONTROL SAMPLE

The days of analyses were taken from a 60-day period in January, February and March of 1976. In addition to the entire routine of sample preparation, the method included bi-weekly detector cleaning, one column replacement, one detector jet replacement and one replacement of septum.

Days of analysis	Total cholesterol (mg%)		Total triacylglycerols (mg%)	
	Range	Mean ± S.D.	Range	Mean ± S.D.
1	153.6—155.7	154.9±0.85	87.6—89.4	88.4±0.75
2	168.6—173.0	170.2±2.33	89.1—96.4	92.5±2.75
3	156.0—159.3	156.2±2.18	83.6—94.0	88.3±3.98
4	161.4—166.0	163.5±2.11	95.8—97.6	96.8±0.84
5	152.6—160.4	154.9±3.63	91.6—106.3	97.7±6.5
6	161.7—165.5	164.6±3.01	99.4—114.0	108.6±5.13
7	166.4—178.7	169.6±2.3	95.7—106.5	102.7±4.6
8	170.0—171.7	171.8±1.34	101.2—104.8	103.3±1.74
9	164.6—169.7	168.7±2.88	96.5—101.7	93.3±2.24
10	166.9—170.5	168.7±1.42	101.1—104.1	102.7±1.35
11	168.3—171.7	169.6±1.42	98.5—102.2	100.8±1.60
12	165.0—170.7	168.3±2.29	96.5—106.7	103.1±4.21
Overall Mean		165.1		98.2
Total S.D.		6.3		6.5
Within-day S.D.		2.2		3.0
Between-day S.D.		6.3 ($P < 0.01$)		6.2 ($P < 0.01$)

tions. On the basis of these data it is concluded that the maximum error observed with the GLC method does not exceed 5%, which is about twice the coefficient of variation of repeat injections.

Fig. 3 shows a plot of the GLC results of duplicate analyses of 227 random samples of plasma for total cholesterol as obtained by analyses extending over a period of two years. A similar plot for total triacylglycerols is shown in Fig. 4. With few exceptions, only one determination was made per sample. However, all the GLC elution patterns were examined for errors in baseline resetting and where necessary, the peak areas were recalculated by cutting out and weighing the paper obtained by xeroxing the appropriate parts of the GLC records. A total of 56 runs were corrected in this way. There is an excellent agreement between the corresponding values, over 90% of which are found to be within ±10% of the ideal correlation line with intercept 0 and slope equal to 1.0. The average A and B values are 200.7±49.5 and 198.4±48.7 mg% for total cholesterol and 139.5±120.4 and 138.4±110.0 mg% for total triacylglycerols, respectively. The correlation coefficients for the cholesterol and triacylglycerol analyses of the A and B samples were 0.9348 and 0.9790, respectively. A calculation of the coefficient of variation of the duplicates gave values of 5.0 and 10.5% for the A and B samples of total cholesterol and triacylglycerols, respectively. These coefficients of variation are consid-

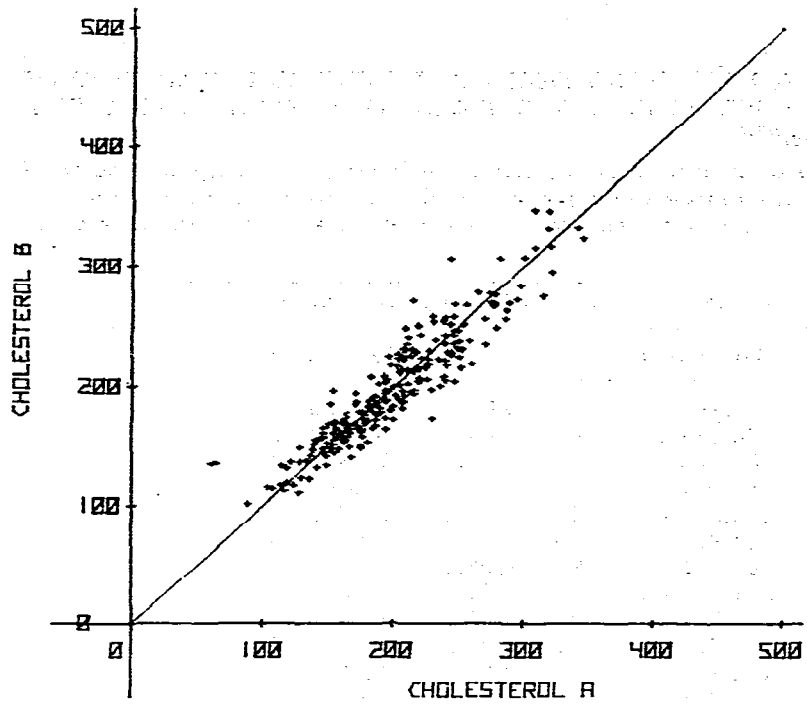

Fig. 3. Comparison of results (mg%) obtained by GLC for duplicate samples of plasma over a period of two years.

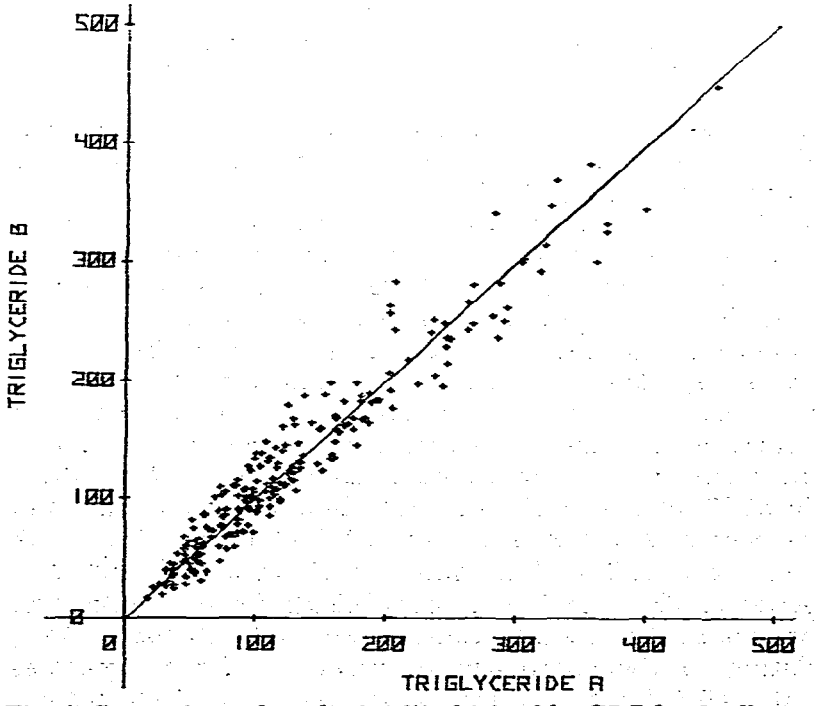

Fig. 4. Comparison of results (mg%) obtained by GLC for duplicate samples of plasma over a period of two years.

erably higher than those of the relative errors calculated from the multiple repeat analyses carried out over a shorter period of time. Nevertheless, it is obvious that the analytical system reproduces its results with high precision, as already noted for a manual version of the method in triacylglycerol analyses [11].

Bias

Any bias in the GLC analysis was assessed by comparing the results with the AutoAnalyzer target values. Table V gives the average differences of 10 repeat analyses of the plasma pools LRC 1, LRC 2 and LRC 3 and the target values at three different times of analysis. The GLC values for both total cholesterol and total triacylglycerols are about 5—10% lower than those obtained by the AutoAnalyzer method for the same samples. The bias (mg%) defined as the average of the errors for each pool, varies from 5—17 mg% in direct proportion to the total lipid level. The results have been compared by calculating the relative error in percentage units.

Fig. 5 shows a plot of the values obtained for total cholesterol on 197 plasma samples selected for maximum range of values by the AutoAnalyzer and the automated GLC procedures over a period of a few months. Although in most instances only a single GLC determination was made, an excellent agreement appears to have been realized over the entire concentration range, with a correlation coefficient of 0.9799 and a regression coefficient of 1.0177. The means and standard deviations of the AutoAnalyzer and GLC estimates were 236.28±92.46 and 236.23±96.03 mg%, respectively. The coefficient

TABLE V

COMPARISON OF RESULTS OBTAINED BY AA11 AND GLC METHODS FOR PLASMA POOLS OF WIDELY DIFFERENT TOTAL LIPID CONTENT

Analyses 1—3 were obtained on three separate days within a 2-week period.

Statistic	Target values					
	Cholesterol			Triacylglycerols		
	185	275	396	82	160	290
Analysis 1						
Bias (mg%)	—5	—15	—17	—7	—6	—14
Rel. error (%)	3.0	3.5	5.0	9.0	4.0	5.0
Ave. rel. bias (%)		4.5			6.0	
Analysis 2						
Bias (mg%)	—9	—12	—17	—3	—8	—14
Rel. error (%)	4.8	4.3	5.0	3.6	5.0	5.0
Rel. bias (%)		4.7			4.5	
Analysis 3						
Bias (mg%)	—6	—1	—18	—8	—6	—13
Rel. error (%)	3.2	0.4	4.5	9.7	3.7	4.5
Rel. bias (%)		2.7			6.0	

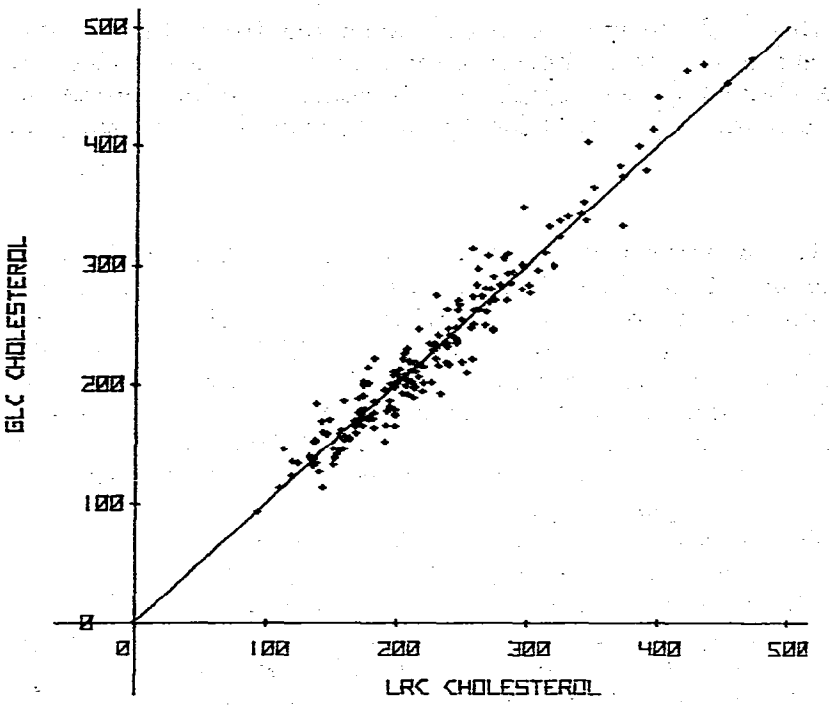

Fig. 5. Comparison of results (mg%) obtained for total plasma cholesterol by GLC and AutoAnalyzer methods over a period of a few months.

of variation between pairs analyzed by the two methods was 4.5%. This indicates that the agreement between the AutoAnalyzer and the automated GLC procedures is about as good as that obtained for repeat analyses of the same sample of plasma by the GLC method alone. Fig. 6 shows a plot of the values obtained for total triacylglycerols on the 197 selected plasma samples. Again with the exception of a few instances only a single determination was made on each sample. The overall correlation coefficient was 0.9837 and the regression coefficient 0.9067. The means and standard deviations of the AutoAnalyzer and the GLC estimates were 345.98±474.08 and 325.16±436.97 mg%, respectively. The extremely high standard deviations arise from the wide range of triacylglycerol concentrations encountered in these samples. The coefficient of variation between duplicates was 8.5%. In contrast to the excellent agreement between the estimates for total cholesterol the AutoAnalyzer and the GLC methods appear to disagree on the values for total plasma triacylglycerols. The GLC method seems to underestimate the total plasma triacylglycerol level by about 20 mg%. An inspection of the plot reveals that there are several plasma samples which differ by up to 50 mg% in the estimated content of triacylglycerols. An examination of the GLC elution patterns and computer print-outs for errors in computation failed to reveal any and suggested that a true bias existed in either of the two methods of analysis. The excellent correlation between the AutoAnalyzer and the GLC estimates, however, indicates that both methods are assessing essentially the same components. It is suggested in discussion that the AutoAnalyzer method may

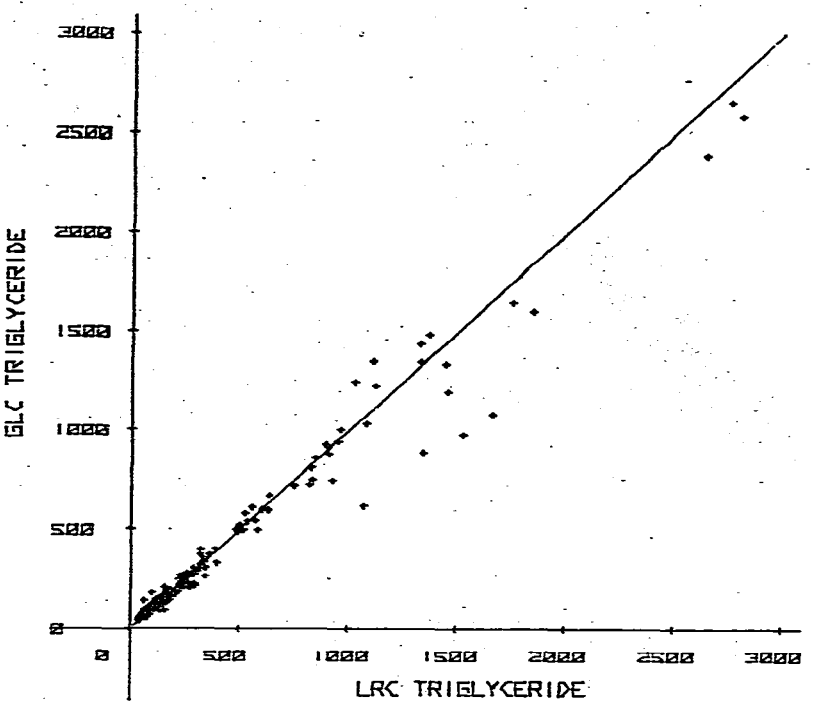

Fig. 6. Comparison of results (mg%) for total plasma triacylglycerols as obtained by GLC and AutoAnalyzer methods over a period of a few months.

give overestimated values due to the manner of expression of data, and due to the presence of non-specific chromogens and partial acylglycerols in the plasma samples.

Fig. 7 shows a plot of the GLC values of total cholesterol versus the AutoAnalyzer target values for 794 random samples of plasma as obtained over a period of two years. Since this collection of samples includes a high proportion of runs recorded using the lower rate of temperature programming there were numerous errors in baseline resetting, the correction of which required the recalculation of the GLC data by cutting out and weighing the peak areas. About 200 samples were corrected in this way. The overall correlation coefficient was 0.98108 with a regression coefficient of 0.9696. The means and standard deviations for total cholesterol of the AutoAnalyzer and the GLC estimates were 217.39±45.93 and 207.03±48.89 mg%, respectively. The coefficient of variation between duplicates was 6.1%. It is seen that over the extended period of time of analysis, involving numerous changes in the operating conditions, columns and liquid phases, as well as recalibrations of the system and dilutions of new batches of internal standard, a good general agreement was realized for the estimates of total plasma cholesterol by the AutoAnalyzer and the automated GLC methods. On the average the GLC method underestimated the total plasma cholesterol by about 10 mg%, while the other parameters of the data remained very much the same as those observed for the short-term correlations.

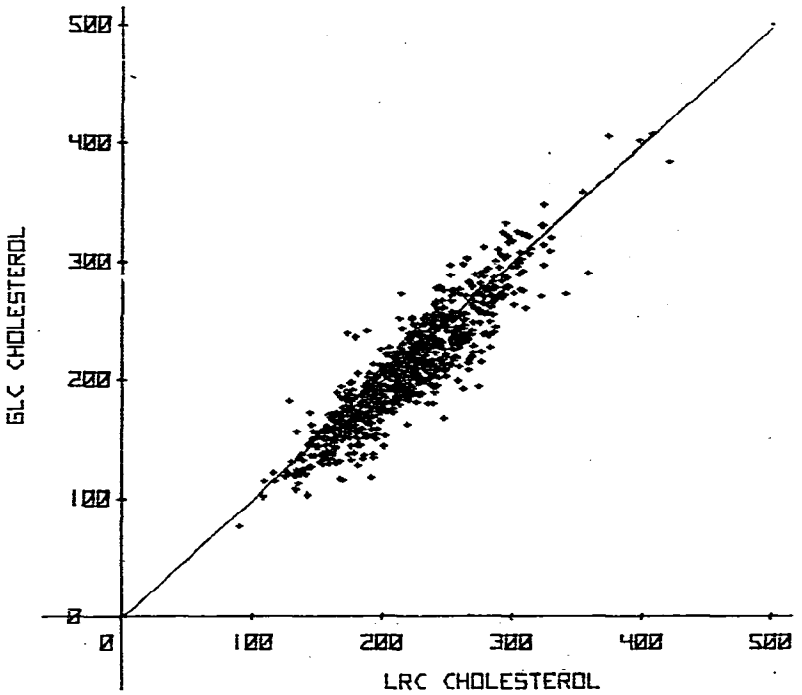

Fig. 7. Comparison of results (mg%) for total plasma cholesterol as obtained by GLC and AutoAnalyzer methods over a period of several years.

Fig. 8 shows a plot of the values for the triacylglycerols as obtained by the AutoAnalyzer and the GLC methods on the 794 random samples of plasma analyzed over a 2-year period. The overall correlation coefficient was 0.9739 with a regression coefficient of 0.9084. The means and standard deviations of the AutoAnalyzer and the GLC estimates were 157.1436±120.9516 and 138.7833±112.8181, respectively. The coefficient of variation between duplicates was 12.7109. It is seen that in the larger number of samples analyzed over the longer period of time, the discrepancy between the GLC and the AutoAnalyzer methods remained about the same as that seen in the smaller number of samples analyzed over the shorter periods of time. In comparison to the AutoAnalyzer, the GLC method underestimated the total plasma triacylglycerols by about 20 mg%.

DISCUSSION

The present large-scale study confirms the general suitability of the automated high-temperature GLC procedure for the analysis of plasma total cholesterol and triacylglycerols claimed previously from analyses of model mixtures of neutral lipids and reference sera. There is evidence that under carefully controlled conditions the plasma lipids can be subjected to a preliminary dephosphorylation with phospholipase C without affecting the estimates for

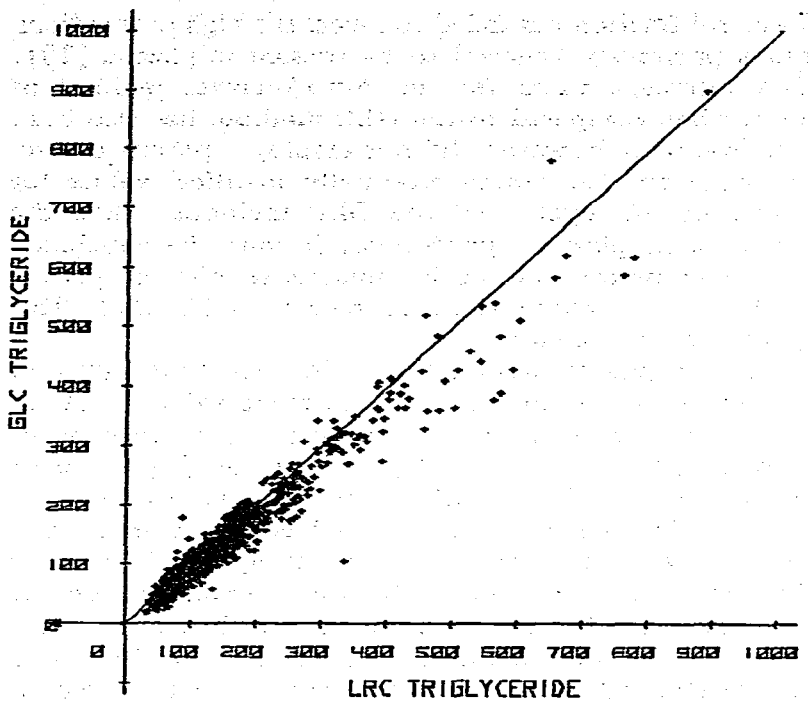

Fig. 8. Comparison of results (mg%) for total plasma triacylglycerols as obtained by GLC and AutoAnalyzer methods over a period of several years.

free cholesterol, cholesteryl esters and triacylglycerols in the digestion residue. We have shown elsewhere [3] that the monoacylglycerols, diacylglycerols and ceramides released by the enzyme treatment yield valid estimates of the plasma lysophosphatidylcholines, phosphatidylcholines and sphingomyelins.

The GLC values obtained for both total cholesterol and total triacylglycerols show excellent correlation with the modified AutoAnalyzer target values, but exhibit a negative bias. Thus, the mass values for total cholesterol are about 5—10 mg% below those of the colorimetric values of the AutoAnalyzer even though a plasma correction has been already made on the data [8, 9]. It is known that the Liebermann-Burchard method gives as much as 20 mg% higher color yield for cholesteryl esters than for free cholesterol and that there are differences in the color yield of different cholesteryl esters [12]. Furthermore, metabolites of cholesterol found in plasma in variable amounts [13] are believed to be responsible for a discrepancy of about 12% between the ferric chloride—sulfuric acid and the GLC or enzymatic methods of analysis of plasma cholesterol in the free form [14]. Since the correction factors applied in the LRC AutoAnalyzer method probably apply only to a narrow range of free cholesterol—cholesteryl ester ratios and to a specific fatty acid composition of the cholesteryl ester, as well as to a specific ratio of cholesterol and its companion sterols, a complete agreement would not be expected between any indirect and direct methods of analysis. It may be noted, however, that a gas chromatography—mass spectrometry (GC—MS) examination

[15] of the plasma free sterol fraction has failed to reveal the high proportions of cholesterol companions previously believed to be present in plasma [13].

A significantly higher intercept value for the AutoAnalyzer method of cholesterol determination, when compared to the GLC method, has also been reported by Watts et al. [5], who, however, did not employ a plasma correction factor. Since they were able to obtain essentially identical values for total cholesterol by both AutoAnalyzer and the GLC methods, when the analyses were made on isolated plasma lipoproteins, it must be concluded that a chromogen is possibly present in the infranatant fraction of plasma lipoproteins obtained after ultracentrifugation at density 1.21 g/ml. This possibility deserves experimental examination.

There is evidence also for plasma interference with the triacylglycerol determination by the AutoAnalyzer method. An intercept value of about 20 mg% obtained in the present experiments compares to an intercept value of 0.58 mmoles/l or about 50 mg% reported by Watts et al. [5] for their AutoAnalyzer—high-temperature GLC comparison. In contrast to the cholesterol determination, the interference for triacylglycerol determination remained relatively constant with the sample concentration, the slope being about 1.0. An examination of the detailed methodology employed by the AutoAnalyzer method reveals that the values are not routinely corrected for a cold alkali blank estimated to be about 3 mg% trioleoylglycerol for the AA11 at Toronto, or for the presence of about 2% mono- and 4% diacylglycerols [16], which could have contributed a maximum of 6—8 mg%, when measured as triacylglycerols. The possibility of occasional contamination of the plasma neutral with polar lipids during the Zeolite adsorption [11] could also have contributed to the higher estimates for total triacylglycerols, as could have the presence of other unidentified components containing actual or potential vicinal hydroxyl groups [8, 11]. Furthermore, the AutoAnalyzer method [8] expresses its results in terms of a C_{54} triacylglycerol or trioleoylglycerol (triolein), which results in a variable overestimation of the content of plasma triacylglycerols averaging 5—8 mg%, since the actual average plasma triacylglycerol is usually a C_{52} [17, 18] or as low as a C_{51} species [18]. Watts et al. [5] reported an average carbon number of 51.8 for the triacylglycerols of normolipemic and hyperlipemic human plasma. The above discrepancies could add up to about 20 mg%, which is the approximate difference observed between the GLC and the AutoAnalyzer methods. Additional discrepancy might arise due to a lack of a hot alkali blank, which for technical reasons also remains uncorrected for in the AutoAnalyzer method. These explanations would account for the discrepancies in the measurements of the unknown plasma samples, as well as would rationalize the lack of disagreement in the measurement of standard trioleoylglycerol from the Lipid Standardization Laboratory, and possibly the smaller differences observed between the GLC and the AutoAnalyzer estimates for triacylglycerols in plasma lipoprotein fractions [5]. The much higher intercept values reported by Watts et al. [5] for the AutoAnalyzer and GLC comparison of plasma total triacylglycerols would require a greater allowance for the above potential interferences or some other basis would have to be found for explanation (subliminal losses of triacylglycerols on GLC during isothermal analysis?). In any event, the present

difference between the GLC and the AutoAnalyzer values is of the order that could be reasonably expected from an examination of the two analytical routines and a knowledge of the plasma lipid composition from independent analyses [16]. On the basis of the above data and the data of Watts et al. [5], it would be desireable to re-examine the AutoAnalyzer methodology of triacylglycerol determination to see if a plasma or serum standardization similar to that employed for cholesterol determination may not be necessary. Certainly the erroneous expression of the final results and the inclusion of the appropriate blanks should be reconsidered for accurate triacylglycerol analyses.

Finally, it should be remembered that the GLC method also could be occasionally in error due to a rapid peroxidation and loss of the more unsaturated glyceryl and cholesteryl esters during isolation, dephosphorylation, derivatization and storage of the plasma lipid samples. Likewise, incomplete dissolution of the plasma lipoproteins from the frozen samples especially after prolonged storage [7] may have contributed more to an underestimation of both total cholesterol and total triacylglycerols than presently appreciated.

The high precision and apparent high accuracy of the present GLC results supports earlier claims in this regard and would seem to recommend it as the method of choice for accurate determination of plasma cholesterol and triacylglycerols. In the present state of development the automated GLC method is capable of determining total cholesterol and total triacylglycerols on a maximum of four samples per hour, which, even when extrapolated to a maximum of 96 samples per day, would not approach the through-put of an AutoAnalyzer (about 300 samples per day). The advantage of the GLC method of plasma lipid determination lies in the definitive nature of the measured components, which is especially important in the analysis of abnormal plasma samples, and in the additional information provided about the composition of the plasma lipids. The separate values for free fatty acids, lysophosphatidylcholines, free and esterified cholesterol, the phosphatidylcholines and sphingomyelins, along with the major molecular species of the cholesteryl esters and the acylglycerols and ceramides are of interest to a variety of clinical conditions and the normal metabolic state of the body.

Obviously, with precise peak area integration the GLC method can provide estimates approaching those sought for plasma cholesterol [19] and plasma triacylglycerols [20] by the absolute or definitive methods of quantitation using stable isotope dilution and combined GC—MS.

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