Results for Serum Cholesterol and Triglycerides by Gas–Liquid Chromatography, as Compared with a Continuous-flow Technique

Rodney Watts, Tim Carter, and Stella Taylor

We assessed the usefulness of gas–liquid chromatography for the routine and reference measurement of cholesterol and triglycerides (triacylglycerols). Compared with results obtained by an AutoAnalyzer II method, correlation was good with both serum specimens (triglycerides, r = 0.88; cholesterol, r = 0.90) and lipoprotein fractions isolated by ultracentrifugation (triglycerides, r = 0.98; cholesterol, r = 0.98). However, none of the comparisons was completely free of analytical bias, and in general the AutoAnalyzer method underestimated the cholesterol value and overestimated triglycerides. The obvious advantage of the AutoAnalyzer is its greater analytical speed, but the chromatographic method appeared to be more accurate and precise and to suffer less from interfering substances, and in addition gave information about the cholesterol ester and triglyceride composition. We expect gas–liquid chromatography to be of major importance in the calibration of other analytical methods.

Additional Keyphrases: intermethod comparison • analytical error • hyperlipoproteinemia

Methods for determining serum cholesterol and triglycerides (triacylglycerols) are legion, and all have been capable of producing precise results in the hands of able workers. However, in practice, particularly for triglycerides, precision and agreement are frequently poor between laboratories, even those applying the same method (7).

Attempts to alleviate this situation have met with only partial success. Enzymic methods (2, 3) for example, are inherently specific, although less satisfactory when applied routinely (1) and are expensive. Similarly, a liquid–liquid phase-partition method that gave good results for triglycerides did not extract all the free cholesterol (4).

It has been the practice in our laboratories to use an AutoAnalyzer II system (Technicon Instruments Corp., Tarrytown, N. Y. 10591) for the routine analysis of triglycerides and cholesterol (5), although results have been neither as accurate nor as precise as we anticipated. We assessed the suitability of a Pye 204 gas chromatograph, previously used only as a research tool (6), for routine lipid analysis by evaluating about 100 serum specimens and 50 ultracentrifuge fractions that previously had been analyzed by the AutoAnalyzer method. The precision of the gas–liquid chromatography (GLC) was assessed by replicate and multiple analyses.

Materials

All reagents and solvents were of AR grade except for heptane, which was redistilled, and were supplied by BDH Ltd., Poole, Dorset, U. K. Standard triglycerides, for use in GLC, were those used previously (6). Cholesterol, cholesterol myristate, palmitate, olete, and linoleate (all 99% pure) for use in GLC and stock solutions of triolein and cholesterol in isopropanol for use in calibrating the AutoAnalyzer were all supplied by Sigma Chemical Co. Ltd., London, U. K.

A natural mixture of triglycerides was prepared from rabbit adipose tissue. The fatty acid composition (in moles per 100 mol) was: myristic, 3.9%; palmitic, 31.4%; palmitoleic, 5.8%; stearic, 6.5%; oleic, 26.6%; and linoleic, 25.8%. These fatty acids were found to be randomly associated to give an intact glyceride structure for which the carbon number (i.e., the total number of carbon atoms in the acyl moieties) distribution was 46, 31.1%; 48, 12.4%; 50, 26.9%; 52, 36.0%; and 54, 21.6%.

Methods

Automated analyses. Automated simultaneous analyses for triglycerides and total cholesterol in serum or in lipoprotein fractions after ultracentrifugation (7) were done essentially according to Technicon method AAII-24 except that the zeolite absorption mixture was replaced by alumina (8).

Ultracentrifugation. Three milliliters of serum was layered on 4.5 ml of 2 mol/liter NaCl and an upper layer of saline (relative density 1.006) was added. Specimens were placed in a Beckman Ti-50 fixed-angle rotor head and spun at about 100 000 × g for at least 20 h in a Beckman Spinco ultracentrifuge (Beckman R.L.I.C. Ltd., Croydon, Surrey, U. K.). Layers of measured vol-

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Y = 1.47 + 0.75X
N = 106
R = 0.89

V = 0.59 + 0.94X
N = 106
R = 0.88

Fig. 1. Chromatograms of (a) standard and (b) lipids from a normal serum (cholesterol, 6.20 mmol/liter, triglycerides 1.39 mmol/liter)

Chromatography on 45 × 0.65 cm 3% SE-30 on 100–200 mesh “Universal” support; N₂ flow rate 37.5 ml/min; 245 °C isothermally for 2 min, then programmed to 330 °C at 40 °C/min. Approximately 15 μg total lipid applied

Fig. 2. Comparison of results obtained from the AutoAnalyzer and GLC methods for (A) Serum cholesterol and (B) serum triglycerides

Units are mmol/liter

Extractions. We used the original method of Dole (9) to extract the serum or lipoprotein fractions for GLC. The heptane extracts were analyzed for phospholipid (10) and 6–8% of the serum total phospholipids were found to be present. When the lipids were extracted with chloroform–methanol–1.0 mol/liter sulfuric acid (50/50/2 by vol), an additional 5–10% of the free cholesterol was obtained. Thus the analytical recoveries of neutral lipids by the Dole method exceeded 97%.

Gas-liquid chromatography. GLC of the intact lipids without prior hydrolysis was done in a manner similar to those methods published previously (6, 11). A model 204 chromatograph (Pye-Unicam Ltd., Cambridge, England) with dual-flame ionization detectors was fitted with a single 45 × 0.65 cm glass column packed with 3% (by wt) SE-30 (methyl polysiloxane silicone gum; Applied Science Laboratories Inc., State College, Pa. 16801) on 100–120 mesh “Universal” support (Jones Chromatography Ltd., Llanbradach, Glamorgan, Wales). The column was originally conditioned by an overnight purge with a nitrogen flow of 50 ml/min at 345 °C, followed by six 1-mg injections of olive oil. At the time this study was commenced the column had been in use for about two years and the nitrogen flow rate had been gradually reduced to 37.5 ml/min to maintain satisfactory resolution. We used a temperature program...
of 245–330 °C at 40 °C/min, with an initial isothermal period of 2 min, when the free cholesterol peak was quantitated by triangulation. However, most of the present study was done during an evaluation load of a Pye DP88 computing integrator (Pye-Unicam Ltd.), when it was possible to shorten the period of analysis by using a temperature program of 265–330 °C, with no isothermal period.

A standard mixture of commercial triglycerides (Standard 1) was used to obtain molar correction factors (12) for each molecular species of triglyceride of carbon numbers 48 to 56. Serum triglycerides having a carbon number of 46 were only separated from the cholesterol esters if a much slower temperature-programming rate was used. However, except for lipids extracted from some Fredrickson type IV and V sera, we have found that the lower triglycerides consistently accounted for only about 4.5 moles per 100 mol of the total, thus extending previous results (6).

To obtain molar correction factors for free cholesterol and cholesterol esters, we prepared a standard mixture (Standard 2) containing the oleate and linoleate esters in amounts comparable to those in normal serum. In addition, cholesterol myristate was added for internal standardization purposes, because its chromatographic behavior is different from that of the phospholipid pyrolysis products, and it occurs in the serum at only the 1 mol/100 mol proportion. To obtain a correction for the interference by the lower serum triglycerides in the measurement of cholesterol esters (which ranged from 0.9% of total cholesterol when the serum triglyceride/cholesterol ratio was 0.25 to 4.0% of total cholesterol when it was 1.00), we added rabbit adipose triglyceride to the cholesterol mixture (Standard 2) and prepared a stock standard (Standard 3) in isopropanol (final concentrations: 1.26 mmol of triglyceride and 5.52 mmol of cholesterol per liter). A working GLC standard (Standard 4) was prepared by diluting the stock 10-fold with isopropanol.

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Table 1. Precision of the GLC Method

<table>
<thead>
<tr>
<th>Serum or lipoprotein fraction</th>
<th>Mean value (mmol/liter) ± SD of duplicates</th>
<th>Deviation of duplicates, %</th>
<th>CV* of duplicates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cholesterol</td>
<td>Triglycerides</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>1</td>
<td>22.18 ± 0.16</td>
<td>2.05 ± 0.08</td>
<td>4.3</td>
</tr>
<tr>
<td>2</td>
<td>9.00 ± 0.42</td>
<td>3.82 ± 0.13</td>
<td>4.7</td>
</tr>
<tr>
<td>3</td>
<td>4.49 ± 0.27</td>
<td>0.57 ± 0.05</td>
<td>6.0</td>
</tr>
<tr>
<td>4</td>
<td>4.88 ± 0.04</td>
<td>1.84 ± 0.02</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>7.24 ± 0.20</td>
<td>1.79 ± 0.03</td>
<td>2.8</td>
</tr>
<tr>
<td>6</td>
<td>10.70 ± 0.20</td>
<td>1.78 ± 0.03</td>
<td>1.9</td>
</tr>
<tr>
<td>7</td>
<td>6.64 ± 0.07</td>
<td>0.31 ± 0.02</td>
<td>1.1</td>
</tr>
<tr>
<td>8</td>
<td>4.15 ± 0.10</td>
<td>0.85 ± 0.05</td>
<td>2.4</td>
</tr>
<tr>
<td>9</td>
<td>8.06 ± 0.28</td>
<td>2.02 ± 0.02</td>
<td>3.5</td>
</tr>
<tr>
<td>10</td>
<td>7.56 ± 0.10</td>
<td>0.61 ± 0.04</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Average deviation
or CV

2.9  4.1  4.1  5.7

* CV = 100 √(Σd²/n) where d is the difference between duplicates and x is the mean.
Table 2. Triglyceride (TG) Composition and Free Cholesterol (Chol) Content of Normal and Hyperlipidemic Sera

<table>
<thead>
<tr>
<th>Total chol mmol/liter</th>
<th>TG mol/liter</th>
<th>Electro-phoretic pattern</th>
<th>TG composition according to carbon no. mol/100 mol</th>
<th>Cn*</th>
<th>Chol mmol/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.13</td>
<td>0.85</td>
<td>Normal</td>
<td>3.3</td>
<td>17.26</td>
<td>22.8</td>
</tr>
<tr>
<td>4.06</td>
<td>0.53</td>
<td>Normal</td>
<td>4.5</td>
<td>17.25</td>
<td>23.0</td>
</tr>
<tr>
<td>6.66</td>
<td>1.08</td>
<td>Normal</td>
<td>4.3</td>
<td>17.25</td>
<td>21.0</td>
</tr>
<tr>
<td>8.77</td>
<td>1.07</td>
<td>Ilia</td>
<td>2.2</td>
<td>17.23</td>
<td>20.8</td>
</tr>
<tr>
<td>9.37</td>
<td>0.83</td>
<td>Ilia</td>
<td>2.3</td>
<td>17.21</td>
<td>21.9</td>
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<td>8.23</td>
<td>1.17</td>
<td>Ilia</td>
<td>3.0</td>
<td>17.34</td>
<td>21.1</td>
</tr>
<tr>
<td>10.80</td>
<td>2.08</td>
<td>IIb</td>
<td>3.0</td>
<td>17.23</td>
<td>22.3</td>
</tr>
<tr>
<td>10.70</td>
<td>3.64</td>
<td>IIb</td>
<td>1.5</td>
<td>17.32</td>
<td>20.4</td>
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<tr>
<td>5.58</td>
<td>2.64</td>
<td>IV</td>
<td>8.6</td>
<td>17.07</td>
<td>22.4</td>
</tr>
<tr>
<td>7.00</td>
<td>2.90</td>
<td>IV</td>
<td>4.3</td>
<td>17.17</td>
<td>25.2</td>
</tr>
<tr>
<td>8.91</td>
<td>6.99</td>
<td>IV</td>
<td>3.1</td>
<td>17.39</td>
<td>24.2</td>
</tr>
</tbody>
</table>

* Cn, average fatty-acid chain length

In the most accurate and simple methods of GLC internal standards are used. Correction factors, relative to the area of the standard peak, can be applied to the areas of the peaks of interest to give true concentrations. This method does not require that accurate amounts of serum extract be applied to the column. However, in order to obtain results and precision that other nonexpert workers might also obtain, we decided to use known volumes of about 10 μl of the Dole heptane layer.

Each day the first analysis was discarded, and the system was calibrated with Standard 4 after the third analysis. Absolute detector responses were determined for triolein and free cholesterol, and the lower limit of detection was about 1 nmol for both cholesterol and triglycerides. The sensitivity limit for our method was therefore 10 μmol of lipid per liter of whole serum or ultracentrifugal fractions. Serum cholesterol values were obtained by automatic peak area determinations by use of the DP88 integrator, and the triglyceride concentrations were obtained manually by triangulation of the peaks.

Results and Discussion

Figure 1 shows typical chromatograms of the cholesterol/triglyceride standard (Standard 4) and the lipids of a normal serum. Hypertriglyceridemic and hypercholesterolemic sera would yield patterns qualitatively similar to the normal.

Figure 2 shows a comparison of the results by AutoAnalyzer vs. GLC for 106 sera; Figure 3 shows a similar comparison for VLDL and LDL fractions. In all cases the correlation coefficient, r, is close to unity and, as expected, the purified lipoprotein fractions exhibit greater r values. These fractions also exhibit intercept values approaching zero; indeed, there is almost complete equivalence between the methods when triglycerides in purified lipoprotein fractions are analyzed (Figure 2B). However, the AutoAnalyzer tends to underestimate cholesterol values in ultracentrifuge fractions and the magnitude of this underestimation increases with the sample concentration (Figure 3A).

For serum specimens, the intercept values of 1.38 and 0.58 mmol/liter for cholesterol and triglycerides, respectively, indicate the presence of interfering substances in the AutoAnalyzer techniques. Whereas for triglycerides in serum (Figure 2B) interference remains relatively constant with sample concentration (i.e., the slope is about 1.0), for cholesterol in serum (Figure 2A) the slope shows the same underestimation, increasing with the sample concentration, as is seen with lipoprotein fractions. The combination of these two sources of interference for the analysis of cholesterol in serum means that the AutoAnalyzer will tend to overestimate at low values and underestimate at high values. Many simple cases of cholesterol overestimation have been cited, notably bilirubin (13) and cholesterol esters (14), but it has also been reported that any water present in the isopropanol extract decreases the production of color in the AutoAnalyzer method (15), leading to underestimation. It would seem that a number of factors are combining to influence the continuous-flow determination of serum cholesterol. Where values are comparable (i.e., cholesterol about 6.0 mmol/liter), our results agree with those of Blomhoff (11).

The precision of the GLC method was assessed in two ways. First, 10 specimens were analyzed in duplicate in seven batches (Table 1). The overall coefficients of variation (CVs) were 4.1% and 5.7% for cholesterol and triglycerides, respectively. Secondly, two sera were analyzed on each of five occasions. Serum 1 gave a CV of 4.2% (mean cholesterol, 7.85 mmol/liter) and Serum

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3 This was done because the first sample acts as a column conditioner (active-site blocker); thus the usual correction factors are incorrect for it.
2 gave a CV of 4.0% (mean cholesterol, 7.80 mmol/liter). Thus the CVs obtained by both methods for cholesterol are in close agreement and apply to a wide range of cholesterol concentrations. For triglycerides, Serum 1 gave a CV of 7.8% (mean triglyceride, 0.64 mmol/liter) and Serum 2 gave a CV of 3.2% (mean triglyceride, 3.12 mmol/liter). Thus error varied inversely with the actual concentration of triglycerides to be measured; the magnitude of errors at the lower concentration is magnified by errors in triangulating the small areas of peaks 48 and 54 (Figure 1). Nevertheless, we believe the results represent realistic maximum bounds of error that any laboratory should be able to achieve or excel.

In spite of the inherent specificity and versatility of GLC methods for the analysis of lipids (6, 11, 16, 17), little use has been made of this technique in the routine laboratory. Reagent costs (currently 2.5 p/test) are very low as compared with other techniques, especially enzymic ones, but it must be admitted that in order to analyze a reasonable number of specimens (at a rate of about four per hour), capital equipment must include, not only the GLC apparatus itself, but an automatic sampling arrangement and integrator. Even with these refinements, GLC is likely to remain an under-used technique when today's ever-increasing workloads are considered. Its very great advantages lie in its accuracy and precision, leading, we predict, to its increasing use in the calibration of suitable materials for the accurate standardization of other methods that are more suited to routine use in the clinical laboratory.

This improved accuracy and precision may help to resolve problems with regard to reference ranges and in the differential diagnosis of hyperlipoproteinemias (18-21). It is tempting to speculate how diagnosis, differentiation, and treatment of such disorders would be improved by such increased analytical accuracy. However, even with the most nearly accurate methods, caution is still needed (21).

In addition to the advantage of accuracy, GLC of lipids is capable of providing further information (i.e., determination of free and esterified cholesterol and triglyceride composition), but its ultimate impact must also remain a matter of speculation at the present time. Table 2 shows the triglyceride composition and free cholesterol contents of some sera representative of various Fredrickson types (22) as determined by our GLC method. These kinds of results have already been shown to be of use in studying lipid metabolism (6), liver disease (11), and the dietary status of patients (6). Whether these additional data will be of use in the classification of lipid disorders is still a matter for the future, but if they are, the disadvantage of the GLC, namely low analysis rate, may well be outweighed by the diagnostic information available. A detailed report of the intact lipid compositions and their relationship to the type of hyperlipoproteinaemia is being prepared.

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References