papers and notes on methodology

Rapid quantitative apolipoprotein analysis by gradient ultracentrifugation and reversed-phase high performance liquid chromatography

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Abstract A new methodology for the analysis of lipoprotein composition using a combination of gradient ultracentrifugation and high performance liquid chromatography was used to determine the differences in lipoprotein composition between non-hyperlipidemic men and women. Lipoproteins from each subject were separated into six subfractions: VLDL, IDL, LDL, and three subfractions of HDL by a single gradient ultracentrifugation spin of less than 5 hr. The HDL subfractions were designated HDL-L (the lightest density subfraction, rich in apoCs and poor in apoA-II), HDL-M (the middle subfraction, rich in apoA-II), and HDL-D (the most dense, relatively poor in both the apoCs and apoA-II). The concentrations of the water-soluble apolipoproteins in each subfraction were determined using reversed-phase HPLC. The concentrations of apoB and the lipid components of the lipoproteins were determined by chemical and enzymatic methods. This methodology proved to be highly reproducible when performed on fresh plasma samples and we were able to identify many sex-associated differences in lipoprotein composition. This methodology is the only non-immunological technique available for analyzing lipoprotein composition that offers such a combination of accuracy, speed, and completeness. — **Hughes, T. A., M. A. Moore, P. Neame, M. F. Medley, and B. H. Chung.** Rapid quantitative apolipoprotein analysis by gradient ultracentrifugation and reversed-phase high performance liquid chromatography. *J. Lipid Res.* 1988. **29:** 363–376.

Supplementary key words lipoproteins • sex differences • high density lipoprotein subfractions

Immunological assays are the most common methods used today for the measurement of apolipoproteins. There have been a number of methods reported for the measurement of apoA-I (1, 2), apoA-II (2, 3), apoC-I (4, 5), apoC-II (5–8), apoC-III (5–7), apoE (9–11), and apoB (12). Although these assays have been extremely useful in expanding our understanding of lipoprotein physiology and hyperlipidemia, there are some inherent difficulties in using immunologic assays. Antibodies must be generated and characterized. The ability of an antibody to recognize its ligand may change depending on the particular lipoprotein with which it is associated. The characteristics of each antibody are unique, making standardization difficult. Each ligand must be isolated and stored for long periods of time. When radioimmunoassays are used, ligands must be labeled and stored. Finally, each apolipoprotein must be assayed separately.

We have developed a technique for analyzing lipoprotein composition using a combination of gradient ultracentrifugation (to isolate lipoprotein subfractions) and HPLC (to quantitate the water-soluble apolipoproteins in each of the purified lipoprotein subfractions). This method is rapid and accurate and allows us to detect subtle changes in lipoprotein concentrations and composition.

METHODS

Subject population

Twelve men and six women with total plasma cholesterol concentrations of less than 220 mg/dl and total

Abbreviations: VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL-L, high density lipoprotein, lowest density fractions, high content of apoCs; HDL-M, high density lipoprotein, middle density fractions, high content of apoA-II; HDL-D, high density lipoprotein, most dense fractions, low content of both apoA-II and Cs; Lp[a], a polymorphic form of LDL; HPLC, high performance liquid chromatography.

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plasma triglyceride concentrations of less than 180 mg/dl were studied. These subjects were selected from a much larger population that was being randomly screened. None of the subjects were on any medication nor did they have any significant illness at the time of sampling. Two men and two women exercised daily and two of the men were smokers (one smoked less than a pack per day). The men ranged in age from 28 to 66 years (48.3 ± 14.0 years, mean ± SD) and had a mean percent ideal body weight of 106 ± 11 (range: 91 to 129%). The women were 33 to 60 years of age (45.7 ± 9.8 years) and were slightly more obese than the men (117 ± 13% IBW, $P = 0.05$, range: 106 to 140%).

Amino acid analysis

N-Terminal amino acid analysis was done manually using the dimethylamino azobenzene isothiocyanate (DABITC) methodology described by Chang (13) and modified by Allen (14). Amino acid analysis was done using reversed-phase HPLC of PTC-amino acids and the Pico-Tag methodology described by Waters Associates.

Statistics

All data are expressed as means ± standard deviation. Differences between data sets were determined using the *t*-test procedure in SAS (statistical analysis system) which computes the '*t*' statistic based on the assumption that the variances of the two groups are equal and also computes an approximate *t* based on the assumption that the variances are unequal.

Lipoprotein isolation

Lipoproteins were isolated using a gradient ultracentrifugation technique briefly described previously (15). Blood was drawn, after at least a 12-hr fast, into tubes containing EDTA and immediately placed on ice. Plasma was separated by centrifugation at 4°C, and NaN_3 and reduced glutathione were added to give final concentrations of 0.02% and 0.5 μg/ml, respectively. Ten ml of plasma was raised to a density of 1.35 gm/ml with 5.94 g of potassium bromide (KBr) and placed in 40-ml Quik-Seal ultracentrifuge tubes. A second layer of buffer (10 ml) (NaCl 0.9%, EDTA 1.0 mM, Tris 10 mM, NaN_3 0.1%, pH 8.5, containing 0.5 μg/ml glutathione and protease inhibitors 6-aminohexanoic acid 100 mM, benzamidine HCl 5 mM, and phenylmethylsulfonylfluoride, 1.0 mM) raised to a density of 1.20 g/ml with KBr was added to the centrifuge tube and, finally, the tube was filled with the above buffer (d 1.006 g/ml) to a final volume of approximately 40 ml. The tubes were sealed and spun to 8.0×10^{11} rad^2/sec at 70,000 rpm at 15°C in a 70 Ti rotor (approximately 4 hr and 20 min).

Tubes were emptied by pumping chloroform into the bottom of the tubes using a Shimadzu LC-6A HPLC pump. The sample was collected from the top of the tube using a Beckman fraction recovery system. The effluent was pumped through a Pharmacia UV-1 detector and the absorbance at 280 nm was recorded (**Fig. 1**). One-ml fractions were collected. **Fig. 2** shows the cholesterol, protein (measured by the method of Lowry et al. (16)), and apoA-I concentrations of each of the fractions. ApoA-I was recovered only in the HDL density fractions (d 1.065 to 1.21 g/ml). Three HDL pools (L, M, and D) were made based on the ultraviolet profile and the fraction densities determined by refractometry. The *rationale* for this division will be discussed later. **Fig. 3** shows a gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the lipoprotein pools of a control plasma sample isolated by this technique. It shows some contamination of the HDL fractions with albumin (especially HDL-D, the most dense HDL) but there is little albumin in VLDL, IDL, or LDL, and there is good separation of LDL and HDL. There was a trace of apoA-I in LDL but this was not detectable by HPLC.

Apolipoprotein analysis by HPLC

One ml of VLDL and each HDL pool and 2 ml of IDL were delipidated, first with 5 ml of hexane–isopropanol 3:2 and then with 4 ml of hexane alone. This delipidation procedure was used because of the extremely low solubility

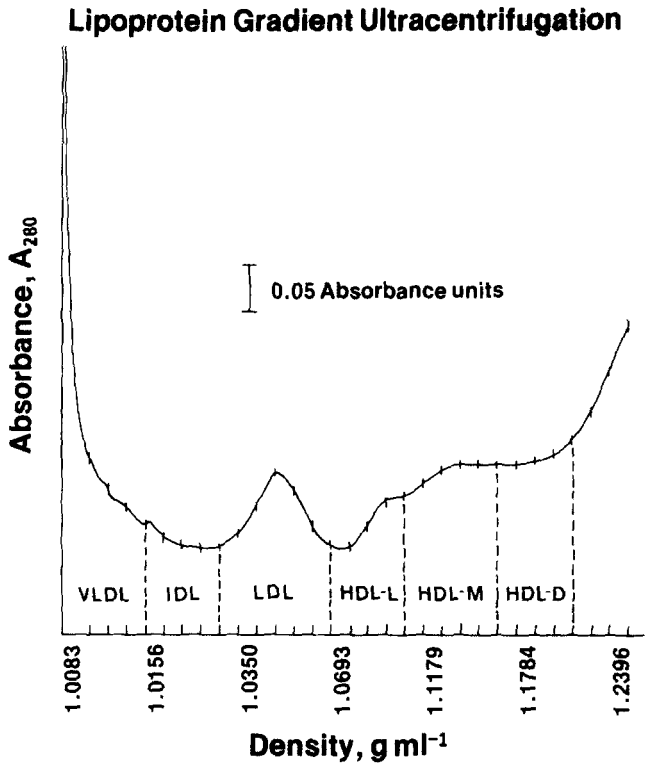

Fig. 1. Typical ultraviolet profile of control plasma showing the usual pools. Each mark is 1 ml. Pools were initially established by the relative apolipoprotein composition (see Methods). Reproducibility of each ultracentrifugation was checked by measuring the density of selected fractions from each tube and comparing them to the original ultracentrifugation.

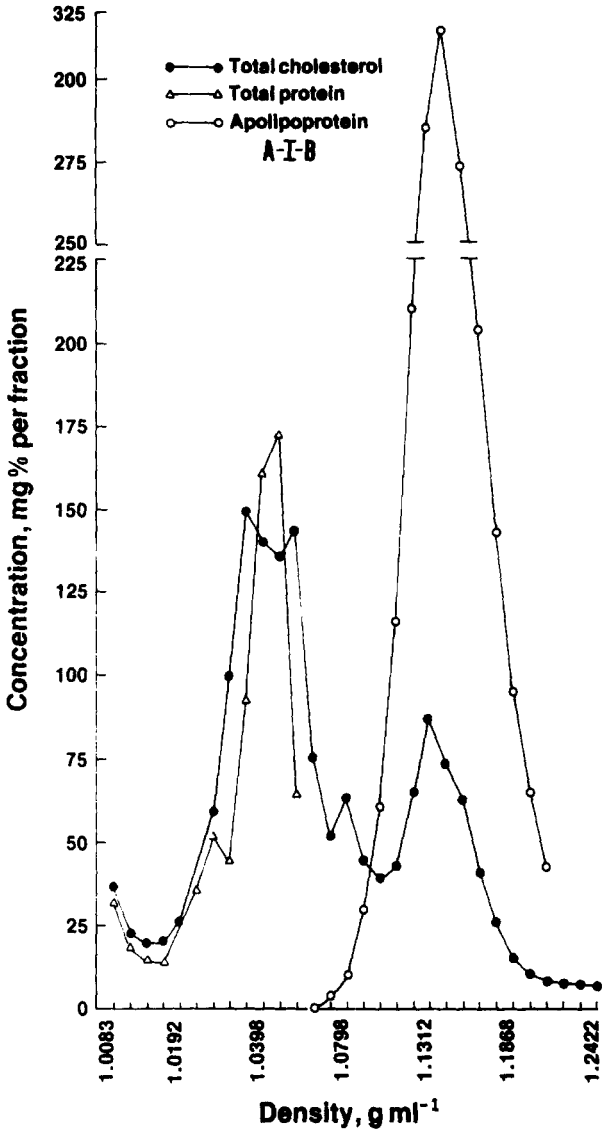

Fig. 2. The apoA-Ib (the major HPLC apoA-I redox-form), enzymatic cholesterol, and Lowry protein concentrations on each 1-ml fraction from a gradient ultracentrifugation separation of a control plasma.

of protein in the hexane layer (17). Human insulin, either Novolin-R or Humulin (100 to 300 μg), was added as an internal standard to each sample prior to delipidation. Insulin was chosen as a standard because of its ready availability in a highly purified and quantified form and because its retention time is shorter than any of the apolipoproteins. The aqueous layer was dried and the proteins were solubilized in 3 M guanidine-HCl, Tris buffer (described above, without the protease inhibitors) and injected onto the HPLC column. A Perkin Elmer Series 4 pump was used with an LC-75 UV detector (detection at 214 nm). Peak areas were quantitated with a Shimadzu Data Processor Chromatopac C-R3A and the data were directly transferred to a VAX computer, via an RS-232 port, for further analysis. A Vydac reversed-phase C18 column (4.6 × 250 nm, no. 218TP54) was used for analysis and the proteins were eluted with an acetonitrile–water gradient (containing 0.1% trifluoroacetic acid) of 25 to 58%, at 1%/min, and a flow rate of 1.2 ml/min. The column was heated to 50°C in a water bath. Representative chromatograms are shown in **Fig. 4** and **Fig. 5.**

Individual peaks were isolated by HPLC and identified by amino acid analysis and N-terminal analysis (**Table 1**). The second residue of apoC-II is reported to be glutamine (20) rather than glutamate. Our result was probably due to deamidation during amino acid analysis. It was found that apoA-I and C-II were each eluted as two peaks and that apoA-II and C-III were eluted as three peaks. The apolipoprotein "redox-forms" were identical by amino acid and N-terminal analysis and by SDS polyacrylamide gradient gel analysis. As we will describe in a subsequent manuscript, these redox-forms appear to be due to oxidation of methionine, probably during preparation of the sample. It appears that the more oxidized redox-forms elute earlier. Each apolipoprotein redox-form is designated by a lower case letter. ApoE-3 was generously provided by Dr. Robert Mahley from the Gladstone Foundation in San Francisco, CA. It was found to co-elute with apoA-IIb (32.0 min). A response factor relative to insulin was determined using this apoE-3. The VLDL from a patient with known Type III hyperlipidemia (Fig. 5) and an E-2/E-2 phenotype was found to have a large broad peak at 30.5 min (between apoA-IIc and A-IIa). This peak was isolated and found to have a molecular weight similar to apoE on gradient SDS-PAGE. These results suggest that reversed-phase HPLC may separate apoE-2 and apoE-3. Unfortunately, these apoE isoproteins appear to co-elute with the apoA-II redox-forms which will make it difficult to quantitate the apoE in HDL. However, this is not a problem in VLDL, as seen in Fig. 5, since this subfraction contains no apoA-II.

Each apolipoprotein was purified by preparative reversed-phase HPLC using a procedure similar to that described above for the analytical analysis: flow rate of 2.0 ml/min, gradient of 0.5%/min, and a 10-mm diameter column with identical packing material. Response factors relative to insulin were determined for each of the apolipoproteins at 214 nm by determining the concentration of each purified protein by quantitative amino acid analysis and injecting a known amount of the apolipoprotein and a known amount of insulin. The linearity of peak area responses was determined by making repetitive injections of increasing amounts of HDL-M. **Fig. 6** shows that the responses were linear for each of the apolipoproteins from approximately 0.1 μg to 50 μg per injection. The sensitivity of this procedure for each apolipoprotein varies somewhat depending on individual response factors (1 μg of apoA-II produces a peak area about 30% less than 1 μg of apoC-II) and peak configurations (apoE elutes as broad peaks that

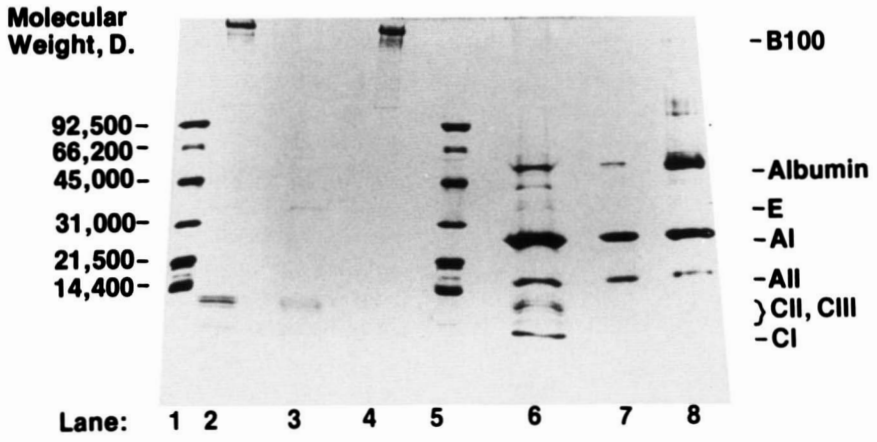

Fig. 3. Gradient SDS-PAGE (3–20%) of the apolipoproteins in each lipoprotein pool (see Fig. 1). Low molecular weight standards are shown in lanes 1 and 5. Lanes: 2, VLDL; 3, IDL; 4, LDL; 6, HDL-L; 7, HDL-M; 8, HDL-D. ApoB-100 in IDL did not enter this particular gel but is usually seen in the appropriate position in other gels. Each lipoprotein subfraction was diluted to a different degree so as not to overload the gel.

are more difficult to integrate than the sharp peaks produced by the other apolipoproteins).

HDL pools

Fig. 7 shows the results of HPLC analysis of each of the HDL fractions (1.0 ml) from a gradient ultracentrifugation separation of plasma from a nonhyperlipidemic subject. Each apolipoprotein concentration is expressed as a percentage of the concentration of apoA-Ib. It shows that the middle fractions (d 1.11–1.16 g/ml) have a high relative concentration of apoA-II, while the lower density fractions were rich in the apoCs and poor in apoA-II. The higher density fractions were relatively poor in all the apolipoproteins except apoA-I. We elected to pool the HDL fractions into three pools (L, M, and D, lowest to highest density) based on these relative protein concentrations

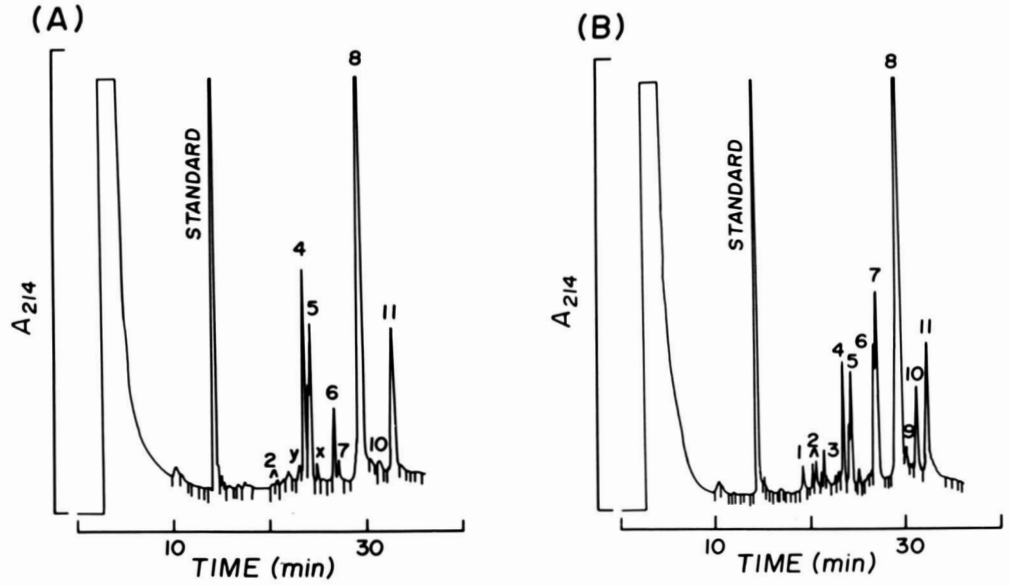

Fig. 4. Typical HPLC chromatographs of human apolipoproteins. These samples are HDL-L subfractions from a nonhyperlipidemic control. Panel A shows an HDL-L subfraction that was stored frozen and panel B shows an aliquot of the same HDL-L subfraction after being stored at 4°C. The usually identified apolipoproteins and their redox-forms are: 1: apoC-IIIa, 2: apoC-IIIb, 3: apoC-IIa, 4: apoC-IIIc, 5: apoC-I, 6: apoC-IIb, 7: apoA-Ia, 8: apoA-Ib, 9: apoA-IIc, 10: apoA-IIa, 11: apoA-IIb. Peaks X and Y were almost always seen but have not been identified. The HPLC conditions used were: acetonitrile–water (with 0.1% TFA) linear gradient of 25 to 58% at 1%/min, a flow rate of 1.2%, and the column heated to 50°C. The standard was human insulin.

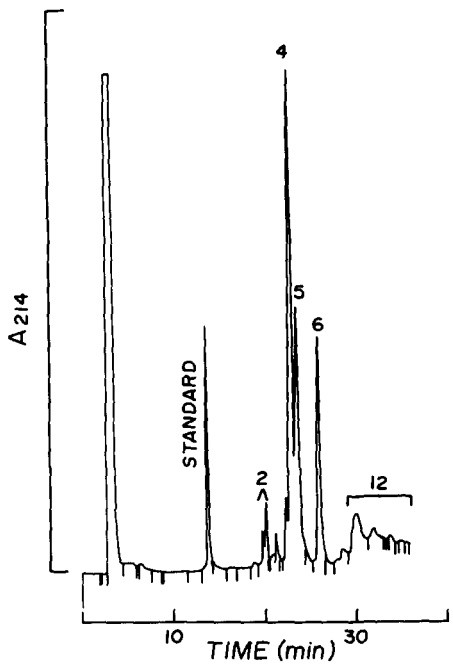

Fig. 5. This sample is VLDL from a patient with Type III hyperlipidemia. The usually identified apolipoproteins and their redox-forms are: 2: apoC-IIIb, 4: apoC-IIIc, 5: apoC-I, 6: apoC-IIb, 12: apoE. The HPLC conditions used were: acetonitrile-water (with 0.1% TFA) linear gradient of 25 to 58% at 1%/min, a flow rate of 1.2%, and the column heated to 50°C. The standard was human insulin.

since they clearly represent different populations of HDL particles. It is possible that each of these pools contains several subpopulations of HDL particles but, using our current techniques, these are the only subfractions that are readily discernible. Our HDL-L is similar to HDL_{2b} with a density range of 1.063–1.11 g/ml and a high relative content of cholesterol and apoCs.

It is possible that the lipoproteins have not achieved equilibrium with the salt gradient at the completion of the centrifugation so that no firm conclusions can be drawn as to the identity of the lipoprotein particles based on these densities. However, there is little change in the lipoprotein positions when the centrifugation time is prolonged. This would suggest that they are essentially at equilibrium. Also, our lipoprotein subfractions have compositions similar to those that would be expected in these density ranges. This question is of little practical concern since the only reason that we measure the fraction densities is to assure the reproducibility of the salt gradient.

Enzymatic and chemical assays

Cholesterol (Autoflo from Boehringer Mannheim Diagnostics), free cholesterol (Boehringer Mannheim food analysis kit), and triglyceride (Sigma) concentrations of the lipoprotein subfractions were determined with commercially available enzymatic assays. We did not measure the free cholesterol in HDL-D because the reagent precipitates during the procedure, which may be due to the high concentration of KBr in this fraction.

The apoB content of LDL was determined by diluting an aliquot of LDL 1:5 with water, and then mixing 100 μl of diluted LDL with 100 μl of 100 mM SDS, 0.2 N NaOH. Protein assay reagents (15) were added and the

TABLE 1. Amino acid and N-terminal amino acid analysis of purified apolipoproteins (residues/molecule)

	ApoA-I		ApoA-II		ApoC-I		ApoC-III		ApoC-II	
	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs	Exp	Obs
Asp	21	20.5	3	3.0	5	5.1	7	6.8	5	4.7
Thr	10	9.7	6	5.3	3	2.9	5	4.7	9	7.2
Ser	15	14.2	6	6.3	7	6.6	11	9.4	9	7.6
Glu	46	48.7	16	15.0	9	9.5	10	10.8	14	13.6
Gly	10	10.4	3	3.8	1	1.5	3	3.3	2	2.4
Ala	19	19.5	5	5.3	3	3.4	10	9.4	6	6.2
Val	13	13.1	6	6.6	2	2.1	6	5.4	4	4.2
Met	3	2.9	1	1.0	1	0.5	2	2.4	2	3.4
Ile	0	0	1	1.0	3	2.7	0	0.4	1	1.0
Leu	37	34.9	8	7.9	6	6.3	5	5.6	8	7.7
Tyr	7	8.3	4	3.8	0	0.2	2	1.9	5	3.9
Phe	6	5.9	4	3.9	3	3.0	4	4.0	2	2.4
His	5	4.8	0	0.1	0	0	1	1.2	0	0.3
Lys	21	21.6	9	9.0	9	7.9	6	6.2	6	5.6
Arg	16	15.7	0	0	3	3.4	2	2.5	1	1.7
N-Terminal										
1)	Asp	Asp	none[a]	none	Thr	Thr	Ser	Ser	Thr	Thr
2)	Glu	Glu			Pro	Pro	Glu	Glu	Gln	Glu
3)	Pro	Pro			Asp	Asp	Ala	Ala	Gln	Gln

References: apoA-I, Brewer et al. (18); apoA-II, Brewer et al. (19); apoC-I, Jackson et al. (20); apoC-III, Brewer et al. (21); apoC-II, Myklebost et al. (22). Abbreviations: Exp, expected; Obs, observed.

[a] ApoA-II has a cyclic amino acid as an amino-terminal that cannot be hydrolyzed off under these conditions; therefore, no amino acid is identified.

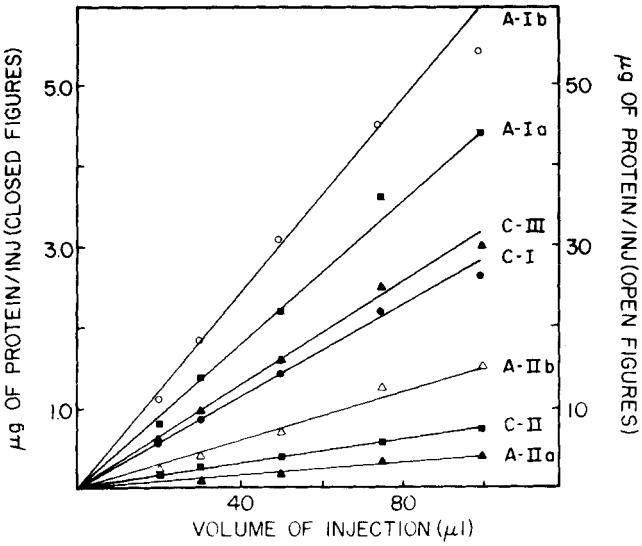

Fig. 6. This figure shows the linearity of the peak integration when different volumes of the same sample are injected. The open symbols represent tenfold higher concentrations of protein so that all the peaks can be displayed together. The range over which proteins can be accurately quantitated with our current system is from approximately 0.1 to 50 μg/peak per injection. This range varies with the response factor of the particular protein and the sensitivity of the detector.

absorbance was measured at 640 nm. Bovine serum albumin, in similar reagents, was used as a standard. It was assumed that essentially the only protein in LDL was apoB. The SDS gel described above supports this assumption.

The apoB concentration of VLDL, IDL, and the HDL pools was determined by precipitating the apoB with 50% isopropanol in water (23). Three aliquots (100 μl) of each sample were washed twice with 1.0 ml of isopropanol-water. The apoB was pelleted by centrifugation (2500 rpm for 20 min) and the supernatant containing the soluble proteins and salt was poured off. The pellet was dried under vacuum and resolubilized in 100 μl of 100 mM SDS, 0.2 N NaOH. This required incubation overnight at 37°C. One hundred μl of water was added and then the protein assay reagents as above. If the solution did not clear after 30 min in the copper reagent, it was discarded and the procedure was repeated. The recovery of protein was linear up to, at least, 10 μg/tube.

The water-insoluble protein in the HDL subfractions was probably Lp[a]. The distribution that we observed was similar to that of Lp[a], that is, the highest concentration was in the d 1.060–1.11 g/ml range (24). Also, individuals with elevated concentrations of this "apoB" had a clearly defined peak between LDL and HDL on their absorbance profile when their sample was fractionated following centrifugation. We have isolated this peak and found that the particles in it had a diameter of approximately 22 nm, similar to Lp[a] but unlike HDL. Gradient SDS-PAGE demonstrated that the protein in this fraction had a molecular weight larger than apoB, consistent with being Lp[a]. Studies are planned to more clearly identify the water-insoluble protein measured in the other HDL subfractions, but until we have verified that it is Lp[a], we will identify it as "apoB." It is not likely that we are measuring some contaminating plasma proteins because these should have their highest concentrations in HDL-D. We always find the lowest values in this fraction, and frequently see no detectable activity. It should be noted that the presence of Lp[a] in these HDL subfractions can significantly increase the concentration of cholesterol and there is no way of differentiating apoA-I-associated cholesterol from Lp[a]-associated cholesterol with this methodology.

RESULTS

Reproducibility of apolipoprotein analysis

Five injections of the same delipidated HDL-M were analyzed by HPLC and the results are shown in **Table 2.** The coefficient of variation was less than 5% in almost all cases. The widest variation occurred with apoC-IIIb. This was due to the presence of albumin in the sample. ApoC-IIIb elutes just before albumin and when there is albumin present in the sample, it interferes somewhat with the integration of apoC-IIIb. Another point of possi-

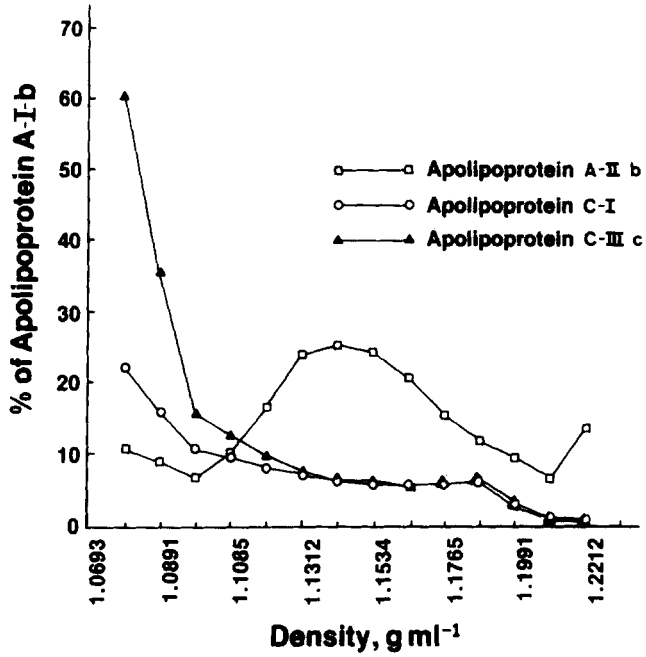

Fig. 7. The ratio of the minor apolipoproteins (apoA-IIb, C-IIIc, and C-I) to apoA-Ib was determined in each 1-ml fraction over the HDL density range of a gradient ultracentrifugation separation of control plasma (Fig. 1). It appears that the lower density HDL is rich in apoCs and poor in apoA-II and the middle density fractions are rich in apoA-II, while the higher density fractions are poor in all the minor apolipoproteins.

TABLE 2. Interassay variation of HPLC analysis

Apolipoprotein	Amount[a]	Coefficient of Variation[b]
	mg/dl	
A-Ia	2.33 ± 0.03	1.2
A-Ib	95.0 ± 0.6	0.6
Total A-I	97.5 ± 0.4	0.4
A-IIa	1.94 ± 0.06	3.2
A-IIb	35.8 ± 1.3	3.6
Total A-II	38.0 ± 1.5	3.9
C-I	5.48 ± 0.30	5.4
C-IIa	1.06 ± 0.03	3.1
C-IIb	0.76 ± 0.02	2.1
Total C-II	1.83 ± 0.04	2.3
C-IIIa		
C-IIIb	0.22 ± 0.02	9.6
C-IIIc	3.29 ± 0.04	1.2
Total C-III	3.47 ± 0.13	3.6
Total protein	148.0 ± 2.1	1.4

Forty μl of delipidated HDL-M from a nonhyperlipidemic male subject (from the same plasma sample reported in Tables 3 and 4) was injected and analyzed five times using the standard HPLC conditions described in Methods.

[a]Mean ± SD.

[b]SD × 100/mean.

ble interference is between apoC-IIb and A-Ia. These proteins elute about 30 sec apart so that if there is a large amount of apoA-Ia present, this will obscure the apoC-IIb peak. This is demonstrated in Figs. 4a and 4b. Fig. 4a shows a sample that was stored frozen and in which there was very little apoA-Ia so that the apoC-IIb is easily discernible. However, an aliquot of the same sample was stored in the refrigerator and accumulated a significant amount of apoA-Ia which obscured the integration of apoC-IIb in Fig. 4b. This has rarely been a problem, however, since we began taking measures to prevent in vitro oxidation of our samples (primarily by analyzing the samples as rapidly as possible and keeping them cold as much as possible). Since then, there have been only minor amounts of apoA-Ia present in the samples.

Intra-rotor variation

Three aliquots of the same nonhyperlipidemic plasma were spun in the same rotor, fractionated, and analyzed as above. **Table 3** and **Table 4** show the mean ± SD for each of the apolipoproteins in each of the lipoprotein pools (this experiment was repeated three times with different plasma samples with similar results). The coefficient of variation for the major redox-forms of each of the apolipoproteins in HDL-M and HDL-D was always less than 5% and frequently in the 1–2% range. This is essentially the same coefficient of variation of repetitive injections, indicating that these lipoprotein pools were analyzed with a high degree of reproducibility. The apoA-I and A-II in HDL-L showed slightly higher variations. This is probably due to very minor differences in the division made between HDL-L and HDL-M. Since HDL-M is very rich in both apoA-I and A-II, any slight variation in where this division is made could produce very sig-

TABLE 3. Intra-rotor variation of the ultracentrifugation-HPLC method (HDL subfractions)

	HDL-L		HDL-M		HDL-D		Total HDL	
Apolipoproteins	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
	mg/dl	%	*mg/dl*	%	*mg/dl*	%	*mg/dl*	%
A-Ia	0.168 ± 0.014	8.3	0.424 ± 0.021	5.0	0.162 ± 0.023	14.2	0.755 ± 0.050	6.6
A-Ib	7.80 ± 0.58	7.4	98.8 ± 0.7	0.7	42.6 ± 0.7	1.6	149.1 ± 1.1	0.1
Total A-I	7.97 ± 0.59	7.4	99.2 ± 0.7	0.7	42.7 ± 0.7	1.6	149.9 ± 1.1	1.1
A-IIa	0.089 ± 0.012	13.5	0.293 ± 0.011	3.8			0.382 ± 0.023	6.0
A-IIb	2.39 ± 0.27	11.3	39.3 ± 0.9	2.3	11.5 ± 1.7	1.7	53.2 ± 0.9	1.7
A-IIc								
Total A-II	2.48 ± 0.27	10.9	39.6 ± 0.9	2.3	11.5 ± 1.7	1.7	53.5 ± 0.9	1.7
C-I	1.04 ± 0.03	2.9	5.94 ± 0.22	3.7	1.68 ± 0.07	4.2	8.65 ± 0.14	1.6
C-IIa	0.067 ± 0.003	4.5	0.699 ± 0.084	12.0			0.766 ± 0.086	11.2
C-IIb	0.231 ± 0.003	1.3	0.898 ± 0.038	4.2	0.195 ± 0.006	3.1	1.32 ± 0.03	2.3
Total C-II	0.298 ± 0.004	1.3	1.60 ± 0.11	6.9	0.195 ± 0.006	3.1	2.09 ± 0.11	5.3
C-IIIa								
C-IIIb	0.059 ± 0.007	11.9					0.059 ± 0.007	11.9
C-IIIc	1.05 ± 0.03	2.9	4.01 ± 0.14	3.5	0.923 ± 0.025	2.7	5.99 ± 0.09	1.5
Total C-III	1.11 ± 0.04	2.9	4.01 ± 0.14	3.5	0.923 ± 0.025	2.7	6.04 ± 0.09	1.5
Total soluble protein	13.1 ± 0.9	6.9	151.7 ± 2.0	1.3	58.0 ± 0.9	1.6	222.8 ± 1.7	0.8
ApoB	19.5 ± 2.6	13.3	2.06 ± 1.16	56.3	0.457 ± 0.421	92.1	22.0 ± 3.9	17.7
Total cholesterol	20.9 ± 0.4	1.9	30.2 ± 0.1	0.3	7.01 ± 0.28	4.0	58.1 ± 0.7	1.2
Free cholesterol	5.11 ± 0.05	0.9	6.57 ± 0.03	0.5				
Triglyceride	3.00 ± 0.03	1.0	4.75 ± 0.08	1.7	1.47 ± 0.24	16.3	9.22 ± 0.18	2.0

Fresh plasma from a nonhyperlipidemic white male was aliquoted into three ultracentrifuge tubes, spun, and analyzed as described in Methods. The lipoproteins were delipidated within 24 hr of the ultracentrifugation. The composition of the HDL subfractions (mean ± SD) and the coefficient of variation (CV) of each parameter measured is shown. Similar results were obtained in two other identical experiments.

TABLE 4. Intra-rotor variation of the ultracentrifugation–HPLC method (VLDL, IDL, LDL, and total plasma)

Apolipoproteins	VLDL		IDL		LDL		Total Plasma	
	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV	Mean ± SD	CV
	mg/dl	*%*	*mg/dl*	*%*	*mg/dl*	*%*	*mg/dl*	*%*
E	0.041 ± 0.070	170.7	0.024 ± 0.024	100.0				
C-I	1.22 ± 0.03	2.5	0.305 ± 0.031	10.2			10.2 ± 0.1	0.1
C-IIa	0.087 ± 0.032	36.8						
C-IIb	1.12 ± 0.03	2.7	0.152 ± 0.013	8.6				
Total C-II	1.21 ± 0.05	4.1	0.152 ± 0.013	8.6			3.45 ± 0.10	2.9
C-IIIa								
C-IIIb	0.481 ± 0.007	6.1	0.010 ± 0.017	170.0				
C-IIIc	2.70 ± 0.06	2.2	0.480 ± 0.039	8.1				
Total C-III	3.18 ± 0.07	2.2	0.490 ± 0.033	6.7			9.71 ± 0.07	0.7
Total soluble protein	5.77 ± 0.17	2.9	1.00 ± 0.12	12.0			229.6 ± 1.5	0.7
ApoB	11.2 ± 4.0	35.7	5.30 ± 0.37	7.0	83.2 ± 0.4	0.5	99.7 ± 4.0	4.0
Total cholesterol	16.9 ± 1.2	7.1	11.4 ± 0.5	4.4	117.1 ± 0.9	0.8	203.5 ± 1.9	0.9
Free cholesterol	6.37 ± 0.29	4.6	3.62 ± 0.14	3.9	21.6 ± 0.1	0.5		
Triglyceride	31.4 ± 0.6	0.6	5.56 ± 0.29	5.2	15.0 ± 0.2	1.3	61.1 ± 0.0	0.0

Fresh plasma (same spin as in Table 3) from a nonhyperlipidemic white male was aliquoted into three ultracentrifuge tubes, spun, and analyzed as described in Methods. The lipoproteins were delipidated within 24 hr of the ultracentrifugation. The composition of the VLDL, IDL, LDL subfractions, and whole plasma (mean ± SD) and the coefficient of variation (CV) of each parameter measured is shown. Similar results were obtained in two other identical experiments.

nificant changes in the concentrations of these proteins in HDL-L. The apoCs, on the other hand, are relatively enriched in the lighter HDL fractions so that a small variation in where the HDL-L and HDL-M division is made will not result in a significant change in their concentrations. Most of the very minor peaks had variations of greater than 5%. These peaks were nearing the sensitivity limit of this procedure and it is not surprising that the variations were somewhat higher.

Similar excellent reproducibility was found with the VLDL apoCs, but IDL apoCs had a variation of 5–10%. Again, this was probably due to the very small peaks that were being analyzed. The apoE variation was extremely large. This protein appears to be particularly difficult to quantitate because of its low concentration in nonhyperlipidemic plasma and because it elutes as multiple broad peaks. At these concentrations, this analysis is more qualitative than quantitative. However, this procedure should be able to accurately identify and quantitate elevated concentrations of this apolipoprotein.

As shown in Tables 4 and 5, the reproducibility of the cholesterol and triglyceride determinations was excellent: less than 2% in the HDL and LDL subfractions and approximately 5% in VLDL and IDL. The only significant variation was seen in the triglyceride measurements in HDL-D, which was probably due to the very low concentrations of triglyceride in these samples. The coefficient of variation of the LDL apoB determination was also excellent (0.5%). The coefficient of variation of the apoB determination in the other lipoprotein subfractions was not so good, ranging from 7% for IDL to 92% for HDL-D. The wide variations in HDL-D were obviously due to very low concentrations of apoB in this fraction. It is not likely that the variations in the other fractions were due to the protein assay itself since we obtained very good reproducibility with LDL. The likely source of this variation was the delipidation and resolubilization of the apoB. We see no way of eliminating this 10–40% variation at this time, so we must remain cognizant of it when analyzing patient samples.

Inter-rotor variation

One hundred ml of plasma (fasting) was obtained from the same nonhyperlipidemic male described above. Nine aliquots of 10 ml each were taken. Three aliquots were centrifuged each day on 3 consecutive days beginning with the day that the plasma was drawn (rotor A, B, and C). The plasma was stored at 4°C with NaN_3 and glutathione until it was spun. The samples from rotor A were compared to those from rotor B and then C to determine whether there were changes resulting from the storage of the plasma. We then determined the coefficient of variation for the first tube in each rotor, the second tube in each rotor, and the third tube in each rotor and averaged them together.

The most significant alteration in lipoprotein composition that was observed was a progressive reduction in HDL triglyceride over this 3-day period (A: 7.38 ± 0.77 mg/dl; B: 3.57 ± 0.18 mg/dl, $P = 0.003$; C: 2.53 ± 0.64 mg/dl, $P = 0.005$). This may have been a finding unique to this experiment since this was not observed in the more prolonged storage experiments reported below. There was also a minor reduction in the percentage of reduced apolipoproteins in HDL in rotor C versus A (A-Ib: 96.8% vs. 97.7%; A-IIb: 93.4% vs. 96.7%; C-IIIc: 85.6% vs. 91.7%)

but only the apoA-II and C-III differences were significant at the $P < 0.05$ level.

Table 5 shows the average inter-rotor coefficient of variation (%). The reproducibility of the total plasma and total HDL determinations appears to be excellent (4–8%) except for apoC-II and "apoB." The relatively poor reproducibility of apoC-II is probably the result of interference from apoA-Ia as discussed elsewhere. As noted above, apoA-Ia concentrations were changing during this experiment, thus having a variable effect on apoC-II. The variability of the "apoB" was again due to the erratic recovery of "apoB" in HDL-M and HDL-D. All of the apolipoprotein determinations in HDL-L and IDL show relatively poor reproducibility. This is most likely due to the low concentrations of these lipoproteins in this individual.

Effect of storing plasma at 4°C

An aliquot of the plasma described in Tables 3 and 4 was stored for 12 days at 4°C and then divided into three samples and analyzed by our usual procedures. During this period there was a significant shift of the apoCs from the VLDL and IDL to the HDL subfractions, an increase in the HDL-D mass, and a reduction in HDL-L mass. There was a 19% reduction in apoC-III ($P = 0.035$) and a 26% reduction in apoC-II ($P = 0.013$) from the combined VLDL and IDL fractions. This resulted in a significant enrichment of apoC-I in VLDL, from 21 to 27% of the soluble protein ($P = 0.0004$). The total HDL had a 16% increase in apoC-III ($P = 0.002$), a 54% increase in apoC-II ($P = 0.019$), and a 7% increase in apoC-I ($P = 0.043$). All three HDL subfractions had significant increases in the ratios of apoC-III, C-II, and C-I to apoA-I. The soluble protein in HDL-L fell 23% ($P = 0.034$) while the HDL-D soluble protein increased 14% ($P = 0.003$). This was due to a 17% reduction of apoA-I ($P = 0.026$) and a 36% reduction of apoA-II ($P = 0.016$) in HDL-L with a 14% increase in apoA-I in HDL-D ($P = 0.002$).

The percentage of oxidized redox-forms of apoA-I, A-II, and C-II increased significantly in HDL. However, the percentage of apoA-I or A-II in the oxidized form was less than 1% prior to storage and only increased to 1–2% after storage. The percentage of apoC-II in the oxidized form (C-IIa) increased from 37% to 49% ($P = 0.036$) with storage.

There was an increase in VLDL + IDL cholesterol (33%, $P = 0.052$) and free cholesterol (43%, $P = 0.012$) while there were decreases in HDL-L (12%, $P = 0.002$) and HDL-M (17%, $P = 0.0001$) cholesterol as well as HDL-L (12%, $P = 0.0002$) and HDL-M (36%, $P = 0.0001$) free cholesterol. There was also a 39% ($P = 0.0009$) increase in HDL-M triglyceride.

It is apparent that there were significant alterations in lipoprotein composition during storage of plasma under these conditions.

Effect of storing plasma at −20°C

Three aliquots of plasma were analyzed fresh while three aliquots of the same plasma were frozen to −20°C

TABLE 5. Inter-rotor variation of the ultracentrifugation-HPLC method (HDL subfractions)

Apolipoproteins	HDL-L	HDL-M	HDL-D	Total HDL
		coefficient of variation, %		
ApoA-I	37.6	5.5	5.7	2.8
ApoA-II	35.7	7.2	15.6	3.0
ApoC-I	34.1	3.7	10.2	3.2
ApoC-II	43.0	14.3	15.8	12.3
ApoC-III	26.5	9.9	14.8	7.1
Total soluble protein	35.6	5.7	7.6	2.4
"ApoB"	9.3	34.9	35.4	13.4
Total cholesterol	4.9	2.7	9.2	2.3
Free cholesterol	4.1	7.4		
	VLDL	IDL	LDL	Plasma
ApoE	42.1	51.9		
ApoC-I	15.3	11.9		4.3
ApoC-II	9.8	20.8		9.1
ApoC-III	7.3	12.0		5.9
Total soluble protein	12.7	20.1		2.4
ApoB	11.3	24.5	5.5	7.7
Total cholesterol	8.1	20.6	6.1	2.9
Free cholesterol	10.9	15.8	3.6	

Plasma (100 ml) was drawn from the same nonhyperlipidemic white male described in the previous tables and aliquoted into nine tubes. Three tubes were spun on each of three consecutive days. The coefficient of variation (%) was determined for tube 1 from each rotor, then for tube 2, and finally for tube 3. These were averaged and are shown below. The values for the individual redox-forms and triglycerides are not shown since these were found to change during sample storage.

for 2 weeks and then analyzed. When the frozen plasma was thawed, it contained a white precipitate that was removed by low speed centrifugation. Unfortunately, during the ultracentrifugation, another precipitate formed that clogged the tubing when one of the samples was being pumped out of the centrifuge tube. This prevented analysis of this sample. This precipitate has been seen frequently when we ultracentrifuge previously frozen samples. Freezing caused a 25% reduction in apoA-I ($P = 0.0002$), a 16% reduction in apoA-II ($P = 0.0007$), and a 16% reduction in total apoC-I ($P = 0.006$). These were due to reductions of these apolipoproteins in the HDL-L and HDL-M subfractions. There were small increases in apoA-I and A-II in HDL-D, but these did not balance the decreases in the other HDL subfractions. There were no changes in the apoC-II and C-III concentrations in any of the HDL subfractions. There was an 11% reduction in total HDL cholesterol ($P = 0.001$) which was due to reductions in the HDL-L and HDL-M subfractions. Freezing apparently did prevent oxidation of the apolipoproteins in that there were no changes in the percentages of the minor redox-forms (apoA-Ia and apoA-IIa,c).

There were no significant effects on the protein compositions of VLDL or IDL but there was a 46% increase in VLDL cholesterol ($P = 0.018$) and a 49% increase in VLDL free cholesterol ($P = 0.018$). LDL had an apparent 43% increase in apoB ($P = 0.0003$).

Freezing the plasma causes many alterations in the lipoprotein composition that are quite different from those seen when the samples are stored at 4°C. From these results, it appears that the plasma must be spun fresh in order to obtain accurate results.

Effect of storing the lipoprotein subfractions at 4°C or −20°C

Aliquots of VLDL and the three HDL subfractions were stored for 2 weeks at either 4°C or −20°C and then delipidated and analyzed by HPLC. These results were compared to results obtained when the same samples were delipidated and analyzed immediately after centrifugation. The refrigerated HDL had a 62% reduction in HDL-L apoC-II ($P = 0.003$), an 8% reduction in HDL-M apoA-I ($P = 0.01$), and a 12% reduction in HDL-D apoC-III ($P = 0.0009$). The reduction in apoC-II appears to be due to an increase in apoA-Ia that obscures the apoC-IIb peak (Fig. 4B). There was no change in the concentration of apoC-IIa, suggesting that the reduction in apoC-II may just be due to integration difficulties. There were no changes in the apoC-III redox-forms distribution in HDL but there were increases in the percentage of apoA-Ia (2.7% of total to 5.9%, $P = 0.02$) and apoA-IIa (4.1% to 10.3%, $P = 0.01$). VLDL showed no change in the concentrations of the apoCs but there were reductions in the oxidized apoC-IIa (34.2% to 9.5%, $P = 0.017$) and apoC-IIIa (22.8% to 2.2%, $P = 0.0009$) with balancing increases in apoC-IIb and C-IIIc.

Storing at −20°C produced no changes in VLDL or HDL apolipoprotein concentrations and prevented oxidation of the apolipoproteins. It appears that lipoprotein subfractions can be frozen for later HPLC analysis. However, these analyses were done with non hyperlipidemic plasma and hyperlipidemic subfractions may not store as well. Before depending on this storage procedure, many more samples need to be tested. Our usual procedure at this time is to delipidate the subfractions within 24 hr of centrifugation.

Effect of freezing the delipidated apolipoproteins

Delipidated HDL-M in 3 M guanidine–HCl was analyzed by HPLC immediately after delipidation and then frozen at −20°C. It was thawed and reanalyzed every week for 4 weeks. There were no changes in any of the apolipoprotein concentrations or redox-form distributions during this time.

Lipoprotein composition in nonhyperlipidemic subjects

Fasting plasma from twelve men and six women without hyperlipidemia was analyzed. **Table 6** shows that there were significant differences in both HDL-L and HDL-M. HDL-L from women had almost twofold greater concentrations of all the apolipoproteins except apoA-II, and also had significantly more free and total cholesterol. In HDL-M, on the other hand, only the apoA-I and free and total cholesterol were elevated. There were no differences in the HDL-D composition between the two groups.

Table 7 shows that the men had significantly greater concentrations of apoC-II and C-III in VLDL than the women. There were no differences in LDL or IDL concentrations or compositions. The total plasma concentrations of apoA-II, apoB, apoCs, total cholesterol, and triglycerides were the same in the two groups. As expected, the total HDL cholesterol and apoA-I concentrations were greater in the women than in the men.

The relative percentages of oxidized apolipoproteins were low in both groups and no differences were observed.

DISCUSSION

We describe in this report a new methodology that allows the rapid and accurate analysis of the apolipoprotein and lipid composition in multiple lipoprotein subfractions. This technique is capable of detecting differences in subfraction composition that are not apparent when measuring total plasma protein or lipid concentrations.

Gradient ultracentrifugation permits the isolation of multiple lipoprotein subfractions in a single spin of less

TABLE 6. Composition of HDL subfractions in nonhyperlipidemic subjects

Subfraction	Men	Women	P
	mg/dl ± SD		
HDL-L			
A-I	13.6 ± 6.0	32.7 ± 15.0	0.025
A-II	3.04 ± 0.83	4.67 ± 2.27	
C-I	1.17 ± 0.33	1.92 ± 0.53	0.002
C-II	0.348 ± 0.123	0.555 ± 0.203	0.016
C-III	1.20 ± 0.29	1.94 ± 0.66	0.037
Total[a]	19.6 ± 7.2	42.2 ± 17.4	0.024
"ApoB"[b]	6.08 ± 6.03	6.85 ± 6.92	
Total chol.	14.3 ± 3.5	23.0 ± 6.2	0.001
Free chol.[c]	3.60 ± 0.79	5.85 ± 1.60	0.017
Triglyceride	3.21 ± 0.71	4.05 ± 1.33	0.094
HDL-M			
A-I	87.9 ± 12.7	105.3 ± 7.3	0.007
A-II	31.2 ± 6.3	33.1 ± 2.8	
C-I	4.26 ± 0.91	4.33 ± 0.82	
C-II	1.40 ± 0.34	1.55 ± 0.36	
C-III	2.89 ± 0.70	3.30 ± 1.19	
Total[a]	129.3 ± 19.2	149.3 ± 9.1	0.029
"ApoB"[b]	1.66 ± 1.30	1.97 ± 1.22	
Total chol.	25.3 ± 3.9	31.4 ± 2.0	0.003
Free chol.[c]	5.35 ± 0.97	6.64 ± 0.52	0.008
Triglyceride	5.50 ± 1.28	5.51 ± 1.73	
HDL-D			
A-I	36.5 ± 7.9	34.2 ± 4.5	
A-II	7.94 ± 2.83	6.09 ± 0.99	0.062
C-I	1.73 ± 0.87	1.83 ± 1.10	
C-II	0.642 ± 0.561	0.550 ± 0.674	
C-III	0.897 ± 0.330	0.972 ± 0.339	
Total[a]	48.9 ± 11.5	44.6 ± 5.9	
"ApoB"[b]	1.19 ± 0.82	0.754 ± 0.896	
Total chol.	5.65 ± 1.19	5.20 ± 0.98	
Free chol.[c]			
Triglyceride	1.75 ± 0.62	1.70 ± 0.49	
Total HDL			
A-I	138.0 ± 17.0	172.2 ± 15.6	0.001
A-II	42.2 ± 8.6	43.8 ± 4.2	
C-I	7.16 ± 1.49	8.08 ± 0.84	
C-II	2.39 ± 0.90	2.65 ± 1.00	
C-III	4.99 ± 1.05	6.22 ± 1.29	0.044
Total[a]	197.7 ± 25.6	236.1 ± 19.3	0.005
"ApoB"[b]	8.93 ± 6.11	9.58 ± 6.20	
Total chol.	45.2 ± 7.6	59.6 ± 7.2	0.001
Free chol.[c]			
Triglyceride	10.5 ± 2.3	11.3 ± 3.2	

Blood was drawn from twelve men and six women without hyperlipidemia and immediately placed on ice. The plasma was separated by centrifugation and stored at 4°C until it was spun, usually within 24 hr but always within 3 days. Lipoprotein fractions were stored at 4°C until they were delipidated (within 24 hr of ultracentrifugation) and analyzed by HPLC. Enzymatic and chemical assays were done within 7 days of drawing the blood.

[a] Total water-soluble protein.

[b] "ApoB," protein insoluble in 50% isopropanol and water, probably represents Lp[a].

[c] Non-esterified cholesterol.

than 4.5 hr. This represents a substantial savings in technician and instrument time when compared to the traditional sequential flotation method which may require up to 60 hr of centrifugation for some analyses. This short spin time makes it less likely that centrifugation artifacts will be introduced. This technique is probably not capable of purifying individual types of HDL particles because it uses a single parameter for separation (density), and it is likely that some particles of different composition have very similar densities (e.g., substituting apoC-II for apoC-III would not change the particle density but could significantly alter biological function). This technique does group HDL particles into density classes, based on their minor apolipoproteins, which makes detecting compositional changes in single particle types more apparent (e.g., differences in the apoCs should be most apparent in HDL-L and differences in apoA-II most apparent in HDL-M).

Chung et al. (25) have used gradient ultracentrifugation in a vertical rotor to separate lipoproteins with even

TABLE 7. Composition of VLDL, IDL, LDL, and total plasma lipoproteins in nonhyperlipidemic subjects[a]

Lipoprotein	Men	Women	P
	mg/dl ± SD		
VLDL			
E	0.380 ± 0.364	0.495 ± 0.151	
C-I	1.39 ± 0.51	0.974 ± 0.474	
C-II	1.15 ± 0.37	0.603 ± 0.353	0.009
C-III	3.35 ± 1.12	2.00 ± 0.96	0.024
Total	6.37 ± 2.11	4.23 ± 1.77	0.049
ApoB	6.18 ± 5.08	6.41 ± 4.04	
Total chol.	16.1 ± 6.4	14.5 ± 5.1	
Free chol.	7.77 ± 2.74	7.11 ± 2.08	
Triglyceride	53.6 ± 24.4	34.8 ± 12.4	0.099
IDL			
E	0.028 ± 0.039	0.061 ± 0.060	
C-I	0.176 ± 0.063	0.147 ± 0.071	
C-II	0.093 ± 0.047	0.064 ± 0.025	
C-III	0.326 ± 0.111	0.267 ± 0.096	
Total	0.640 ± 0.229	0.565 ± 0.190	
ApoB	2.37 ± 1.34	2.46 ± 1.57	
Total chol.	7.26 ± 2.11	5.27 ± 3.35	
Free chol.	2.68 ± 0.64	2.01 ± 1.08	
Triglyceride	6.15 ± 2.35	4.52 ± 2.38	
LDL			
ApoB	62.8 ± 16.9	62.5 ± 27.0	
Total chol.	90.4 ± 20.2	80.3 ± 25.7	
Free chol.	18.5 ± 2.8	17.7 ± 4.2	
Triglyceride	14.4 ± 4.5	12.3 ± 3.3	
Total plasma			
C-I	8.72 ± 1.60	9.16 ± 1.04	
C-II	3.63 ± 0.81	3.64 ± 1.19	
C-III	8.66 ± 1.49	9.30 ± 0.87	
ApoB[b]	71.3 ± 18.3	71.3 ± 28.0	
Total chol.	159.0 ± 27.8	159.6 ± 30.0	
Triglyceride	84.6 ± 30.5	63.0 ± 20.1	

[a] Same subjects as in Table 6.
[b] This is the total apoB contained in VLDL, IDL, and LDL only. The nonsoluble protein in HDL probably represents Lp[a] and is, therefore, not included.

shorter spin times; however, their lipoprotein subfractions were more heavily contaminated with plasma proteins (15) making apolipoprotein determinations more difficult.

HPLC determination of apolipoprotein concentrations is the only technique currently available that can simultaneously quantitate the five major water-soluble apolipoproteins. This reduces the interassay variability typical of radioimmunoassays which may be particularly important in the study of lipoproteins since protein ratios may be very important in determining lipoprotein function. If we can improve the sensitivity of our method for apoE analysis and develop a technique for separating it from apoA-II in HDL, this technique should accurately measure this apolipoprotein as well. Since HPLC does not depend on antibody recognition, it eliminates many of the problems associated with using different antibodies to assay a single protein. It also eliminates the need for radioisotopes. HPLC is not as sensitive as a radioimmunoassay and, in many cases, differences in antibody recognition are desirable (as in lipoprotein structural studies). However, the sensitivity of RIAs is generally not required when measuring these apolipoprotein concentrations. In addition, HPLC is capable of routinely detecting differences in apolipoprotein structure that may not be apparent with an RIA (such as the apolipoprotein redox-forms which we have identified).

HPLC obviously cannot be used to assay whole plasma. However, when lipoprotein subfractions are to be analyzed, this technique is faster and, since it is easily automated, less labor-intensive than multiple immunoassays. It is also much simpler to assay just a few samples by HPLC than by immunoassay. Since we are only measuring the apolipoprotein content of the lipoprotein subfractions, the apolipoproteins in the free protein fraction are not included in our "total" plasma concentrations. We have RIA data indicating that less than 5% of the total apoA-I is found in the free protein fraction after a single ultracentrifugation, but we currently have no RIA data on the other apolipoproteins. We plan to investigate the extent of apolipoprotein losses with our procedure as well as compare our measurements with established RIA measurements in the future.

It is apparent that each methodology has its own strengths and weaknesses and it is likely that they will be used to complement one another. However, at this time, HPLC is the only readily available alternative to immunological measurement of apolipoproteins. We are currently attempting to identify several small peaks that may represent minor apolipoproteins (such as apoA-IV or D). When this is accomplished, this methodology should be an even more powerful tool for the analysis of the apolipoprotein composition of lipoproteins.

Storage of plasma samples appears to offer problems. When plasma is frozen, there is significant loss of HDL apolipoproteins. However, when plasma is refrigerated, within 2 weeks there are significant shifts of apolipoproteins and lipids from one subfraction to another and an increase in oxidation of the proteins. It appears that plasma should be ultracentrifuged within a few days of collection and should not be frozen at all. We do have evidence that the isolated subfractions may be relatively stable when they are frozen. However, further investigation is needed before this storage procedure can be considered reliable. Once the apolipoproteins have been delipidated, they appear to be very stable. Our current recommendation would be to ultracentrifuge and delipidate samples within 3 days of blood drawing. The HPLC analysis could then be done as machine time is available.

The apolipoprotein concentrations that we obtained with this HPLC methodology appear to be very close to those of immunoassays. Steinberg et al. (1) have recently reviewed apoA-I immunoassays and found a range of total plasma values from 100 to 147 mg/dl in men and 111 to 168 mg/dl in women. Our values of 138 mg/dl and 172 mg/dl, respectively, are very similar to their values. Musliner et al. (3) reported apoA-II concentrations of 39.0 and 42.0 mg/dl for men and women, respectively, which are very close to our values of 42.3 and 43.8 mg/dl.

Carlson and Holmquist (5) have reviewed the results of various immunoassays for the apoCs. They found values that ranged from 6.3 to 7.0 mg/dl for apoC-I, 2.21 to 5.54 mg/dl for apoC-II, and 10.4 to 15.4 mg/dl for apoC-III. We found slightly higher concentrations for apoC-I and lower concentrations for apoC-III but our values for apoC-II were the same. They also reported values for apoB ranging from 72.0 to 98.0 mg/dl while we obtained 71.3 mg/dl in both men and women. However, we excluded the "apoB" in HDL from our total apoB and, if included, this would have added about 9 mg/dl to our total apoB and given us a result in the midrange of their values.

As simpler techniques are developed that allow more precise quantitation of lipoprotein composition, many questions can be addressed that would have been very difficult with previously available techniques. We believe that our methodology may contribute to these investigations by offering several advantages in both speed and accuracy over commonly used techniques.

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