methods

A comparison of anion-exchange and steric-exclusion HPLC assays of mouse plasma lipoproteins

Jonathan Neyer, Christian Espinoza, Luppe Luppen, Terence M. Dohety, Subhash C. Tripathi, Hiroyasu Uzui, Pinky V. Tripathi, Gerald Lee, Prediman K. Shah, and Tripathi B. Rajavashisth[1]

Atherosclerosis Research Center, Division of Cardiology, Department of Medicine, and the Burns and Allen Research Institute, Cedars-Sinai Medical Center and David Geffen School of Medicine at University of California Los Angeles, Los Angeles, CA 90048

Abstract We compared two HPLC methods (anion exchange [AE] and steric exclusion [SE]) for analysis of mouse lipoprotein profiles by determining coefficients of variability (CVs) under varying conditions. CVs for AE and SE were comparable on fresh samples. There was an inverse relationship between subfraction curve area and CV [$r = -0.65$ (AE) and -0.50 (SE)], consistent with the interpretation that as curve area decreased, error variance increased and signal-to-noise ratio decreased. Sample storage did not affect SE. In contrast, with AE, alterations in measured lipoproteins were apparent after storage, including a decrease in the HDL subfraction [66.8% (baseline) vs. 15.9% (1 week); $P < 0.01$] and an increase in areas under LDL and VLDL peaks. Concomitant with decreasing HDL area, reproducibility deteriorated with the duration of storage. Analysis of the effects of decreasing sample injectate volume to <25 μl on SE lipoprotein subfractions revealed that areas under LDL and VLDL peaks decreased and persisted as volume was decreased further. Areas under all lipoprotein subfractions measured with either AE or SE were linearly correlated with the amount of cholesterol [$r = 0.69$ (AE) and 0.87 (SE)]. Both AE and SE yield reproducible, accurate, and rapid measurements of lipoproteins from small amounts of serum. AE yields more sensitive, high-amplitude, well-defined peaks that can be easily distinguished and necessitates the use of smaller sample volumes compared with SE, but sample storage causes alterations in the chromatogram. SE appears better suited to serial analyses involving stored samples.—Neyer, J., C. Espinoza, L. Luppen, T. M. Dohety, S. C. Tripathi, H. Uzui, P. V. Tripathi, G. Lee, P. K. Shah, and T. B. Rajavashisth. **A comparison of anion-exchange and steric-exclusion HPLC assays of mouse plasma lipoproteins. *J. Lipid Res.* 2005.** 46: **1786–1795.**

Supplementary key words lipoproteins • high-performance liquid chromatography • low density lipoprotein • high density lipoprotein • very low density lipoprotein • cholesterol measurement

Cardiovascular disease continues to cause the majority of morbidity and mortality in developed countries and will soon overtake infection as the leading cause of global death and disability (1–4). Epidemiological studies have clearly defined cardiac risk factors such as hypercholesterolemia and deleterious relative proportions of lipoprotein subfractions, such as increased LDL and/or decreased HDL cholesterol levels, as pivotal determinants of cardiovascular disease (5, 6). Although the overall relationships between serum lipoproteins and cardiovascular disease risk is well established (1, 7–12), there is wide variability in clinical outcomes associated with specific levels of LDL or HDL cholesterol, and there are no "cut point" levels that distinguish those who will suffer a cardiovascular disease event from those who will not. Thus, the precise nature of the relationship of lipoproteins with atherosclerotic disease remains incompletely understood. Furthermore, a detailed elucidation of homeostatic cholesterol metabolic pathways and trafficking has not yet been achieved.

Analysis of lipoprotein subfractions and their relationship to both normal and pathologic conditions is essential to a diverse set of ongoing investigations, but current analytic methods suffer from a number of disadvantages (13–15). Traditional assays are especially tedious, slow, and require high sample volumes. HPLC shows promise in addressing some of these difficulties (16–19), but no study has fully evaluated the reproducibility of the assay or the effects of technical considerations, such as the duration of storage, freeze-thaw cycles, or sample size, on HPLC performance. Sample volume in particular is an important practical consideration, because many studies involving lipoprotein measurement use small rodent models such as mice, which have a limited (0.5–2 ml) serum volume. Lipoprotein analysis methods suitable for such animal models require sample blood volumes small enough to al-

Manuscript received 14 July 2004 and in revised form 13 January 2005 and in re-revised form 23 February 2005.

Published, JLR Papers in Press, March 16, 2005.
DOI 10.1194/jlr.D500002-JLR200

Abbreviations: AE, anion exchange; apoE, apolipoprotein E; CV, coefficient of variability; SE, steric exclusion.

[1] To whom correspondence should be addressed.
Division of Endocrinology, Metabolism and Molecular Medicine, Charles R. Drew University of Medicine and Science, 1731 E. 120th St., Los Angeles, CA 90059. e-mail: rajavashisth@ucla.edu

low serial measurements in the same animal. Therefore, we compared the reproducibility and sample storage duration of two HPLC analytic methods: anion-exchange (AE) (17–19) and steric-exclusion (SE) (20) chromatography.

METHODS

Sample collection

Fasting (overnight) blood samples were collected from the orbital sinus of mice. Plasma was separated by centrifugation (1,500 *g* for 10 min) and stored in individual aliquots, with half stored at 4°C for both immediate use and degradation studies and the other half at −80°C for analysis of the effects of long-term storage.

AE

Figure 1A shows the AE application. Each sample is introduced into the mobile phase via a six-port injection valve with a 20 μl sample loop (7725i; Rheodyne, Rohnert Park, CA). The mobile phase solutions are mixed and delivered by two LC-10AT pumps (Shimadzu, Kyoto, Japan). Downstream from the two pumps and the injection valve, a PCX-5100 module (Pickering Laboratories, Mountain View, CA) contains a pressure transducer, a ProtEx-DEAE column packed with MCI gel (Supelco, St. Louis, MO), and pumps for the two postcolumn reagents. These two reagents are delivered under positive pressure from a helium tank. The first reagent, an enzyme mixture described below, is delivered before the primary reaction coil. The primary reaction coil (15 m) is housed in a Shimadzu CTO-10A column oven at 45°C. The second reagent (0.1 M NaOH) is delivered via a low dead-volume mixing T immediately after the samples exit the column oven. After the introduction of NaOH, the samples enter the secondary reaction coil and pass through a Shimadzu RF-10AXL fluorescence detector at an excitation wavelength of 325 nm and an emission wavelength of 420 nm, the obtained data are processed using proprietary software and a desktop personal computer (Shimadzu; see below for a more detailed description), and the samples are then collected in a waste container. A schematic diagram of the signal-producing reactions for AE is shown in Fig. 1A.

All mobile phases and reagents were made with OmniSolv HPLC-grade water (EM Science, Gibbstown, NJ). Mobile phase A consisted of 20 mM sodium phosphate buffer; mobile phase B consisted of 500 mM NaCl with 1 mM EDTA. The enzyme-signaling reagent consisted of cholesterol oxidase (20 μg/ml), peroxidase (50 μg/ml), cholesterol esterase (5 μg/ml), homovanillic acid (500 μg/ml), and 0.2% Triton X-100 in 0.1 M sodium phosphate buffer. The second reagent, 0.1 M NaOH, alkalized the reaction. All mobile phases and reagents were filtered and degassed with 0.22 μm, 1 liter filter systems (Corning, Inc., Corning, NY). The pumps (both A and B) maintained a constant total eluent flow rate of 1.0 ml/min. The concentrations of the mobile phases followed a stepwise elution program. Mobile phase B initiates the step gradient. The gradient for mouse serum starts

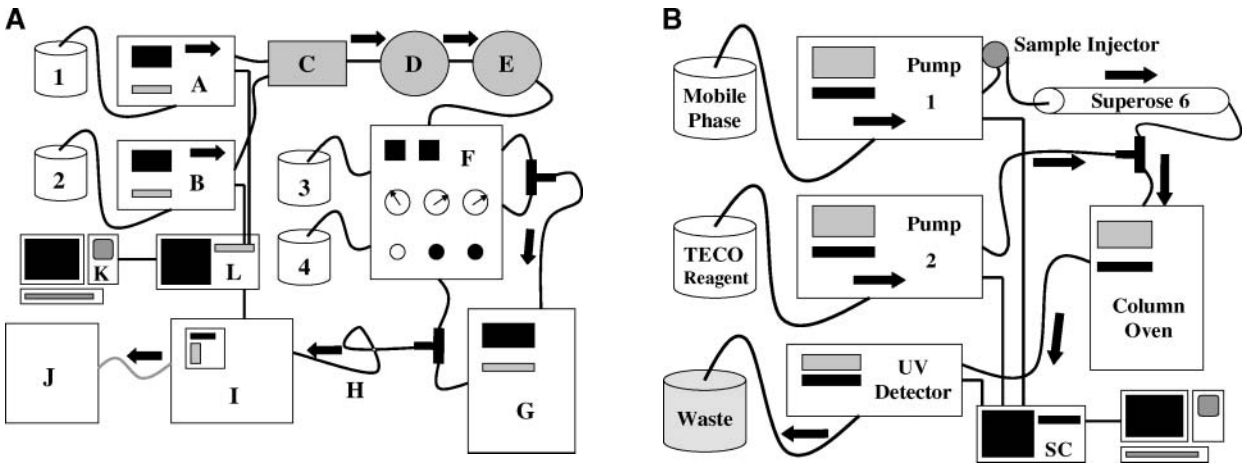

Fig. 1 A: Schematic diagram of anion-exchange (AE) HPLC instrumentation. Eluents [20 mM sodium phosphate (1) and 500 mM NaCl (2)] from the two pumps (A and B) join at a mixer (C), then pass by a stepwise gradient through a pressure transducer (D) and the injector (E). The sample then enters the column [housed in the Pickering machine (F)] and joins up with the oxidase reagent (3) in a mixing T [also housed in the Pickering machine (F)]. After the mixing T, the sample enters the primary reaction coil housed in the column oven (G). When the sample leaves the column oven, NaOH (4) entering from a second mixing T alkalizes the sample. After the mixing T, the sample enters the secondary reaction coil (H), the fluorescence detector (I), and finally a waste container (J). The entire AE system is controlled through a desktop computer (K) using proprietary software (EZSTART version 7.1.1; Shimadzu) that directs the system controller (L), which in turn coordinates the rest of the system. 1, 20 mM sodium phosphate; 2, 500 mM NaCl; 3, oxidase; 4, 100 mM NaOH; A, Shimadzu LC-10ATvp pump; B, Shimadzu LC-10ATvp pump; C, mixer; D, Pickering pressure transducer; E, Rheodyne 7725i manual sample injector; F, Pickering PCX-5100 postcolumn reaction module; G, Shimadzu CTO-10A column oven; H, 0.5 m secondary reaction coil; I, Shimadzu RF-10Axl fluorescence detector; J, Shimadzu FRC-10A fraction collector; K, desktop computer; L, Shimadzu SCL-10AVP system controller. B: Schematic diagram of steric exclusion (SE) HPLC instrumentation. After the sample is introduced into the sample injector (model 7725i; Rheodyne), pump 1 (Shimadzu LC-10ATvp) pushes the sample out of the sample injector with a mobile phase composed of 9% NaCl, 0.01% NaN_3, and 1 M EDTA (pH 7.4) through the Superose 6 10/300 GL column (Amersham Biosciences). Pump 2 (Shimadzu LC-10ATvp) introduces the signaling reagent [composed of 95% Teco signaling reagent (Teco Diagnostics) and 5% Brij solution (Sigma-Aldrich)] via a low dead-volume mixing T, shortly before the 15 m reaction coil housed inside the column oven. Upon exiting the reaction coil at a temperature of 55°C, the sample enters the ultraviolet (UV) detector and finally exits to a waste collector. The signal produced by the UV detector (absorption at 505 nm) is relayed to the desktop computer, which produces the chromatographic peaks and integrates the areas under the peaks using proprietary software (EZSTART version 7.1.1; Shimadzu). The entire system is coordinated by the system controller (SC; Shimadzu SCL-10AVP), which is directed by the software on the desktop computer.

with 0% mobile phase B for the first 2 min of a run, 45% mobile phase B for min 2–7, 55% mobile phase B for min 7–12, and 100% mobile phase B for the remainder of the separation. Both reagents were delivered to the reaction mixture at a flow rate of 0.5 ml/min. The enzyme-signaling reagent was stored and delivered into the reaction mixture at 4°C. The column oven maintained a temperature of 45°C to allow the enzyme-signaling reaction to take place near physiological temperature. The secondary reaction coil was kept at room temperature.

SE

Figure 1B shows the instrument setup for SE. A single pump (Shimadzu LC-10AT) delivered the mobile phase. Samples were introduced into the mobile phase by an injector (Rheodyne 7725i) with a 50 μl sample loop. Downstream from the injector was a Superose 6 10/300 GL column (Amersham Biosciences, Piscataway, NJ). A second pump (Shimadzu LC-10AT) delivered the postcolumn reagent immediately after the column, and downstream, a column oven (Shimadzu CTO-10A) housed a 15 m reaction coil. From there, samples entered an ultraviolet (UV) detector (Shimadzu SPD-10AVP). All electronic devices were controlled as described above. No fractions were collected.

All mobile phases and reagents were made with OmniSolv HPLC-grade water (EM Science). The mobile phase was 0.9% NaCl with 2 mM EDTA and 0.01% sodium azide at pH 7.4. The colorimetric signaling reagent was 95% Cholesterol-10 (Teco Diagnostics, Anaheim, CA) and 5% Brij 35 solution (Sigma-Aldrich, St. Louis, MO). Because this reagent has not been used in HPLC, we determined the optimal concentration of the reagent for our system and found that a concentration of 52.8 g/l produced a good signal strength at low cost. The mobile phase maintained a constant flow rate of 0.4 ml/min, the signaling reagent entered the flow line at a flow rate of 0.2 ml/min, the reaction coil was kept at a constant temperature (55°C), and a UV detector collected data at 505 nm.

Derivation of subfractions

Subfraction relative proportions were derived for both AE and SE using proprietary software (EZSTART version 7.1.1; Shimadzu). Briefly, two integration events are required for each run: width and threshold. These events are used to detect peak start, stop, and apex and to distinguish true peaks from noise. The system uses default values of width = 0.2 min and threshold = 50. Sampling rate can be varied and must be adjusted to optimize how much information the integration algorithm collects for drawing and integrating the chromatogram. The software has features to detect and avoid undersampling or oversampling. Manual adjustment of width and threshold values can be performed as well to optimize the sampling and frequency of data acquisition. Adjustments to integration can be input manually as well. The software reports chromatographic areas in absolute (but undefined) units, and output data include calculation of relative proportions of peaks obtained by comparison with the summed values under all peaks detected.

Ultracentrifugation analysis

Plasma was collected from 25 mice for lipoprotein separation through ultracentrifugation. For this purpose, mice heterozygous for apolipoprotein E deficiency (apoE$^{+/-}$) were used because wild-type mice have very low LDL and VLDL subfractions but apoE$^{+/-}$ mice have moderate hypercholesterolemia and correspondingly increased VLDL and LDL levels. Ultracentrifugation was performed essentially as reported previously (20–23). Plasma was separated by centrifugation (1,500 g for 10 min) and stored in individual aliquots at −80°C, and the serum was later pooled and used for repeated ultracentrifugations with progressively increasing solvent density. The separated fractions were analyzed by HPLC using the SE method. The density of the serum was increased or decreased by the addition of concentrated (3.5 M KBr; d = 1.346 g/ml) and diluted (0.15 M NaCl; d = 1.003 g/ml) salt solutions (22). Preservatives, proteases, and antibiotics were used to prevent serum degradation and microbe growth [1 mM EDTA, 1% (v/v) aprotinin, 100 μg/ml kanamycin, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), and 8 mg/ml chloramphenicol; all obtained from Sigma-Aldrich]. The densities of plasma and preservatives were adjusted according to the following formula:

$$10\ (\mathrm{LD}) + \mathrm{A}\ (\mathrm{HD})/10 + \mathrm{A} = 1.022\ \mathrm{g/ml}$$

where LD is the low-density solution, HD is the high-density solution, and A is the amount in milliliters of high- and low-density solution that needs to be added to 10 ml of water. The first spin was performed using a Beckman NVT90 rotor for 4 h at 15°C at 80,000 rpm. The resulting serum was collected and adjusted to a density of 1.065 g/ml. The next spin was performed with a Beckman SW50 rotor at 50,000 rpm at 10°C for 24 h to isolate the lipoproteins, and sequential 1 ml samples were obtained from the top down and densities were measured. Lipoprotein identification was according to sequential densities: the first two 1 ml samples were LDL, the next two were VLDL, and the last two were HDL. These identities were confirmed by comparison of measured densities with previously reported lipoprotein densities in the mouse [HDL, 1.09–1.10 g/ml; LDL, 1.023–1.060 g/ml; VLDL, 1.017–1.023 g/ml (23)].

Reproducibility

We compared the precision of AE and SE methods by determining retest reproducibility. For both methods, we performed sequential analyses at least three times under varying sample conditions as described below. To determine the intermethod reproducibility, we performed sequential AE and SE analyses on the same sample in variable order. For AE, coefficients of variability (CVs) were obtained by performing chromatography on eight serum samples obtained from eight different mice (one sample per mouse), and all samples were replicated in triplicate. For SE, CVs were derived from chromatographic analyses of serum obtained from six mice, each run at least in triplicate. For comparison of the reproducibility of specific lipoprotein fractions, the individual percentage areas under each lipoprotein peak were obtained from the same set of chromatograms, and CVs of the lipoprotein peaks were calculated and compared. Serum samples for the evaluation of both AE and SE reproducibility were obtained from mice exhibiting a wide range of total serum cholesterol levels ranging from normal (<100 mg/dl) to greatly increased (>2,000 mg/dl) and included mice deficient in urokinase plasminogen activator, LDL receptor, macrophage colony-stimulating factor, paraoxonase-1, leptin receptor, and apoE.

Sample storage

Samples were analyzed immediately after being drawn using both AE and SE methods, and results and reproducibility were compared. Because storage time may potentially affect SE and AE in distinct ways, we analyzed serum samples, then stored aliquots of the same samples at 4°C for up to 2 weeks before reanalysis.

Injection volume

Garber, Kulkarni, and Anantharamaiah (20) have reported that injection volumes of 10 μl produce acceptable SE results; however, recommendations from the Superose 6 column manufacturer suggest injectate volumes between 25 and 250 μl. On the other hand, Haginaka, Yamaguchi, and Kunitomo (18) reported

that only 2–10 μl of serum yield reproducible lipoprotein subfraction peaks using AE. This smaller sample volume reported with AE (18) might represent an advantage of the AE method, but it is not known whether smaller sample volumes than those recommended (20) deleteriously affect SE lipoprotein measurements. Therefore, we evaluated the influence of decreasing injectate volume on the areas under the LDL, VLDL, and HDL lipoprotein peaks using the SE method. Identical serum samples were evaluated using SE and sample injection volumes of 10, 20, and 25 μl.

Total cholesterol assay

To determine the relationship of the SE and AE methods to an independent method of cholesterol measurement, we compared SE and AE HPLC total subfraction areas (VLDL + LDL + HDL) to total cholesterol measured with a manual colorimetric assay (Teco Diagnostics). Samples were run at least in duplicate and compared with a 200 mg/dl chemical standard (Teco Diagnostics). Absorbance was measured at 520 nm.

Statistical analysis

Values are expressed as means ± SD. Mean values were compared using Student's *t*-test (unpaired or paired, as appropriate). Comparisons of continuous variables were performed by calculating Pearson correlation coefficients. CVs were calculated, and $P < 0.05$ was considered significant.

RESULTS

Qualitative comparison

Figure 1 depicts the instrument setups for AE and SE HPLC applications, and the relevant reactions are shown in **Fig. 2**. Representative chromatograms from the AE and SE methods run with serum from nonfasted mice are shown in **Fig. 3**. Sample injection volumes for AE were 8 μl, whereas SE chromatograms used 25 μl injections. Serum from a normocholesterolemic mouse with a genetic deficiency in the gene encoding urokinase plasminogen activator ($uPA^{-/-}$) that has no effect on lipid metabolism yielded the blue chromatogram using AE in Fig. 3A. Serum from a hypercholesterolemic mouse with a genetic deficiency in both uPA and apoE ($uPA^{-/-}$; $apoE^{-/-}$) yielded the red chromatogram (Fig. 3A). Figure 3B (blue chromatogram) shows a chromatogram using SE that used a serum sample obtained from normocholesterolemic mice that were deficient in the gene encoding carbonic anhydrase II ($CA\text{-}II^{-/-}$), a genetic mutation that has no effect on lipid metabolism. For an example of a hypercholesterolemic mouse, we examined serum from apoE null mice using SE (Fig. 3B; red chromatogram). On the AE chromatograms (Fig. 3A), the most polar cholesterol group, HDL, elutes first from the column, whereas VLDL elutes first from the SE column (Fig. 3B). Lipoprotein subfractions were derived and integrated as described in Methods.

Ultracentrifugation

VLDL, LDL, and HDL were extracted and purified by ultracentrifugation. Briefly, the single peaks that eluted validated the time integrations for VLDL, LDL, HDL, and bottom fraction protein (BFP) that were reported previously using SE (20). The densities of these were 0.925, 1.040, and 1.086, respectively. CVs for duplicate determinations were 0.96% (LDL), 1.07% (HDL), and 3.46% (VLDL).

Reproducibility

Tables 1–3 summarize the precision of the AE and SE methods. The CVs for AE and SE methods on freshly drawn samples averaged 7.7 ± 6.2% (range, 0.8–20.9) and 5.8 ± 2.7% (range, 1.2–11.0), respectively. The differences in overall CVs are summarized in Table 1. There was a trend for lower CVs with SE, but this trend did not reach statistical significance ($P = 0.2$). As shown in Tables 2, 3, for the lipoprotein subfractions, average CVs for LDL (4.4 ± 3.2% and 3.8 ± 2.3% for AE and SE, respectively) were somewhat better than those for HDL (6.2 ± 5.7% and 7.4 ± 2.3%) and VLDL (12.3 ± 6.7% and 6.2 ± 2.3%). The variability in lipoprotein subfraction CVs was likely a result of the fact that area percentage values for VLDL were comparatively low. Consistent with this interpretation, there was an inverse linear correlation between CVs and percentage area of peaks representing the lipopro-

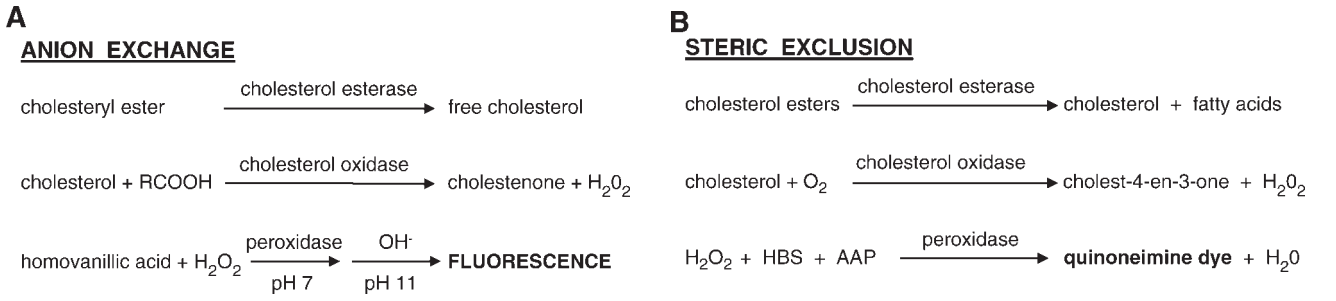

Fig. 2. A: Postcolumn reactions that produce signals for AE. A fluorescent signal is produced by the reaction of homovanillic acid with hydrogen peroxide in the presence of peroxidase, with an excitation wavelength of 325 nm and an emission wavelength of 420 nm. Sources of reagents are as follows: homovanillic acid (H0339; Tokyo Kasei Kogyo Ltd., Tokyo, Japan); cholesterol esterase (COE-311; Toyobo Co. Ltd., Osaka, Japan); cholesterol oxidase (COO-321; Toyobo Co. Ltd.); and peroxidase (PEO-302; Toyobo Co. Ltd.). B: Postcolumn reactions that produce signals for SE. Cholesterol esterase, cholesterol oxidase, and peroxidase are obtained premixed but undiluted in a kit (Cholesterol 10 Reagent Set; Teco Diagnostics) that contains 4-aminoantipyrine (AAP; 0.6 mM), sodium cholate (8.0 mM), cholesterol esterase (>150 U/l), cholesterol oxidase (>200 U/l), horseradish peroxidase (>1,500 U/l), *p*-hydroxy benzene sulfonate (HBS; 20 mM), buffer (125 mM, pH 6.8), nonreactive stabilizers, and fillers. The dilution procedure is detailed in Methods.

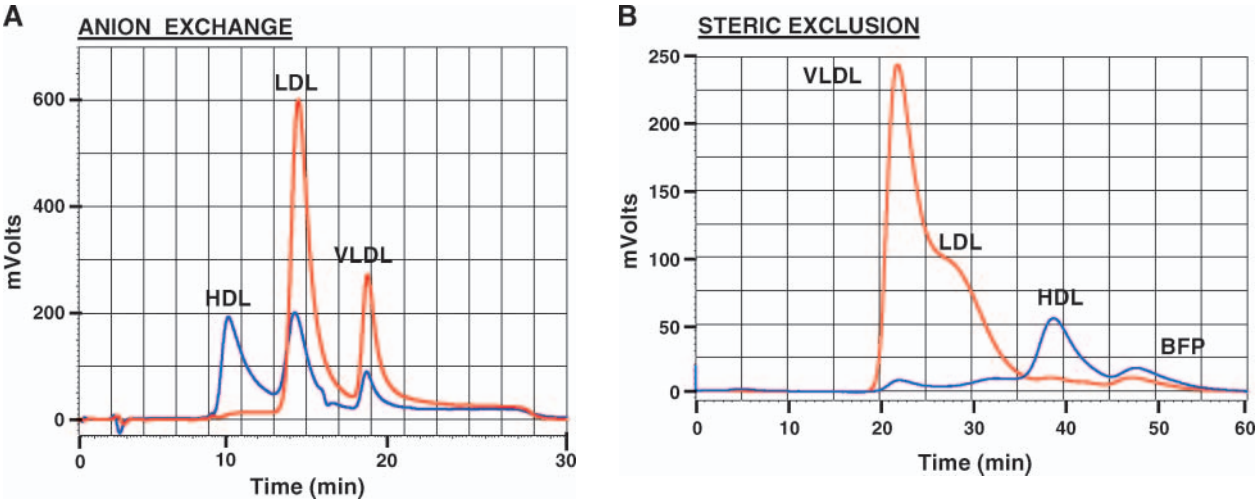

Fig. 3. Representative AE and SE chromatograms showing the ability of each method to resolve variable lipoprotein subfraction quantities. Using the AE method (A), the blue chromatogram was obtained from a mouse with homozygous genetic deficiency of urokinase plasminogen activator ($uPA^{-/-}$). This defect does not alter lipid metabolism; therefore, $uPA^{-/-}$ mice are normocholesterolemic. The red chromatogram was obtained from a double-knockout mouse that was genetically deficient in both urokinase plasminogen activator and apolipoprotein E ($uPA^{-/-}$; $apoE^{-/-}$). This genotype manifests severe hypercholesterolemia secondary to the apoE mutation. Using the SE method (B), the blue chromatogram was obtained from a mouse with a homozygous genetic deficiency of carbonic anhydrase II ($CAII^{-/-}$), which has no effect on lipid metabolism, and yields mice that are normocholesterolemic. The red chromatogram was obtained from an apoE-deficient mouse. The bottom fraction protein (BFP) peak does not represent cholesterol or any lipoprotein subfraction but instead reflects a mixture of low molecular weight proteins that react with the signaling reagent.

tein subfractions (**Fig. 4**). For AE, the correlation between CV and percentage area was –0.65 ($y = -0.2x + 13.8$; $r^2 = 0.42$), and for SE, the correlation was −0.50 ($y = -0.1x + 8.0$; $r^2 = 0.25$). Generally, the reproducibility of AE appeared inferior to that of SE. As seen in Fig. 4, AE precision appeared to be more affected by subfraction curve area than was SE precision: as the area under the subfraction decreased, AE CVs increased (Fig. 4A), but little increase in CV was observed in SE as subfraction area decreased (Fig. 4B).

In wild-type mice and other common laboratory animals, HDL subfractions tend to be large, whereas LDL and VLDL fractions are relatively small. Therefore, SE would be anticipated to be a more precise method to evaluate LDL and VLDL subfractions in such animals. Furthermore, in serial studies, the ability to detect changes over time will be increasingly limited as the retest reproducibility increases. This is a simple statistical consequence of the error variance inherent in the measurement method. Thus, in performing serial measurements of lipoproteins, a retest reproducibility of 25% would require a subsequent measurement to be at least 50% different from the baseline measurement to exceed the 95% confidence interval limits and enable one to conclude that any change

TABLE 1. Comparison of overall AE and SE CVs

Variable	AE	SE
Mean	7.7[a]	5.8[a]
SD	6.2	2.7
Median	4.9	5.9
Maximum	20.9	11.0
Minimum	0.8	1.2
n	23	18

AE, anion exchange; CV, coefficient of variability; SE, steric exclusion. For AE, CVs were obtained by performing chromatography on eight serum samples obtained from eight different mice (one sample per mouse), and all samples were replicated in duplicate or triplicate. For SE, CVs were derived from chromatographic analyses on serum obtained from six mice, each run at least in duplicate. Mice with variable serum levels of cholesterol ranging from normal (<100 mg/dl) to markedly increased (>2,000 mg/dl) were selected for these analyses and included genotypes deficient in urokinase plasminogen activator, LDL receptor, macrophage colony-stimulating factor, paraoxonase, leptin receptor, and apolipoprotein E.

[a] $P = 0.2$ (NS).

TABLE 2. AE CVs

Sample	n	HDL	LDL	VLDL
1	3	ND[a]	3.8	4.7
2	3	6.4	4.1	20.9
3	6	6.1	3.5	3.1
4	5	18.4	6.3	13.9
5	5	4.7	11.4	11.1
6	3	0.9	0.8	7.9
7	3	2.5	2.7	17.2
8	3	4.6	2.4	19.3
Mean		6.2	4.4	12.3
SD		5.7	3.2	6.7
Median		4.7	3.7	12.5
Maximum		18.4	11.4	20.9
Minimum		0.9	0.8	3.1
n		7	8	8

From the same samples used to generate the CVs in Table 1, individual CVs for the percentage areas under each of the three lipoprotein fractions were obtained by repeating AE chromatography at least three times.

[a] ND, not determined.

TABLE 3. SE CVs

Sample	n	HDL	LDL	VLDL
1	3	6.8	3.4	6.2
2	3	8.9	7.6	5.9
3	3	4.5	3.2	8.6
4	4	7.4	5.5	9.1
5	3	5.9	2.0	3.8
6	3	11.0	1.2	3.8
Mean		7.4	3.8	6.2
SD		2.3	2.3	2.3
Median		7.1	3.3	6.0
Maximum		11.0	7.6	9.1
Minimum		4.5	1.2	3.8
n		6	6	6

From the same samples used to generate the CVs in Table 1, individual CVs for the percentage areas under each of the three lipoprotein fractions were obtained by repeating AE chromatography at least three times.

at all had occurred. Taking these considerations in light of our findings that SE retest reproducibility generally appears lower, SE would be the preferred HPLC method in studies evaluating serial changes in lipoproteins.

Sample storage

The two HPLC methods do not produce comparable results when samples are stored at 4°C. For samples run on fresh blood using SE, the percentage areas under each lipoprotein fraction averaged 33.3, 38.4, and 19.2% for VLDL, LDL, and HDL subfractions, respectively. After 7 days of storage at 4°C, the percentage areas of all three lipoprotein peaks measured with SE remained similar to those measured on freshly drawn blood (33.8, 33.2, and 24.1% for VLDL, LDL, and HDL fractions, respectively; P = NS for all compared with fresh samples) (data not shown). The CVs after 1 week of storage also remained similar in magnitude. In contrast, with AE, significant alterations in measured lipoproteins were apparent after 7 days of storage at 4°C compared with those measured at baseline on freshly drawn blood. Lipoprotein subfraction areas on freshly drawn samples using AE averaged 9.9, 23.4, and 66.8% for VLDL, LDL, and HDL, respectively. However, after 7 days of storage at 4°C, these proportions changed to 41.9, 42.1, and 15.9% ($P < 0.03$, 0.02, and 0.01 for VLDL, LDL, and HDL, respectively, compared with fresh samples) (**Fig. 5A–C**). Particularly obvious was the diminution of HDL (Fig. 5C). After 2 weeks of storage at 4°C, the HDL peak had all but disappeared in samples measured with AE. Although the HDL curve area decreased sharply, the areas under the LDL and especially the VLDL peaks increased significantly (Fig. 5). Reproducibility deteriorated as the percentage area under the HDL fraction decreased. After 1 week of storage, CV had increased to 22.7% compared with 5.6% at baseline, but this trend did not reach statistical significance ($P = 0.09$) (Fig. 5C). Reproducibility for VLDL and LDL was not altered by storage duration. We conclude that storage of serum samples for 1 week at 4°C does not significantly alter the results obtained with SE, but, in contrast with AE, there is significant attenuation of the area under the HDL peak and increases in the areas under the LDL and VLDL peaks compared with values measured at baseline. The alterations in AE measurement appear to be accompanied by a deterioration of reproducibility for HDL subfraction measurements, as reflected in the CVs.

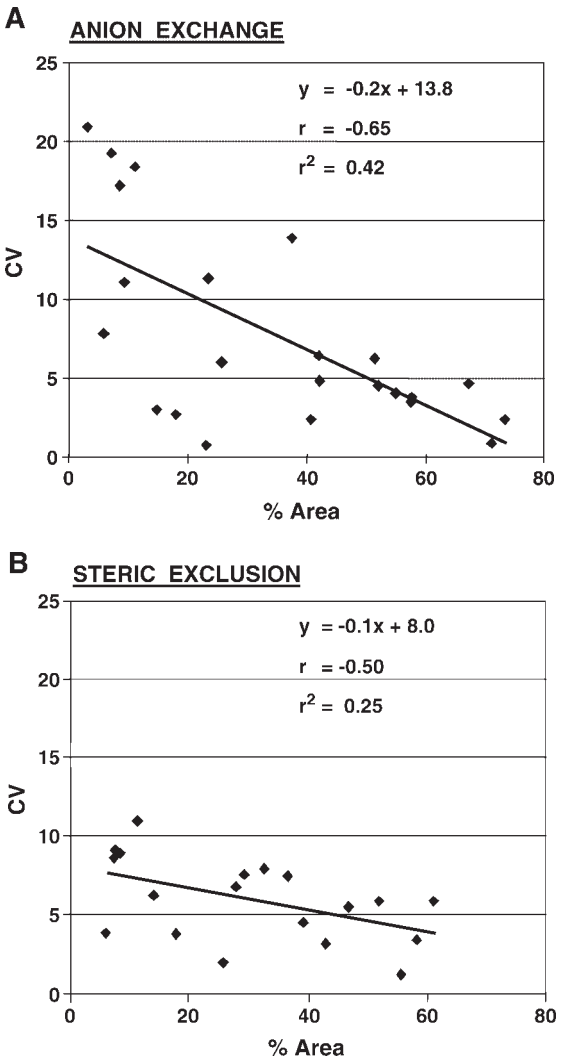

Fig. 4. Precision of AE (A) and SE (B) measurement of lipoprotein subfractions relative to percentage area under the lipoprotein subfraction peaks. Each data point represents the coefficient of variability (CV) for duplicate determinations of the percentage area under a lipoprotein subfraction peak (VLDL, LDL, or HDL). Retest reproducibility was an inverse function of the size of the peaks for both methods, as shown by the negative correlation coefficients and inverse linear trend lines. However, reproducibility for SE was generally superior to that of AE. Retest reproducibility was consistently <10% for subfraction percentage areas of less than ~15% for SE (B); however, for AE (A), similarly precise retest reproducibility required subfraction curve areas of greater than ~35%.

Sample integrity with AE also appears to be affected by storage conditions. **Figure 6** shows three runs of the same serum sample stored under different conditions. The red chromatogram was obtained from serum run the day after sacrifice; the black chromatogram was obtained from serum stored at −20°C for 7 days; the blue chromatogram

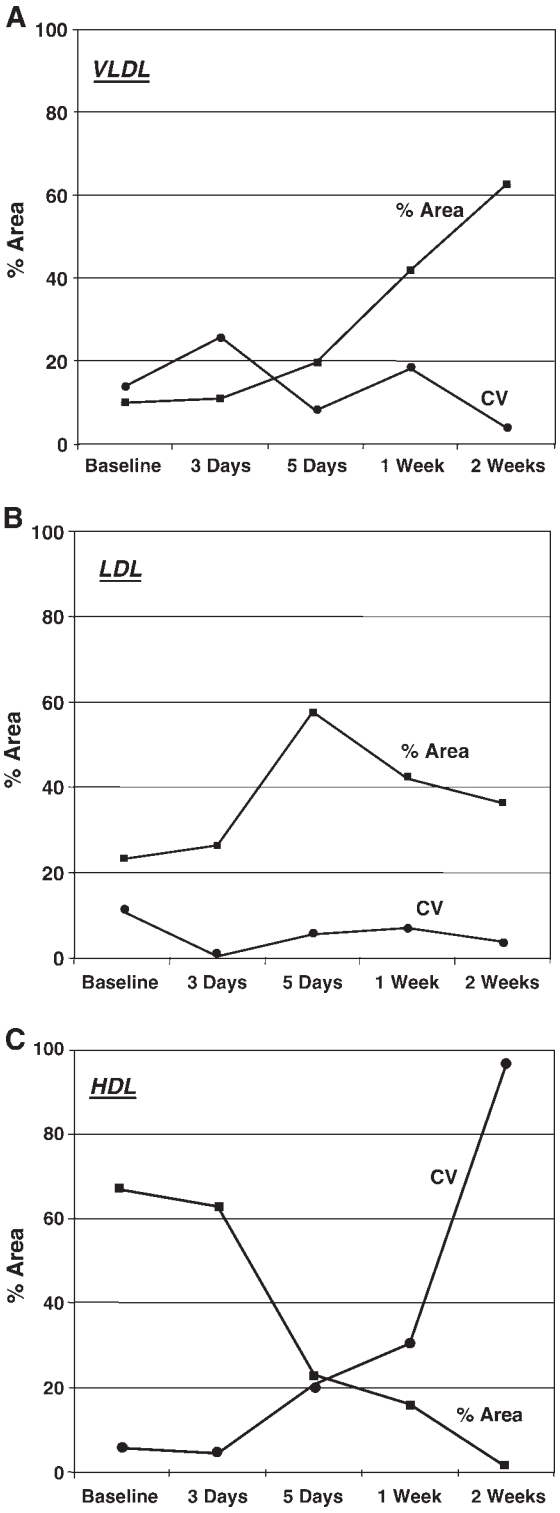

Fig. 5. Effects of storage duration on AE chromatography lipoprotein subfraction percentage areas and reproducibility. The percentage areas (line with squares) under both the VLDL (A) and LDL (B) subfractions appear to increase with storage duration, and this is accompanied by a marked decrease in HDL peak amplitude (C). Concomitantly, the reproducibility (line with circles) of HDL subfraction measurements deteriorates markedly (C); however, the reproducibility of the VLDL (A) and LDL (B) subfractions appears unaffected by storage duration.

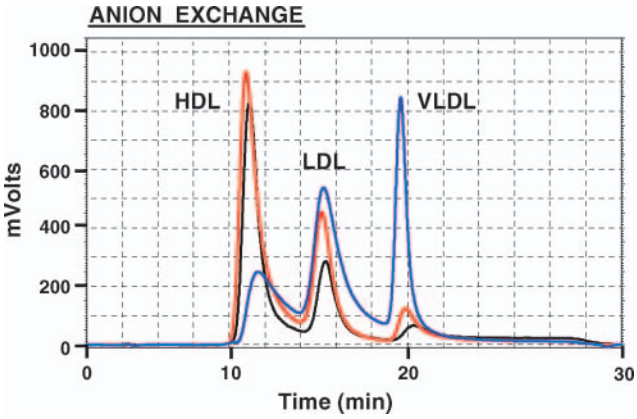

Fig. 6. AE sample degradation. Three runs of the same serum sample obtained from a macrophage colony-stimulating factor-deficient heterozygous mouse stored under different conditions. The red chromatogram was obtained from serum run the day after sacrifice; the black chromatogram was obtained from serum stored at −20°C for 7 days; the blue chromatogram was obtained from serum stored at 4°C for 7 days. This example suggests that AE chromatography results can be markedly altered by storage temperature and time.

was obtained from serum stored at 4°C for 7 days. It again appears that as storage time increases and/or with storage at higher (4°C) temperatures, there is marked diminution of the HDL peak, with a concomitant increase in the LDL and particularly the VLDL peaks (Fig. 6). Collectively, these results suggest that AE is sensitive to storage temperature and duration, and if samples are not run fresh, significant deterioration in both the precision and reproducibility of the obtained results may occur.

Injection volume

Decreasing the injection volume to <25 μl had a significant effect on lipoprotein analysis using SE, as shown in **Fig. 7**. With an injection volume of 25 μl, the mean percentage area under the lipoprotein peaks was 37.7, 35.9, and 1.9% for VLDL, LDL, and HDL, respectively. When injectate volume was decreased to 20 μl, significant decreases were seen in the percentage area under both the LDL and VLDL peaks (to 26.8% and 25.5% for VLDL and LDL, respectively; $P < 0.001$ for both). The effect of decreased injection volume on the relative sizes of the VLDL and LDL lipoprotein subfractions appeared to persist when injectate volume was decreased further. Using an injection of 10 μl, the percentage areas under the LDL and VLDL peaks were less than those measured using 25 μl, but in the case of VLDL, this difference did not quite reach statistical significance [27.6% ($P = 0.08$) and 22.5% ($P < 0.001$) for VLDL and LDL, respectively, compared with 25 μl injection volume]. The relative area under the HDL peak increased with a 20 μl injection (from 1.9% to 4.5%; $P < 0.05$), but with a 10 μl injection it was not significantly different from the percentage area obtained with a 25 μl sample volume. Thus, our results are consistent with the interpretation that the use of sample injection volumes less than that recommended (25 μl) will alter the results of lipoprotein analysis using SE.

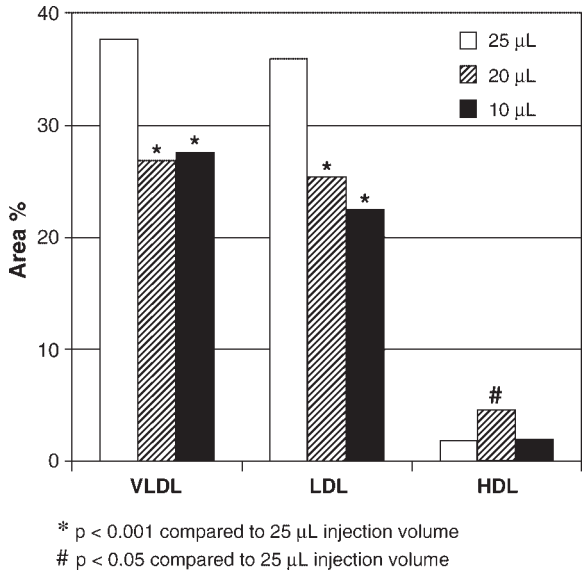

Fig. 7. Effects of injectate volume on SE measurement of lipoprotein subfractions. As injectate volume was decreased to <25 µl, there was a significant decrease in the relative percentage area under both the VLDL and LDL peaks. The area under the HDL peak increased significantly with a 20 µl injectate volume, but using an injection volume of 10 µl, it was not significantly different from that obtained with a 25 µl sample injection. * $P < 0.001$; # $P < 0.05$ (both comparisons relative to 25 µl injections).

Total cholesterol assay

Before comparing the colorimetric cholesterol assay with AE and SE HPLC, we first evaluated the linearity of the colorimetric assay by preparing and analyzing serial dilutions of the standard. The colorimetric total cholesterol assay was linear over the entire range of cholesterol values assessed (data not shown). Both AE and SE HPLC methods produced accurate total cholesterol values. **Figure 8** shows the relationship between total cholesterol values obtained with the manual colorimetric assay and the AE and SE chromatographic total areas. There was a strong linear relationship between the amount of cholesterol measured by the colorimetric assay and the total area of the chromatograms using either AE or SE ($r = 0.69$ and 0.87 for AE and SE, respectively; linear functions were $y = 0.4x + 33$ and $y = 0.1x + 13$ for AE and SE, respectively).

DISCUSSION

HPLC has begun to be used to measure small amounts of serum lipoproteins (18–20), paving the way for more widespread use in laboratory investigations involving small animals. However, it is not yet clear what the advantages and disadvantages of AE and SE HPLC methodologies are and which might be optimal for specific experimental situations. Furthermore, it is not known how AE and SE results compare with one another, nor have the precision, accuracy, and effects of storage duration on the results obtained been adequately investigated.

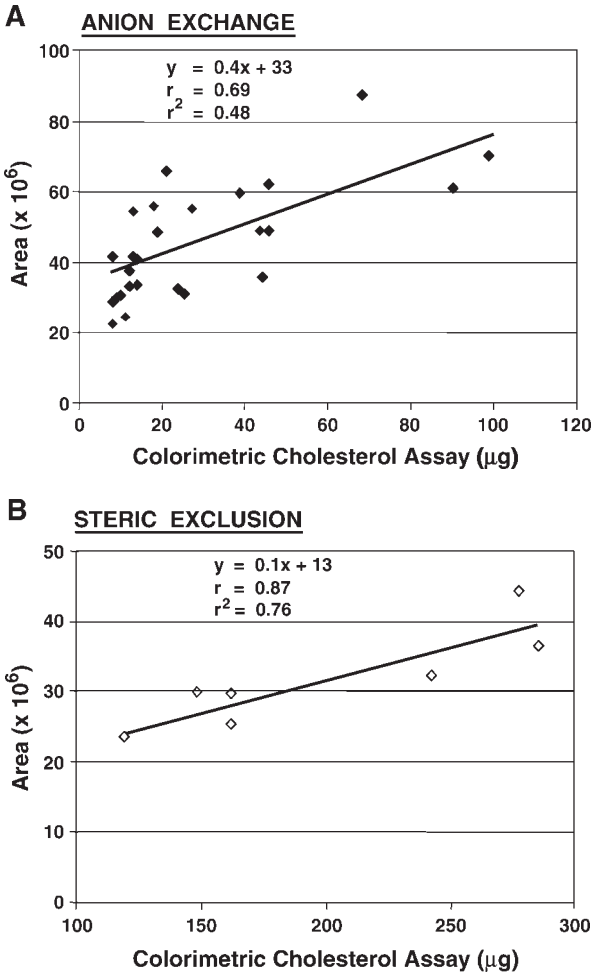

Fig. 8. Comparison of AE (A) and SE (B) measurement of cholesterol (*y* axis) with manual colorimetric assay results (*x* axis). HPLC results represent the integrated areas under the peaks (units are in nanometer-millivolts).

Our results show that both AE and SE HPLC applications provide reproducible, accurate, and comparatively quick assays of individual mouse lipoproteins. Both methods correlate well with a manual colorimetric assay (Fig. 8), and retest reproducibility is within acceptable limits for both methods as well, averaging 7.7% and 5.8% for AE and SE, respectively (Tables 1–3). Although AE and SE each has unique limitations, the ease and elegance of these techniques make them attractive alternatives to traditional, direct lipoprotein assays of pooled serum. In addition, determination of HDL and LDL contents of individual mouse serum samples can only be achieved using AE or SE. This capability, a product of HPLC's high sensitivity, allows researchers to efficiently and cost-effectively explore cholesterol subfraction variation between genetically altered mice and after subjecting mice to treatment conditions of interest. SE in particular enables serial HPLC analysis of mouse lipoprotein subfractions because it requires small amounts of serum yet retains adequate precision in measurement.

The AE method, the more sensitive of the two applica-

tions, appears to yield superior results, producing high-amplitude, well-defined peaks that can be easily distinguished. This application's ProtEx-DEAE column and fluorescent signaling mixture of this procedure achieve better separation and produce a higher signal-to-noise ratio than the SE method. The AE method produces a signal approximately seven times stronger than that obtained with the SE method, and the software can more easily integrate the chromatogram when there is strong signal and a clear separation between peaks. Moreover, the AE application produces these superior results with smaller injection volumes and shorter separation times than the SE method. The AE setup requires only 2–10 μl of serum and 30 min to produce sharp peaks, whereas the SE method requires injection volumes between 10 and 25 μl and ~55 min run times. Thus, AE produces more sensitive results with lesser serum volumes in a shorter time than the SE method. For these reasons, AE might be the HPLC method of choice for some applications, particularly those in which fresh samples will be analyzed and when it is important to minimize serum injection volume.

Although the AE method possesses these advantages, our results demonstrate that AE may not be the optimal HPLC analysis method for some types of mouse studies. We found that the AE column appears to be more susceptible to sample degradation than the more durable SE procedure. Charge, a rather transient physical property used to achieve separation of lipoprotein subfraction moieties in the AE column, can dissociate or otherwise vary, and this will tend to distort the AE chromatogram over time, as our results with frozen serum samples demonstrate (Figs. 6, 7). By contrast, the size of lipoprotein particles does not change appreciably over time, and this likely explains why SE yields more dependable results from stored samples. As shown in Fig. 4B, SE appears to produce very reproducible results even when peak areas are small. In some mouse genotypes, the lipoprotein fractions of interest might be small, and in these instances SE would be preferable, particularly when there are insufficient sample volumes available to allow repetitive analysis of the same sample. An additional disadvantage of AE is the more complicated instrument setup and the greater attendant level of technical expertise required. Although the SE method needs only two pumps, the AE method requires four HPLC pumps (two to provide the mobile phase concentration gradient, one for the signaling reagent, and one to alkalize the signaling mixture). Thus, AE requires a greater initial investment and more maintenance during operation, has more possible sources of breakdown and/or error variance, requires greater technical expertise, is less suitable for stored samples, and appears to yield less reproducible results when peak areas are small.

Both AE and SE applications produce accurate resolution of lipoprotein subfractions, and both have specific advantages and disadvantages. The small sample injection volume required with the AE column makes it well suited to research involving small animals such as mice and rats, particularly in applications in which it is desirable to analyze serial serum samples and when minimal injectate volumes are required. By contrast, the dependability and simplicity of the SE method make it an attractive option for use with larger animals or in cases in which stored samples of serum are used.

This work was supported by grants from the National Heart, Lung, and Blood Institute (HL-51980 and HL-58555 to T.B.R.), the National Institutes of Health, and Philip Morris USA, Inc. (T.B.R.). The authors thank Cole Scientific, Inc., the Mirisch Foundation, United Hostesses Charities, the Eisner Foundation, the Grand Foundation, the Ornest Family Foundation, and the Heart Fund at Cedars-Sinai Medical Center for their generous support.

REFERENCES

1. American Heart Association. 2003. Heart Disease and Stroke Statistics—2004 Update. American Heart Association, Dallas, TX.
2. Tunstall-Pedoe, H., K. Kuulasmaa, P. Amouyel, D. Arveiler, A. M. Rajakangas, and A. Pajak. 1994. Myocardial infarction and coronary deaths in the World Health Organization MONICA Project. Registration procedures, event rates, and case-fatality rates in 38 populations from 21 countries in four continents. *Circulation.* **90:** 583–612.
3. Murray, C. J., and A. D. Lopez. 1997. Alternative projections of mortality and disability by cause 1990–2020: Global Burden of Disease Study. *Lancet.* **349:** 1498–1504.
4. World Health Organization. 1999. The World Health Report. Making a Difference. World Health Organization, Geneva, Switzerland.
5. Kannel, W. B. 2000. Epidemiologic contributions to preventive cardiology and challenges for the twenty-first century. In Preventive Cardiology. N. D. Wong, H. R. Black, and J. M. Gardin, editors. McGraw-Hill, New York. 3–20.
6. Gordon, T., and W. B. Kannel. 1971. Premature mortality from coronary heart disease: the Framingham Study. *J. Am. Med. Assoc.* **215:** 1617–1625.
7. Keys, A. 1980. Seven Countries. A Multivariate Analysis of Death and Coronary Heart Disease. Harvard University Press, Cambridge, MA.
8. Kannel, W. B., W. P. Castelli, and T. Gordon. 1979. Cholesterol in the prediction of atherosclerotic disease. New perspectives based on the Framingham Study. *Ann. Intern. Med.* **90:** 85–91.
9. Libby, P. 2002. Inflammation in atherosclerosis. *Nature.* **420:** 868–874.
10. Glass, C. K., and J. L. Witztum. 2001. Atherosclerosis. The road ahead. *Cell.* **104:** 503–516.
11. Steinberg, D. 2002. Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. *Nat. Med.* **8:** 1211–1217.
12. Libby, P. 2001. The vascular biology of atherosclerosis. In A Textbook of Cardiovascular Medicine. 6th edition. E. Braunwald, D. P. Zipes, and P. Libby, editors. W. B. Saunders, Philadelphia, PA. 995–1009.
13. Usui, S., M. Nakamura, K. Jitsukata, M. Nara, S. Hosaki, and M. Okazaki. 2000. Assessment of between-instrument variations in a HPLC method for serum lipoproteins and its traceability to reference methods for total cholesterol and HDL-cholesterol. *Clin. Chem.* **46:** 63–72.
14. Yoshida, A., M. Naito, M. Kodama, and H. Nomura. 2001. Comparison of direct methods and HPLC for the measurement of HDL- and LDL-cholesterol with ultracentrifugation. *J. Atheroscler. Thromb.* **8:** 84–90.
15. Yamaguchi, Y., M. Kunitomo, and J. Haginaka. 2002. Assay methods of modified lipoproteins in plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **781:** 313–330.
16. Yamaguchi, Y., M. Kunitomo, and J. Haginaka. 1998. AE high-performance liquid chromatographic assay of plasma lipoproteins of rabbits, rats and mice. *J. Chromatogr. B Biomed. Sci. Appl.* **716:** 57–64.

17. Yamaguchi, Y., S. Kagota, M. Kunitomo, and J. Haginaka. 1998. Evidence of modified lipoprotein in the plasma of Watanabe heritable hyperlipidemic rabbits by AE high-performance liquid chromatographic assay. *Atherosclerosis.* **139:** 323–331.
18. Haginaka, J., Y. Yamaguchi, and M. Kunitomo. 2001. AE high-performance liquid chromatography assays of plasma lipoproteins and modified low-density lipoproteins using a ProtEx-DEAE column. *J. Chromatogr. B Biomed. Sci. Appl.* **751:** 161–167.
19. Haginaka, J., Y. Yamaguchi, and M. Kunitomo. 1995. AE high-performance liquid chromatographic assay of plasma lipoproteins. *Anal. Biochem.* **232:** 163–171.
20. Garber, D., K. Kulkarni, and G. M. Anantharamaiah. 2000. A sensitive and convenient method for lipoprotein profile analysis of individual mouse samples. *J. Lipid Res.* **41:** 1020–1026.
21. Jiao, S., T. G. Cole, R. T. Kitchens, B. Pfleger, and G. Schonfeld. 1990. Genetic heterogeneity of plasma lipoproteins in the mouse: control of low density lipoprotein particle sizes by genetic factors. *J. Lipid Res.* **31:** 467–477.
22. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
23. Camus, M. C., M. J. Chapman, P. Forgez, and P. M. Laplaud. 1983. Distribution and characterization of the serum lipoproteins and apoproteins in the mouse, *Mus musculus*. *J. Lipid Res.* **24:** 1210–1228.