A sensitive and convenient method for lipoprotein profile analysis of individual mouse plasma samples

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Abstract **A simple and convenient method to determine plasma cholesterol profiles in individual mouse plasma samples is not presently available. With commonly used methods, plasma samples from several animals in a study group must often be pooled and analyzed, usually by the fast phase liquid chromatography (FPLC) method. The Column Lipoprotein Profile (or CLiP) method described here is a modification of the FPLC method that provides a simple and convenient procedure for determining plasma lipoprotein cholesterol profiles in small sample volumes, allowing determination of profiles from individual animals rather than from pooled plasma. The CLiP method is reproducible; a human sample measured five times over several days produced coefficients of variation as follows: VLDL, 10.0%; LDL, 0.93%; and HDL, 2.51%. CLiP-derived total cholesterol values of five different human samples (with total cholesterol levels ranging from 198 to 263 mg/dL) differed from VAP-II by −1.88% ± 2.57%. Linearity of differing concentrations for each of the lipoprotein classes was determined by measuring the same sample with different aliquot sizes. The linear regression from VLDL had an *r* value of 0.996, while LDL, HDL, and total cholesterol all had *r* values of greater than 0.999. We present a direct comparison of plasma cholesterol profiles from several mouse models with gene modification or expression of transgenic proteins. In conclusion, the CLiP method provides a simple, reliable, and reproducible procedure for determination of plasma cholesterol profiles from individual plasma samples with very low sample volumes, using readily available equipment and reagents.**—Garber, D. W., K. R. Kulkarni, and G. M. Anantharamaiah. **A sensitive and convenient method for lipoprotein profile analysis of individual mouse plasma samples.** ***J. Lipid Res.*** 41: **1020–1026.**

Supplementary key words cholesterol • atherosclerosis • column chromatography

The mouse model has been extensively used in understanding the mechanism of action of lipoproteins or apolipoproteins on atherosclerosis (for reviews, see refs. 1, 2). Several mouse models with gene modification or transgenic expression of particular proteins have been developed. Presently, a simple and convenient method to compare the plasma cholesterol profile of these mouse models is not available. Furthermore, with commonly used methods, a large amount of blood must be taken from a single animal or plasma samples from several animals in a study group must be pooled, followed by analysis by either the fast phase liquid chromatography (FPLC) method or other methods normally used to screen human samples. Thus, determination of plasma cholesterol profiles in individual mouse samples obtained by retro-orbital bleeding has presented a challenge to researchers. The recent method of choice, FPLC, uses separation of mouse plasma samples by size-exclusion chromatography, using one Superose 6 (Pharmacia, Piscataway, NJ) column (3), or two in series (4, 5). Fractions are collected, and lipid analyses are done on the individual fractions.

More recently, the VAP-II method has been developed in our laboratory (6), allowing separation and analysis of cholesterol profiles on relatively small sample volumes with high resolution. Based on the original vertical auto profile (VAP) method (7), VAP-II uses density gradient ultracentrifugation to separate lipoprotein classes and subclasses. While VAP-II is the method of choice for analyzing the species and subspecies of plasma lipoproteins in human samples (8), the somewhat higher sample volume required renders this method less suitable for analyzing individual mouse plasma samples.

With the need for a convenient and sensitive method to analyze 10 μl or less of plasma from individual mice, we have modified a method previously reported by Kieft, Bocan, and Krause (9). This method combines separation of lipoproteins by size-exclusion FPLC followed by on-line enzymatic cholesterol analysis, eliminating the need to pool samples and collect fractions. We report linearity and

Abbreviations: CLiP, Column Lipoprotein Profile; FPLC, fast phase liquid chromatography; HPLC, high pressure liquid chromatography; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; VAP, vertical auto profile; EDTA, ethylenediaminetetraacetic acid; apo, apolipoprotein; KBr, potassium bromide.

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reproducibility of the total cholesterol values, and compare results obtained with this method (which we call CLiP, for Column Lipoprotein Profile) with those obtained by VAP-II. In addition, we present a direct comparison of plasma cholesterol profiles from several mouse models with gene modification or expression of transgenic proteins.

METHODS

CLiP method

Plasma cholesterol lipoprotein profiles (CLiP) were determined using a modification of the method of Kieft et al. (9).

A schematic diagram of the CLiP apparatus is presented in **Fig. 1**. The apparatus consists of two biocompatible HPLC pumps (Model 2150, Pharmacia, Piscataway, NJ), an injector (Model 9125, Rheodyne, Cotati, CA) with a 200-μL sample loop, a heating block (TC-50 controller and FH-40 reactor, Eppendorf, Brinkmann Instruments Inc., Westbury, NY) containing 15 m of Teflon tubing (1/16" o.d., 0.02" i.d.), a 30 × 1 cm Superose 6 column (Pharmacia), and a spectrophotometric detector reading at 500 nm, usually at a sensitivity setting of 0.1 (Model UA-5 with 0080-012 10 μm flow cell, Isco, Lincoln, NE). The running buffer was 0.9% NaCl/2 mm EDTA/0.01% sodium azide (pH = 7.4). Plasma samples (5 or 10 μL) were injected onto the Superose 6 column at a flow rate of 0.4 mL/min. Injections were done using a 25-μL syringe (#1702, Hamilton Co., Reno, NV). Immediately after the column, cholesterol reagent (8 g Cholesterol 1000 [Sigma Diagnostics; St. Louis, MO] and 5 mL BRIJ-35 [Sigma] brought to a total volume of 100 mL with distilled water, at a flow rate of 0.2 mL/min) was mixed with the column eluent through a low-dead-volume mixing tee, and the mixture entered the heating block (set at 55°C). Outflow from the heating block entered the spectrophotometric detector, and the cholesterol profile was collected into a computer at the rate of four data points per min.

A back-pressure regulator (40 psi, Upchurch, Apple Valley, MN) was required in the outflow line from the detector to avoid air bubble formation. Both running buffer and cholesterol reagent were degassed thoroughly to further minimize bubble formation in the detector.

Profiles were decomposed into component peaks and analyzed for relative area using PeakFit (SPSS Science, Chicago, IL), and absolute cholesterol values for total cholesterol and each component peak were determined by comparison with a control sample of known values. Controls were initially human plasma samples whose total cholesterol values had been determined by measurement from three separate CLIA-certified clinical laboratories; later, controls were obtained commercially (Cardiolipid 2, Sigma Diagnostics, St. Louis, MO).

FPLC method

Analysis of cholesterol profiles by the FPLC method were done using a Superose 6 (Pharmacia) column on a Biologic chromatography system (Bio-Rad; Hercules, CA). A 200-μL sample of plasma pooled from three LDL-receptor-deficient mice was injected, then eluted with phosphate-buffered saline at a flow rate of 0.4 mL per min. Fractions (0.25 mL) were collected. Choles-

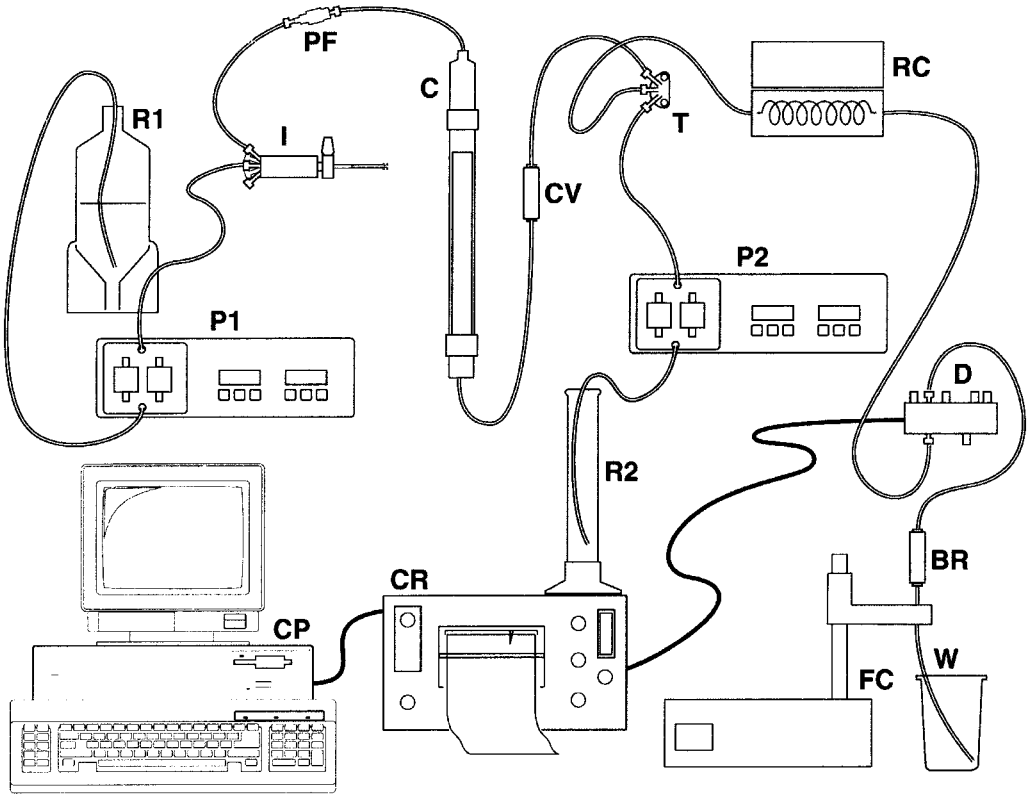

Fig. 1. Schematic diagram of the column lipoprotein profile (CLiP) apparatus. Abbreviations are as follows: R1, column running buffer reservoir; P1, column pump; I, sample injector; PF, column prefilter; C, column; CV, inline check valve; T, low-volume mixing tee; R2, cholesterol reagent reservoir; P2, cholesterol reagent pump; RC, heated reaction coil; D, spectrophotometric detector; BR, backpressure regulator; CR, chart recorder; CP, computer; W, waste; FC, optional fraction collector.

terol reagent (4 g Cholesterol 1000 [Sigma] dissolved in 100 mL deionized H_2O; 1 mL per fraction) was added and mixed, and the absorbance at 500 nm was read in a spectrophotometer after 15 min incubation at room temperature. Peak decomposition was again done using PeakFit (SPSS Science); peak smoothing by the program was required.

Animals

Mice were purchased from Jackson Laboratory (Bar Harbor, ME). In most cases, mice were fed standard mouse chow and were not fasted. Blood was collected by retro-orbital bleeding under anesthesia using heparinized capillary tubes and placed in microcentrifuge tubes containing dry EDTA (Eppendorf); then plasma was separated by microcentrifugation. The Institutional Animal Care and Use Committee of the University of Alabama at Birmingham approved all use of animals.

RESULTS

Optimal length of tubing in the reaction coil (in the heating block)

Tubing length in initial experiments was set at 2 m, similar to that of VAP-II. However, at 2 m and 5 m, insufficient reaction of the sample with the reagent occurred, resulting in attenuated peaks (data not shown). At 15 m of tubing length, reaction was complete, and linearity of response with changes in sample volumes was achieved.

Optimization of temperature in the reaction coil

Optimal temperature setting of the reaction coil heating block was determined by increasing the temperature incrementally between column runs until the total area became constant and before it became reduced due to enzyme degradation (data not shown). The temperature setting of 55°C was selected.

Optimization of reagent

Initial reagent composition was derived from that used in the VAP-II system (6). In order to determine the minimal concentration of enzymatic reagent required, reagent was produced with 5 to 10 g of Cholesterol 1000 enzymatic reagent per 100 mL (Brij-35 content remained constant at 5 mL per 100 mL total volume). Aliquots of Cardiolipid 2 (305 mg/dL cholesterol; Sigma Diagnostics) were analyzed and the results are shown in **Fig. 2**. Maximum peak area was achieved at 7 g of enzymatic reagent per 100 mL. No difference was seen when comparing profiles at 8, 9, and 10 g reagent per 100 mL (data not shown). In order to provide an element of confidence, 8 g of enzymatic reagent per 100 mL was used thereafter.

Linearity

Linearity of differing concentrations for each of the lipoprotein classes was determined by measuring the same (human) sample (total cholesterol = 124 mg/dL) with different aliquot sizes. The profiles were decomposed into lipoprotein fractions; cholesterol levels were calculated assuming that the 10 μL aliquot total cholesterol = 124 mg/dL. **Fig. 3** shows linearity of total cholesterol, as well as of each lipoprotein fraction. The cholesterol ranges (in mg/dL) shown in Fig. 2 are, VLDL, 4.8 to 58; LDL, 36 to 438; HDL, 21 to 251; total cholesterol, 62 to 747. The linear regression from VLDL had an *r* value of 0.996, while LDL, HDL, and total cholesterol all had *r* values of greater than 0.999.

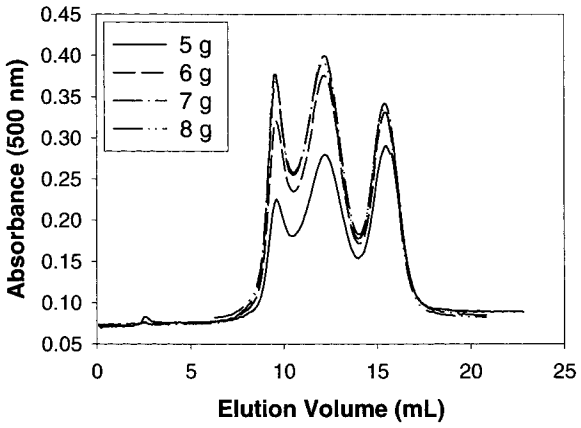

Fig. 2 Effect of enzymatic reagent concentration. Enzymatic reagent was made at proportions of 5 to 8 g reagent powder (Cholesterol 1000; Sigma Diagnostics) and 5 mL BRIJ-35 (Sigma Diagnostics), brought to a total volume of 100 mL. A 10-μL aliquot of a control sample (Cardiolipid 2; Sigma Diagnostics) with total cholesterol of 305 mg/dL was injected with each of the proportional reagent mixtures. Elution volume is expressed as the output from the column eluent pump only, rather than the combined output of both the eluent and reagent pumps.

Reproducibility

The CLiP method was reproducible; a human sample (total cholesterol of 196 mg/dL) measured five times over several days produced coefficients of variation as follows: VLDL (11.9 mg/dL), 10.04%; LDL (124.2 mg/dL), 0.93%; and HDL (60.2 mg/dL), 2.51%. CLiP-derived total choles-

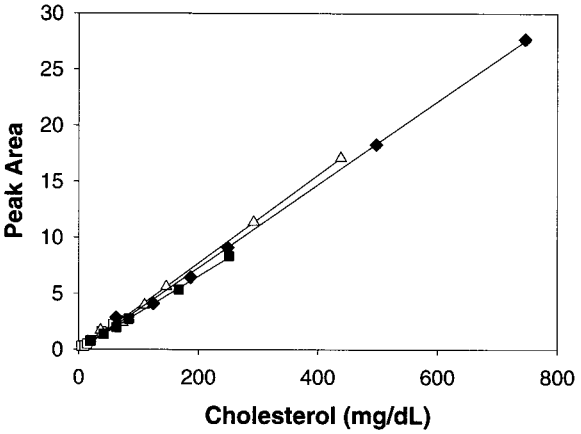

Fig. 3. Clip Linearity. A human plasma sample (total cholesterol = 124 mg/dL) was analyzed for cholesterol profiles at aliquots of 5, 10, 15, 20, 40, and 60 μL. Profiles were decomposed into lipoprotein fractions; cholesterol levels were calculated assuming that the 10 μL aliquot total cholesterol = 124 mg/dL. Symbols are ◆, total cholesterol (TC); □, very low density lipoprotein (VLDL); Δ, low density lipoprotein (LDL); and ■, high density lipoprotein (HDL).

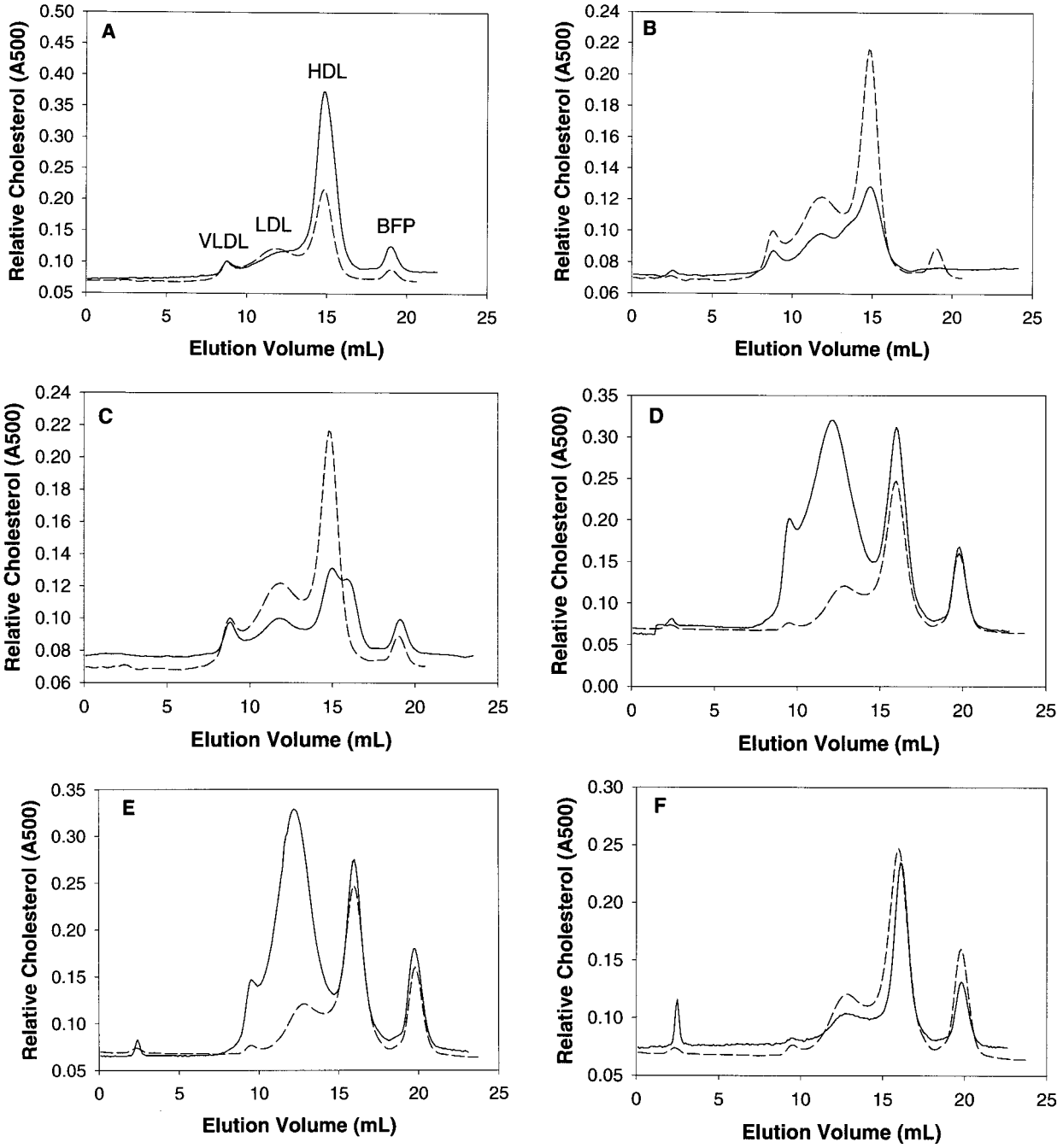

Fig. 4. Representative CLiP profiles of selected mouse strains. Aliquots of plasma from selected mouse strains were analyzed by CLiP. All aliquots were 10 μL. In all panels, the dashed line represents a profile from a normal C57BL/6J mouse. All animals were fed standard rodent chow. Panels are: A) human apoA-I expressing; B) apoA-I knock-out; C) human apoA-II expressing; D) LDL receptor knockout; E) LDL receptor/receptor-associated protein (RAP) double knockout; F) RAP knockout. Abbreviations are as in Fig. 3. Elution volumes are expressed as in Fig. 2.

terol values of five different human samples (with total cholesterol levels ranging from 198 to 263 mg/dL) differed from VAP-II values by −1.88% ± 2.57%.

Identification of total-volume peak

A peak was observed to elute at about 19 mL (after HDL) in nearly every mouse plasma sample (**Fig. 4**). This peak was not present in any human samples separated by the CLiP method. When a partially hemolyzed sample was separated without cholesterol reagent, no peak appeared at that location when detected at a wavelength of 500 nm (data not shown); thus, this peak was not due to absorption by hemoglobin (the peak was present in that sample when the cholesterol reagent was included). The peak was present in samples from both male and female mice (data not shown).

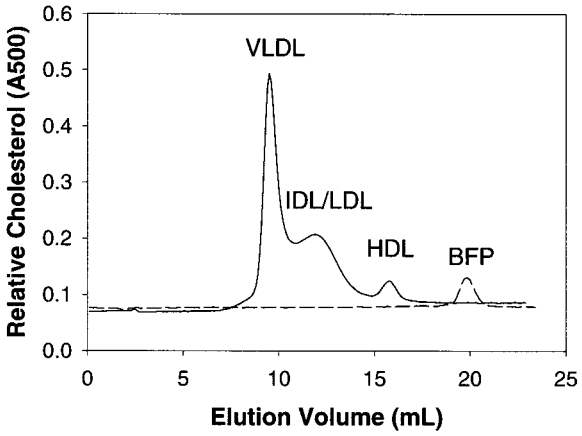

Fig. 5. Characterization of total-volume peak. A 250-μL aliquot of mouse plasma (apoE knock-out) was diluted to 2.5 mL with phosphate-buffered saline, adjusted to a density of 1.21 g/mL with solid KBr, ultracentrifuged (100,000 rpm, 24 h, 4°C, Beckman TL100 rotor), then 250-μL sequential samples were taken from the top. Aliquots of 10 μL from the top (solid line) and bottom (dashed line) fractions were analyzed by CLiP. Abbreviations are as described in the caption for Fig. 3, except that BFP signifies the bottom-fraction peak and IDL signifies intermediate density lipoprotein. Elution volume is expressed as in Fig. 2.

In order to further clarify the nature of the peak, an apoE knock-out mouse plasma sample was centrifuged to isolate lipoproteins from other plasma materials. A 250-μL aliquot (pooled from four mice) was diluted to 2.5 mL with phosphate-buffered saline, adjusted to a density of 1.21 g/mL with solid KBr, centrifuged (100,000 rpm, 24 h, 4°C, Beckman TL100 rotor), then sequential 250 μL samples were taken from the top. The top and bottom fractions were analyzed by CLiP, and the results shown in **Fig. 5** demonstrate that the unknown peak was present in the bottom fraction, but not in the lipoprotein (top) fraction.

Representative transgenic or gene-modified mouse profiles

Figure 4 presents lipoprotein profiles from a number of different transgenic or gene-modified mouse models. All profiles were done using 10 μL of plasma from non-fasting animals fed standard mouse chow. The profile from a C57BL/6J mouse fed normal rodent chow is presented in each panel for reference. Mice expressing human apoA-I were reported to have two new HDL subclasses, one larger and one smaller than normal chow-fed C57BL/6J mouse HDL (10). The CLiP profile (Fig. 5A) demonstrates an increased and broader HDL peak, suggesting the presence of HDL subclasses, although separate HDL subclasses are not resolved. All plasma lipoprotein fractions are reduced in apoA-I knock-out mice (Fig. 5B), consistent with previously reported results (11). Mice expressing human apoA-II have a smaller HDL fraction containing apoA-II only, in addition to larger apoA-I-containing HDL (12); this smaller peak is apparent in the CLiP profile (Fig. 5C). Mice with the LDL receptor knocked out have CLiP profiles similar to those previously reported (13), with elevated VLDL and LDL (Fig. 5D). Disruption of both the LDL receptor and the receptor-associated protein (RAP) gene (14) did not markedly alter the cholesterol profile compared with LDL receptor-deficient mice (Fig. 5E), although it should again be noted that plasma was taken from mice fed normal chow. In mice lacking RAP alone, no significant differences were seen compared with normal, chow-fed mice (Fig. 5F), consistent with results previously described (14).

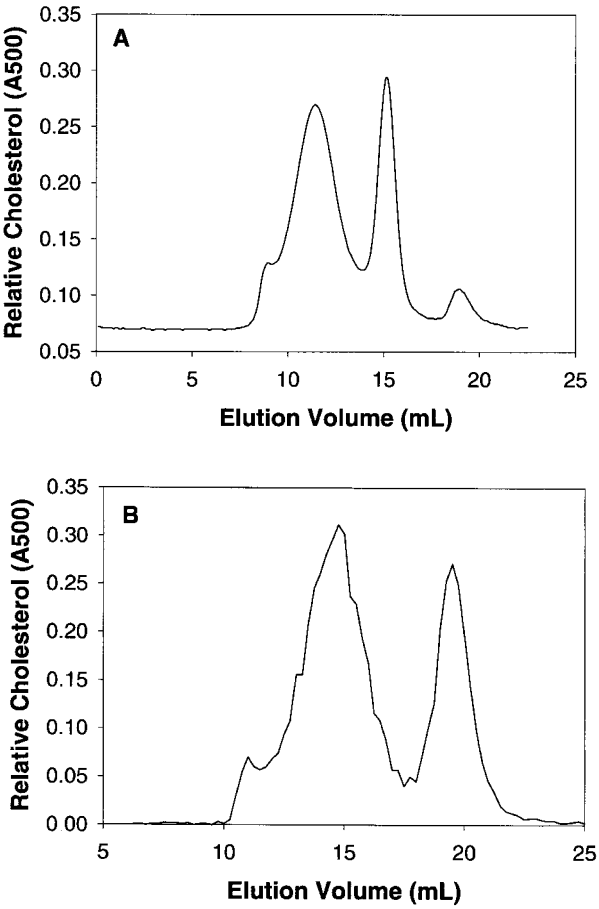

Fig. 6. Comparison of CLiP with FPLC. Plasma was taken from three LDL-receptor knockout mice after an overnight fast and was pooled. The CLiP profile was done using 10 μL of the pooled plasma (panel A), and the FPLC was done using 200 μL of the same pooled plasma (panel B). Although the same type of chromatography column (Superose 6; Pharmacia) was used with both systems, each system had its own separate column.

Comparison of CLiP with FPLC

Plasma was taken from three LDL-receptor knock-out mice after an overnight fast and pooled. Analysis was done by the CLiP method using 10 μL (**Fig. 6A**) and by FPLC using 200 μL (Fig. 6B). Although the same type of chromatography column was used with both systems, each system had its own separate column. Fraction collection in the FPLC method was stopped before the bottom-fraction peak was evident. Raw data from the CLiP method demonstrated much smoother curves due to the greater number of data points collected. After decomposition (pre-

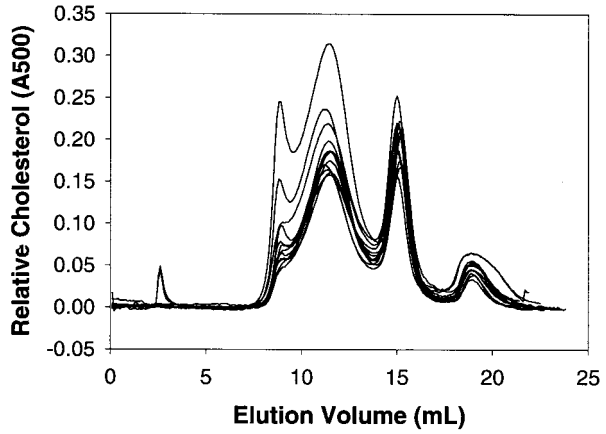

Fig. 7. Comparisons of CLiP profiles from several LDL-receptor knockout mice. Plasma was taken from 12 age-matched female LDL-receptor knockout mice after an overnight fast. Shown are CLiP profiles from each mouse. Baseline correction was applied to each profile using PeakFit (SPSS Science); no other processing was done.

ceded by smoothing in the case of FPLC data), peak areas as a percent of total area (neglecting the bottom-fraction peak in the CLiP results) were for the CLiP, VLDL = 9.29%, LDL = 62.28%, and HDL = 28.43%; while for the FPLC, VLDL = 5.45%, LDL = 65.92%, and HDL = 28.63%.

Similarity of profiles between animals of the same type

Plasma was taken from 12 age-matched female LDL-receptor knock-out mice after an overnight fast, and analysis was done by the CLiP method on plasma from each animal. As shown in **Fig. 7**, considerable variation occurred between mouse profiles, especially in the VLDL levels.

DISCUSSION

The CLiP method described here provides a simplified procedure for determining plasma lipoprotein cholesterol profiles in small sample volumes, allowing determination of profiles from individual animals rather than from pooled plasma. The method would therefore better allow investigators to study correlations of changes in plasma lipoprotein profiles with other factors, such as extent of atherosclerotic lesions. The method is reliable and reproducible, although care must be taken to deliver samples accurately into the injector, due to the low sample volumes used. We are currently using a 10-μL syringe for most injections. We use a 200-μL sample loop on the injector; use of a 10-μL loop is not recommended due to the phenomenon of partial filling caused by laminar flow (injection of a larger sample volume would be required to completely fill the loop). Addition of an auto-injector would facilitate ease of use and further ensure accurate sample injection volumes.

The pumps used in our laboratory are no longer manufactured. However, pumps are currently available which appear to be suitable. In particular, the pumps must be capable of accurate flow rates at relatively low backpressures and must be resistant to corrosion (common in so-called biocompatible pumps). It is also extremely useful to have wash ports behind the piston seal, especially in the cholesterol reagent pump. A steady flow of wash solution (water is sufficient) greatly extends the life of the seals and maximizes pump performance.

Measurement of distribution in plasma lipoproteins of radiolabeled materials, such as peptides or drugs that may affect lipoprotein profiles, is easily accomplished by adding a fraction collector after the spectrophotometric detector (Fig. 1). Thus, the plasma distribution of radiolabeled materials can be directly compared with the cholesterol profile.

This method could conceivably be modified to provide analysis of plasma triglyceride or free cholesterol profiles by the use of customized enzymatic reagents. For instance, a reagent lacking cholesterol esterase could be made using a modification of the reagent reported by Kieft et al. (9). Similarly, commercial enzymatic triglyceride reagents could perhaps be used for triglyceride profiles.

This method produces plasma lipoprotein profiles comparable in resolution to the original VAP method (7), while using a much smaller sample volume, 10 μL rather than 1.4 mL. Although the VAP-II method does use as little as 40 μL (15) and provides superior resolution compared to the CLiP method, the presence in mouse plasma of bottom-fraction material that reacts with the cholesterol reagent would cause VAP-II to overestimate the level of HDL in these samples. However, VAP-II is more appropriate for human clinical sample analysis, due to more rapid sample processing and greater resolution, including resolution of Lp[a].

Sample CLiP profiles from a number of gene-modified or transgenic mouse strains are presented in Fig. 5. The CLiP method produces profile results from these mouse strains that are generally consistent with those previously reported (10–14).

As shown in Fig. 7, individual mice of a specific strain (female age-matched LDL-receptor knock-out mice) have considerable variations in cholesterol profiles. It is likely that pooling of these samples would have resulted in an underestimation of the variations between animals. Availability of individual profiles would greatly improve correlations of lipoprotein cholesterol levels with other experimental results, such as degree of atherosclerosis.

In conclusion, the CLiP method provides a simple, reliable, and reproducible procedure for determination of plasma cholesterol profiles from individual plasma samples with very low sample volumes, using readily available equipment and reagents.

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