Direct Determination of the Linoleate/Oleate Ratio in Serum Cholesterol Esters by Liquid Chromatography

John T. Bernert, Jr., James R. Akins, and Dayton T. Miller

We describe a convenient method for the direct determination of the serum cholesterol linoleate/cholesterol oleate (L/O) ratio by reversed-phase "high-performance" liquid chromatography. After removal of phospholipids by silicic acid chromatography, a serum extract is analyzed on a 5-μm particle size Ultrasphere-ODS column, eluted isocratically with acetonitrile/isopropanol (30/70, by vol). Detection is at 200 nm. Cholesterol palmitoleate interferes with the measurement when the analysis is based on peak area, but not when peak height is used. The overall precision of L/O measurements by this method was very similar to that observed with a gas–liquid chromatographic procedure, in which the cholesterol esters are first isolated and transesterified to the methyl esters. In both cases, the within-run CV for six replicate analyses was less than 2%. Analysis of 53 human serum samples by both methods yielded very similar L/O ratios. A plot of the data (our method = y) vs the usual gas–liquid chromatographic procedure gave a correlation coefficient of 0.988 and a regression equation of y = 1.03x + 0.013. Furthermore, direct analysis of serum cholesterol ester L/O ratios by our liquid–chromatographic method is simpler, quicker, and more readily adaptable to automation.

Additional Keyphrases: liquid chromatography, reversed-phase • diet-related effects on fatty acid esters • peak area vs peak height measurement

In studies involving modification of dietary fat intake in humans, it is important to determine the extent to which the results may be influenced by the degree of adherence to the diet. This variable may be directly monitored when institutionalized groups are involved, but in free-living populations it must be estimated indirectly, e.g., by nutritional interviews and biochemical analyses. In the latter case, a common approach has been to monitor the fatty acid composition of serum or tissue lipids. Generally, linoleate is measured, because it is an important dietary constituent and an essential fatty acid of strictly exogenous origin (1, 2). The influence of variations in total lipid concentration is conveniently minimized by expressing the linoleate concentration as a ratio to that of another fatty acid—commonly, the nonessential oleic acid.

Although current estimates of serum lipid turnover are difficult and somewhat controversial (3), there is general agreement that the order of half-life is probably cholesterol esters > phospholipids > triglycerides > free fatty acids (4–7). The relatively slow plasma cholesterol ester (CE) fractional turnover rate, reported unaffected by diet (8), is undoubtedly one reason why the fatty acid composition of this lipid class has often been chosen for monitoring dietary studies. Plasma CE composition is clearly responsive to long-term dietary change (9–13) and is therefore useful in reflecting established nutritional patterns. As with the other serum lipids, CE composition is susceptible to influence by relatively recent dietary trends (13), but this influence tends to be less for CE than for the other lipids. Thus, the main advantages of analyzing serum CE are the gradual dietary response resulting from the relatively long CE half-life and an enhanced sensitivity of the linoleate/oleate (L/O) ratio, owing to the strongly selective fatty acid incorporation pattern in this lipid (14, 15).

Measuring L/O ratios in serum CE has generally required the preliminary isolation of the cholesterol esters by thin-layer chromatography, followed by elution, transesterification, and analysis of the resulting fatty acid methyl esters by gas–liquid chromatography (GLC). We have recently used "high-performance" liquid chromatography (HPLC) on silica columns for the preliminary separation and recovery of neutral lipid fractions (16), which may then be directly transesterified and analyzed by GLC. However, these approaches are laborious, time consuming, and only partly adaptable to automation. In addition, the transesterification of CE is relatively slow and potentially selective (17). Recently, Duncan et al. (18) described a sensitive and reliable reversed-phase HPLC procedure for the analysis of serum cholesterol and other neutral lipids. Because a direct determination of L/O ratios by HPLC would offer a number of important advantages over current procedures, we have investigated the application of reversed-phase HPLC to this analysis.

Materials and Methods

Materials. Chromatographic standards were obtained from the Sigma Chemical Co., St. Louis, MO 63178, or from Applied Science Labs, Inc., State College, PA 16801. Hexane, tetrahydrofuran, acetonitrile, and isopropanol were HPLC-grade solvents from Burdick and Jackson Labs., Inc., Muskegon, MI 49442; all other solvents were reagent grade. Unisil (200–325 mesh) was a product of Clarkson Chemical Co., Williamstown, PA 17701. LiChrosorb Si 60 (10-μm particle size) was purchased from Universal Scientific, Atlanta, GA 30341. Human serum samples were generally assayed within two weeks of their collection and were stored frozen (−20 °C) before being analyzed.

Sample preparation. Serum aliquots (0.5 mL) were extracted by the procedure of Folch et al. (19). After the extract was dried under a stream of N2, the lipids were dissolved in 0.5 mL of chloroform and applied to a 250-μL column of chloroform-washed Unisil packed in a Pasteur pipette. The neutral, nonpolar lipids were then eluted with 5 mL of chloroform. The overall recovery of esterified cholesterol was >99% throughout these procedures, as determined by measurement of cholesterol [1-14C]oleate (New England Nuclear, Boston, MA 02118) added to the original sample.

Isolation of cholesterol esters. Half of the eluate from the Unisil column was dried and reconstituted in hexane. This sample was fractionated by HPLC (16) on a 25-cm column of
LiChrosorb Si 60, with tetrahydrofuran/hexane (2/98, by vol) as the eluent and a flow rate of 3 mL/min. Detection was by ultraviolet absorption at 206 nm. After triglyceride elution, the solvent composition was changed to tetrahydrofuran/hexane (6/94, by vol) over a 1-min interval; this composition was then maintained until free cholesterol had been eluted.

The cholesterol ester peak was collected directly from the effluent stream and dried under N₂. Under these conditions, the cholesterol esters were eluted as a single unresolved peak with a retention time of approximately 1.5 min. Recovery of CE at this step averaged 94% as monitored with radiolabeled cholesterol oleate, and the total fatty acid profile of this fraction was indistinguishable from that of samples isolated by thin-layer chromatography on silica gel G plates (Analtech, Newark, DE 19711), with hexane/ethyl ether/acetic acid (90/10/1, by vol) as the developing solvent.

Transesterification and GLC. The cholesterol esters were isolated as described above and transesterified to the methyl esters by the procedure of Glass (20) as modified by Tuckey and Stevenson (21), except that anhydrous methanolic HCl (22) was substituted for the methanolic sulfuric acid to minimize the incidence of chromatographic artifacts. The methanolic HCl was prepared by dissolving 30 g of HCl gas in a liter of dry methanol. A series of analyses with CE standards, in which the time course and the extent of reaction were monitored chromatographically, confirmed that this procedure provided for a complete and nonselective conversion of the cholesterol esters. The methyl esters were then analyzed by GLC on a 2 m × 2 mm i.d. glass column packed with 10% Silar 10C on 100/120 mesh Gas-chrom Q (Applied Science Labs.). The temperature was programmed from 150 to 185 °C, and helium was the carrier. The injection port temperature was 230 °C, and the detector was maintained at 250 °C. We used a gas chromatograph equipped with flame-ionization detectors (Model 5840A; Hewlett-Packard Co., Palo Alto, CA 94304) and an automatic sampler for these determinations.

Reversed-phase HPLC. The remaining half of the neutral lipid fraction eluted from the Unisil column was dried under N₂, and the residue was dissolved in 2.0 mL of isopropanol. Aliquots (20 µL) of this solution were then analyzed, essentially by the procedure of Duncan et al. (18). For this analysis we used a 25-cm C-18 column (UltraspHERE-ODS, 5-µm particle size; Altex Scientific, Berkeley, CA 94710), eluting components isocratically at 0.8 mL/min with acetonitrile/isopropanol (30/70, by vol) and using a Model 110A pump (Beckman Instruments, Fullerton, CA 92634). A variable-wavelength detector (SF 770; Schoefel Instruments Div., Kratos, Inc., Westwood, NJ 07675) was used, monitoring at 200 nm. Quantitation was by peak height, with a Hewlett-Packard 3390A recording integrator calibrated in the external standard mode.

Results

A typical chromatogram from the analysis of a standard mixture of cholesterol linoleate and cholesterol oleate by HPLC is given in Figure 1A, and a representative serum extract is shown in Figure 1B. Initial analyses were performed on a 30-cm C-18 column packed with 10-µm irregularly shaped particles, which only partly resolved cholesterol oleate from cholesterol palmitate. However, as indicated in Figure 1B, the use of a high-efficiency column containing 5-µm diameter spherical packing provided for the near-baseline separation of that pair. The identity of the components in Figure 1B was confirmed by collection of the peaks and analysis by thin-layer chromatography, and by GLC after transesterification of the lipids. Under these analytical conditions no contaminating triglycerides or other lipid species were detected in peaks 6 through 9. However, the retention time of cholesterol palmito-
Table 1. Influence of Cholesterol Palmitoleate on the L/O Ratio as Determined by HPLC

<table>
<thead>
<tr>
<th>Cholesterol palmitoleate added, µg</th>
<th>Wt. ratio</th>
<th>L/O, mean (and SD)</th>
<th>Height</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (4)</td>
<td>0</td>
<td>1.91 (0.01)</td>
<td>1.94 (0.04)</td>
<td></td>
</tr>
<tr>
<td>0.89 (3)</td>
<td>0.15</td>
<td>1.91 (0.01)</td>
<td>2.17 (0.02)</td>
<td></td>
</tr>
<tr>
<td>1.78 (3)</td>
<td>0.30</td>
<td>1.92 (0.04)</td>
<td>2.37 (0.08)</td>
<td></td>
</tr>
<tr>
<td>2.67 (3)</td>
<td>0.45</td>
<td>1.92 (0.02)</td>
<td>2.56 (0.02)</td>
<td></td>
</tr>
</tbody>
</table>

* Aliquots (20 µL) of a standard mixture containing 5.98 µg of cholesterol linoleate and 3.14 µg of cholesterol oleate and the indicated amount of cholesterol palmitoleate were assayed under the conditions described in Materials and Methods. The number of analyses for each group is given in parentheses. ° Ratio (by weight) of cholesterol palmitoleate to cholesterol linoleate in the sample.

Fig. 2. Quantitative analysis of cholesterol linoleate and cholesterol oleate by HPLC

Aliquots of a standard mixture in isopropanol containing 288.8 µg of cholesterol linoleate and 130.3 µg of cholesterol oleate per milliliter of solution were analyzed by reversed-phase HPLC. “Counts” (y-axis) refers to the deflection of the electronic integrator used in the print-out of results.

Table 2. Analytical Precision for the Analysis of Serum Cholesterol Ester L/O Ratios by HPLC/ GLC and by Reversed-Phase HPLC

<table>
<thead>
<tr>
<th></th>
<th>GLC</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run* (n = 6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean L/O</td>
<td>2.54</td>
<td>2.60</td>
</tr>
<tr>
<td>SD</td>
<td>0.044</td>
<td>0.038</td>
</tr>
<tr>
<td>CV, %</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>L/O range</td>
<td>2.51–2.62</td>
<td>2.55–2.64</td>
</tr>
<tr>
<td>Day-to-dayb (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean L/O</td>
<td>2.40</td>
<td>2.48</td>
</tr>
<tr>
<td>SD</td>
<td>0.045</td>
<td>0.045</td>
</tr>
<tr>
<td>CV, %</td>
<td>1.9</td>
<td>1.8</td>
</tr>
<tr>
<td>L/O range</td>
<td>2.35–2.47</td>
<td>2.42–2.59</td>
</tr>
</tbody>
</table>

* For within-run analyses, six aliquots of a single serum sample were extracted and carried through the entire analysis by HPLC/GLC and by reversed-phase HPLC as described in Materials and Methods. ° For day-to-day determinations, duplicate aliquots of a serum pool were extracted and analyzed by GLC and by reversed-phase HPLC over a five-day period.

Discussion

In 1960, Hirsch et al. (27) first proposed analyzing the linoleate content of adipose tissue samples obtained by needle biopsy as a means of monitoring long-term dietary history. Several subsequent studies indicate that adipose concentrations of linoleate are reliable dietary compliance indicators in studies of at least three years' duration (14, 28–30). Bynen...
et al. (31) analyzed a number of reports and calculated an overall regression coefficient of 0.80 for dietary vs adipose tissue polyunsaturates in groups maintaining a long-term dietary pattern. However, the slow turnover of adipose fatty acids precludes their use in studies lasting less than several years (15, 29, 32), and their analysis requires a biopsy. Thus, when an alternative assay of serum lipid fractions is indicated, CE analysis may be quite useful.

The simple reversed-phase HPLC procedure described here offers a number of potential advantages over the usual GLC determination of serum L/O ester values. For GLC analyses, CE must first be isolated and then transesterified to their methyl esters before analysis. The HPLC procedure determines CE directly, thereby obviating both of these preliminary steps, and also avoids any selective fatty acid losses during transesterification. The entire HPLC procedure, including the chromatographic analysis, is conducted at room temperature, further minimizing the loss of relatively labile polyunsaturated fatty acids. Moreover, for the routine analysis of large numbers of samples, the HPLC procedure is readily adaptable to automation if an automatic injector is used, requiring only an initial serum extraction and phospholipid removal on a silicic acid column before analysis.

The recording integrator we used was capable of simultaneously determining peak height and area counts, and many of the analyses were monitored by both methods. We found that quantitation based on peak area generally gave somewhat larger L/O ratios and was less precise than peak height, probably in part because of the contribution of cholesterol palmitoleate to the linoleate peak area. Moreover, in contrast to GLC, peak height measurements may be inherently more reliable for quantitation during HPLC (33, 34).

Although there was good agreement between the GLC and HPLC values in this study, the L/O ratios by HPLC tended to be slightly higher overall. The difference was small but statistically significant (a t-test of the data in Figure 3 yields p <0.001 with 51 degrees of freedom). The reason for this slight difference is not apparent. A contribution of cholesterol palmitoleate to the ratio as determined by HPLC does not appear to be the cause, no significant effect of cholesterol palmitoleate addition being detected during standard analyses (quantitated by peak height), even at a weight ratio of cholesterol palmitoleate to cholesterol linoleate of 0.45. A contribution from other polyunsaturated esters to the cholesterol linoleate value is similarly unlikely, none being detected (by GLC) in that peak; moreover, except for cholesterol arachidonate, their concentrations in serum are very low.

The slightly higher HPLC ratios may have resulted from decreased losses of cholesterol linoleate, although no significant losses could be detected during the GLC procedure when standards were analyzed. Furthermore, we obtained the same results whether we calibrated the HPLC analysis with weighed-in standards or with standards quantitated by GLC after transesterification. Despite this slight difference between the two methods, the overall agreement as indicated by the regression parameters was good and suggests that the direct HPLC procedure may be useful in the routine determination of L/O ratios in serum CE.

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References

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Partition Affinity Ligand Assay (PALA) for Quantitation of Triiodothyronine in Serum

Bo Mattilsson and Håkan Eriksson

Radioimmunoassay of triiodothyronine was performed with a homogeneous incubation system. Bound and free ligand were separated by partitioning in an aqueous two-phase system. The agreement between the results obtained with this technique and conventional heterogeneous radioimmunoassay was very good (r = 0.981).

Additional Keyphrases: radioimmunoassay • incubation with homogeneous ligands

Since the introduction of competitive-binding assays (1), efforts have been made to apply them in clinical analysis (2, 3). Today a broad spectrum of macromolecular antigens and haptenas can be quantified with such procedures.

Although acute sensitivity is expected from the binding constants of the participating molecular entities, only rarely is such sensitivity achieved, in part because the time needed for achieving equilibrium is often too long for routine assays and in part because of the experimental procedures used. In many cases a low sensitivity is sufficient, so that more rapid methods have been developed. The speed of the immunochromical reaction itself may not be changed, but the experimental procedure may have briefer or fewer separation and washing steps. This problem has been approached from two angles: Large, automated analytical machines have reduced or eliminated much of the time-consuming manual handling of samples and thereby have also improved the reproducibility of the assay (4). Alternatively, use of a homogeneous assay, so far applied only in an enzyme-linked immunoassay, eliminates both the separation and the washing steps, by using homogeneous solutions involving the specific steric conditions necessary for macromolecular interactions (5–7).

Recently a new method for radioimmunoassay (RIA) was described, in which no separation was required: the whole heterogeneous assay was carried out in the same reaction vessel. The solid-phase binding of one of the two populations of antigens, antibody-bound or free, included an attenuator that quenched all the 125I radiation emanating from material bound to the solid phase (8).

We describe here the use of still another recently developed binding assay, Partition Affinity Ligand Assay (PALA) (9–11), to quantitate triiodothyronine (T3).

Biomolecules have long been separated routinely in aqueous two-phase systems (12). Recently, it was shown that, if the reactants in the binding assay had an asymmetric and dissimilar distribution between the two phases, the difference could be utilized in a quick and simple analytical procedure (10).

Materials and Methods

Phadebas® T3 RIA-kit and antiserum against T3 raised in sheep were generous gifts from Pharmacia Diagnostics AB, Uppsala, Sweden. We used the standard sera containing T3 (0, 0.6, 1.5, 3.0, 8.0 and 16.0 nmol/L) and the 125I-labeled T3 from the kit. 8-Anilino-1-naphthalene sulfonic acid was purchased from Sigma Chemical Co., St. Louis, MO 63178.

Polyethylene glycol 4000 (PEG-4000) was purchased from BDH, Poole, U.K.

![Fig. 1. Schematic presentation of the assay procedure: A, distribution of labeled and unlabeled hapten (T3) in the partition system; B, distribution of antibodies in the partition system; C, distribution of the antibody–hapten complex in the partition system](image-url)