Apolipoprotein A-I assayed in human serum by isotope dilution as a potential standard for immunoassay

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Abstract We measured the amount of apoA-I in serum by isotope dilution, finding 1.33 mg/ml (standard deviation 0.177) in six normolipidemic, healthy subjects. We developed this method by adapting published techniques to purify apoA-I from 3 ml of serum in two steps: density gradient ultracentrifugation and high performance liquid chromatography gel filtration. The ^{125}I-labeled apoA-I tracer was first screened, by incubation with serum, to select labeled apoA-I which retained the ability to exchange with native apoA-I and bind to HDL. A known amount of ^{125}I-labeled apoA-I-labeled HDL was added to unknown serum samples; apoA-I was reisolated from the serum and its specific radioactivity was used to calculate the dilution of the added, labeled apoA-I by the unlabeled apoA-I in the unknown serum. By not relying on immunochemical techniques, the isotope dilution assay provided results that are independent of the expression of individual apoA-I antigenic sites. Therefore, sera that have been assayed by isotope dilution can serve as standards to evaluate the accuracy of immunoassays for serum apoA-I and provide primary standards for such immunoassays. — **Weech, P. K., D. Jewer, and Y. L. Marcel.** Apolipoprotein A-I assayed in human serum by isotope dilution as a potential standard for immunoassay. *J. Lipid Res.* 1988. **29:** 85–93.

Supplementary key words high density lipoprotein • high performance liquid chromatography

Apolipoprotein A-I (apoA-I) is the apolipoprotein that is present in highest concentration in normolipemic human plasma: average reported mean 1.38 g/l in women, 1.24 g/l in men (1). ApoA-I associates almost entirely with the high density lipoproteins (HDL) and its concentration in plasma has been a good, negative correlate of atherosclerotic disease or hyperlipoproteinemia in several studies (2–13). Many immunoassays have been developed for apoA-I and were reviewed by Steinberg et al. (1) as part of a project of the Standardization Committee of the International Union of Immunological Societies. That report described the methods that contribute to the immunoassay of apoA-I and some of the problems of accuracy, which seem to be related to the heterogeneity of apoA-I-containing lipoproteins, and the differences in expression of some antigenic sites in lipoproteins when compared with purified apoA-I standards. Evidence of interest in the accuracy and standardization of apoA-I immunoassays is seen in the report of a National Institute of Health Workshop (12), a collaborative study between laboratories in the United States (13), a call from The National Institutes of Health in 1985 to participate in a second collaborative study in the United States, and a collaborative study recently sponsored by the European Economic Community.

Immunochemical studies of apoA-I have shown that the problems of immunoassay stem, in the main, from the presence of many different antigenic sites on apoA-I, which are not all expressed equally, or at the same time, or on all HDL. This has been demonstrated with polyclonal antisera (14) and monoclonal antibodies (15–17), and is probably responsible for the need to denature serum, plasma, and lipoprotein samples before use in most immunoassays where purified apoA-I is used as the standard (1). Use of a reference serum as a secondary standard was shown to minimize the variation in electroimmunoassay results on unknown samples, when the latter were assayed using a variety of antisera and primary standards (18).

We have recently found that monoclonal antibodies can be prepared and selected to have the property of reacting with antigenic sites that are expressed on all normal apoA-I and HDL. These sites are unaffected by storage of the samples (16, 17). Using these antibodies, or other antibodies and antisera selected to have the same properties, the inherent advantages of immunoassay can be exploited for apoA-I, i.e., the ability to assay apoA-I specifically, in plasma or serum, on many samples together. Nevertheless, the problem remains of choosing a suitable, stable primary

Abbreviations: HDL, high density lipoproteins; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; apoA-I, apolipoprotein A-I.

or secondary standard, and we have addressed this by developing an isotope dilution technique for assay of apoA-I in serum. This serum can serve as the immunoassay standard, because its apoA-I content is known, independent of any particular immunoassay. The isotope dilution assay, as described here, is intended for the validation of standard sera, rather than the assay of apoA-I in all unknown samples of a clinical study.

MATERIALS AND METHODS

Materials

Phenyl methylsulfonyl fluoride (PMSF) was obtained from Sigma Chemical Company (St. Louis, MO). Sodium dodecyl sulfate, sequanal grade, was obtained from Pierce Chemical Company (Rockford, IL). Sephadex G-25 and Sephacryl S-300 were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Reagents for SDS polyacrylamide gel electrophoresis were electrophoresis purity grade from Bio-Rad (Richmond, CA). For labeling of apoA-I, $Na^{125}I$ (IMS.30) was obtained from Amersham (Arlington Heights, IL), chloramine-T (sodium N-chloro-*p*-toluenesulphonamide) from Eastman Kodak (Rochester, NY), and KI from Merck (Montreal, Canada). All other chemicals were purchased from Fisher Scientific (Montreal, Canada).

Subjects and samples

Blood was drawn from normolipidemic healthy subjects and patients by venipuncture into plain Vacutainer tubes. ApoA-I was measured in sera of three patients having low concentration of HDL-cholesterol. Before treatment, patients 1 and 2 (see Table 3) had Type IV lipoprotein phenotype, and patient 3 had a Type IIa phenotype. All three patients were treated with Probucol and Atromid; patient 2 was also treated for hypothyroidism, and patient 1 had peripheral vascular disease. Blood was allowed to clot at 20°C for 1 to 2 hr, and the serum was clarified by centrifugation at 1000 *g* for 15 min at 5°C. Disodium ethylenediaminetetraacetate (Na_2EDTA), sodium azide, and phenylmethylsulfonyl-fluoride (PMSF) were added to final concentrations of 1 mM, 0.02% (w/v), and 1 mM, respectively. Concentrations of triacylglycerols, total cholesterol, and HDL-cholesterol were measured in the serum (19).

Procedure

An outline of the scheme for assay of apoA-I in serum is shown in **Fig. 1.** There were three stages to the procedure: *i*) isolation and radioactive labeling of pure apoA-I; *ii*) screening of this labeled apoA-I to select only the protein that retained the ability to exchange with native apoA-I and bind to HDL; and *iii*) measurement of the

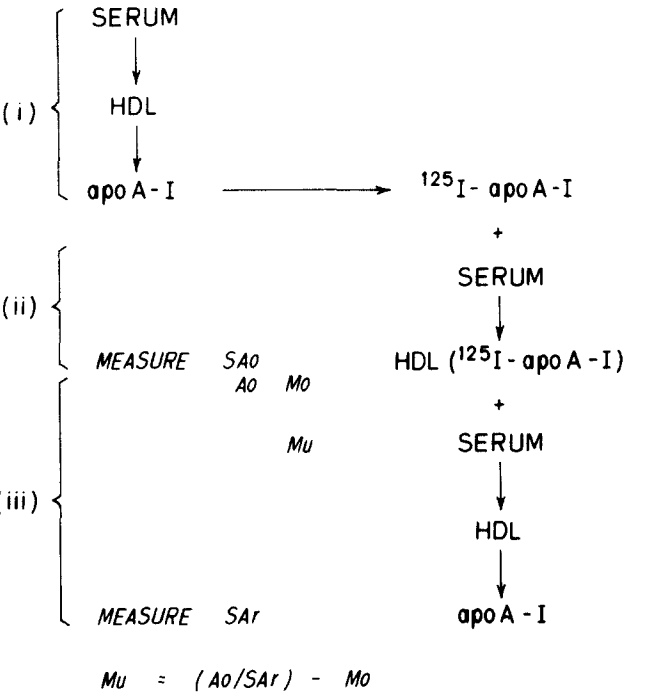

Fig. 1. Procedure for the isotope dilution assay of apoA-I, where: SAo is the specific activity (cpm/μg of apoA-I) of the HDL added to the unknown serum; Ao is the amount of radioactivity (cpm) added to the unknown serum; Mo is the mass of apoA-I added to the unknown serum; Mu is the mass of apoA-I in the unknown serum, to be determined; and SAr is the specific activity (cpm/μg of apoA-I) of the recovered apoA-I.

dilution of specific activity of ^{125}I-labeled apoA-I by the apoA-I in the serum under test.

At step *ii*), 42–84 μg of apoA-I ($19.8–26.6 \times 10^6$ cpm) was added per 3 ml of serum, the radioactive apoA-I being 1–2% of the unlabeled apoA-I in the serum. At the isotope dilution step *iii*), 194–500 μg of apoA-I ($0.39–2.58 \times 10^6$ cpm) was added to each 3 ml of serum. This radioactive plus unlabeled apoA-I in the HDL was 5–11% of the apoA-I in the unknown serum.

Isolation of HDL

The technique of Terpstra (20) was used for density gradient ultracentrifugation as follows. One g of KBr and 50 mg of sucrose were dissolved in 3.0 ml of serum in a polyallomer tube for the SW41 rotor (Beckman Instruments Inc., Palo Alto, CA). Using a Buchler Densiflo apparatus (Searle Instruments, Fort Lee, NJ), solutions of KBr adjusted to the following densities were layered over the sample: 2.0 ml at 1.210 g/ml, 3.0 ml at 1.080 g/ml, and approximately 4.0 ml of water. All solutions contained 1 mM Na_2 EDTA, 0.02% (w/v) NaN_3, and were brought to pH 7.0 with $NaHCO_3$. A blank gradient containing KBr solution (d 1.006 g/ml) in place of the sample was used for measuring the density gradient after centrifugation. Densities were measured at 20°C using an Anton Paar DM40 digital density meter (Mettler, Fisher Scien-

tific, Canada). Samples were centrifuged at 40,000 rpm, for 20 hr at 12°C in an L5 or L8 ultracentrifuge (Beckman).

Samples were removed from the centrifuged gradients by puncturing the bottom of the tubes (Beckman Fraction Recovery System) and collecting fractions of 0.5 ml with a Superac fraction collector (LKB-Produkter, Bromma, Sweden). Fractions of 1.0 ml were collected from the blank gradients for density measurements.

Isolation and labeling of apoA-I

HDL was isolated from 45 ml of serum from a normolipidemic subject by applying to a density gradient the infranatant of serum previously centrifuged at 1.050 g/ml. The HDL were dialyzed against 10 mM NH_4HCO_3, 1 mM Na_2EDTA, 0.02% (w/v) NaN_3, lyophilized, and delipidated with $CHCl_3$–CH_3OH (21). The proteins were redissolved in SDS buffer: 0.1% SDS in 0.1 M NaH_2PO_4, 0.02% NaN_3 (w/v), pH 7.0, at 5.5 mg of protein/ml. The HDL proteins were separated on a column of Sephacryl S300 1.5 × 100 cm (Pharmacia Fine Chemicals AB, Uppsala, Sweden) equilibrated with the above SDS buffer, and eluted at 15 ml/hr with a Pharmacia peristaltic pump. Aliquots of 5.5 mg of HDL-protein were chromatographed and the purest fractions of apoA-I were selected and pooled, following analysis by SDS-PAGE (22). These fractions corresponded to the center of the major peak on the 280 nm absorbance tracing.

ApoA-I from the chromatography above was dialyzed against 0.15 M NaCl, 0.05 M NaH_2PO_4, 0.001 M Na_2EDTA, pH 7.5, (PBS) and 1 mg in 1 ml was labeled with 0.2 or 0.5 mCi of ^{125}I by the chloramine T technique (23). Free I^- was removed by gel filtration through a column 1 × 20 cm of Sephadex G-25 (Pharmacia) equilibrated with PBS.

HPLC

The apparatus for HPLC consisted of three columns in series: TSK-GSWP precolumn 7.5 × 75 mm, TSK-G3000SW 7.5 × 300 mm, and TSK-G2000SW 7.5 × 300 mm, HPLC controller 2152, pump 2150, Uvicord SD 2158 absorbance monitor with 276 nm filter, 2210 recorder, and Superac 2211 fraction collector (LKB-Produkter, Bromma, Sweden). The solvent for column elution was the SDS buffer above, as used with the Sephacryl S300 column and used by Kinoshita et al. (24–26). The HDL isolated from a 3-ml serum sample by density gradient ultracentrifugation was dialyzed against 10 mM NH_4HCO_3, 1 mM EDTA, 0.02% NaN_3 (w/v) for several hr, lyophilized, and redissolved in 400 μl of elution buffer containing 1% SDS (w/v). Samples were heated to 60°C for 5 min and filtered through a 0.22-μm Millex GV filter (Millipore, Bedford, MA). The chromatography was essentially as described by Kinoshita et al. (24–26): 50–90 μl of sample was injected and eluted at 300 μl/min with SDS buffer gassed with He. Fractions of 250 μl were collected.

Other analyses

Radioactivity was measured with an RIAgamma gamma counter (LKB, Turku, Finland) with correction for absorption by KBr where necessary, and protein was measured by the method of Lowry et al. (27). Amino acid composition was determined by the method of Fauconnet and Rochemont (28) with some modifications (29) using a Beckman 121 MB analyzer with a microbore column (0.28 × 28 cm) packed with Beckman type W3H spherical resin. Amino acids were eluted using a temperature (42.6–69°C) and salt gradient. Solvents used were: *1*) 0.2 N citrate, pH 3.25, containing 2% isopropanol; *2*) 0.2 N citrate, pH 4.25; and *3*) 1.4 N citrate, pH 4.95, containing 2% isopropanol. Samples were hydrolyzed for 24 hr, and no corrections were made for loss of threonine or serine.

RESULTS

The G3000SW and G2000SW columns were each able to separate two major peaks of HDL proteins, but the combination of the two columns was superior to either alone, and so was used for all subsequent HPLC experiments. By monitoring absorbance at 276 nm, two major and three minor peaks were obtained from chromatography of HDL denatured with SDS on these HPLC columns (**Fig. 2a**), and also on a larger column of Sephacryl S300 using delipidated HDL in the same solvent. The main peak contained almost all of the apoA-I, and the following peak contained apoA-II together with lower molecular weight proteins, as found by Kinoshita et al. (24, 25). The two central fractions of the apoA-I peak contained only one band on SDS-PAGE (**Fig. 3**) which was identified as apoA-I by its M_r value (28,200 by gel filtration and 25,300 by SDS-PAGE, compared with the major band in HDL, M_r 26,000), immunoreaction with our monoclonal antibody 4H1 (16), and by its amino acid composition (**Table 1**).

In routine HPLC experiments we injected 50 μl of sample, but equal resolution was obtained with 90 μl. The amount of SDS relative to protein in the injected sample was more important in obtaining good resolution. For example, with an SDS–protein weight ratio of 0.95, amounts of up to 315 μg of HDL protein gave good resolution of the apoA-I peak; 420 μg or more of protein gave a trailing apoA-I peak unless the ratio of SDS–protein was increased. With a ratio of 1.29, up to 620 μg of protein gave good resolution, but 930 μg of protein gave poor resolution at this ratio. The pattern of elution of radioactivity from the column showed that apoA-I trailed into the apoA-II peak whenever the column's capacity was ex-

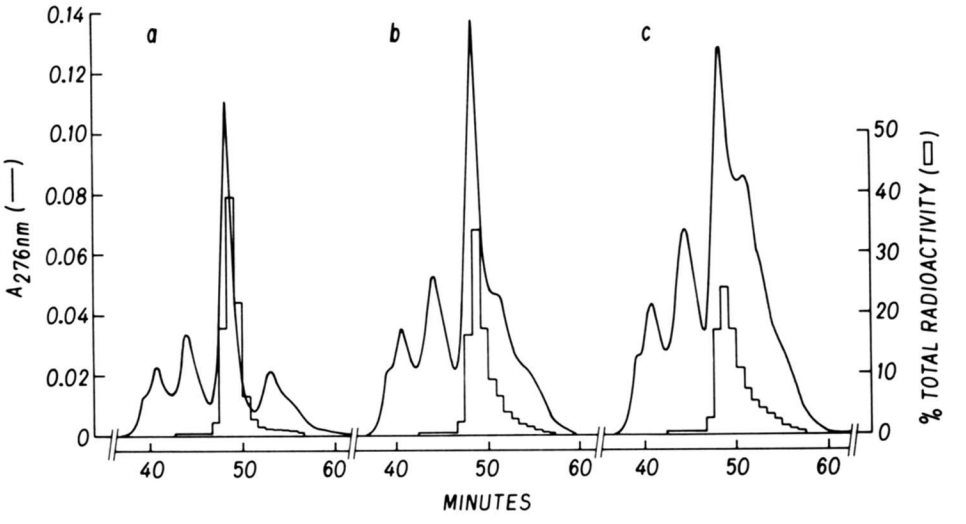

Fig. 2. HPLC gel filtration of HDL denatured with SDS. The elution of protein is shown by the line, tracing the absorbance at 276 nm. The elution of ^{125}I-labeled apoA-I is shown by the histogram; (a) 620 μg of HDL protein was chromatographed; (b) 930 μg of protein; (c) 1240 μg of protein. The SDS protein ratio was 1.29 (w/w) in each case. Chromatograms similar to (a) were obtained with up to 315 μg of protein at an SDS-protein ratio of 0.95. Only chromatograms like (a) were used for measurement of apoA-I specific radioactivity. Trailing of apoA-I radioactivity and appearance of an artefactual peak at 51 min were evident in the chromatograms (b) and (c), which were overloaded with sample.

ceeded, or whenever the SDS-protein ratio was too low (Fig. 2b, c). In these situations an additional peak appeared on the absorbance tracing between the usual apoA-I and apoA-II peaks. This peak spoiled the resolution between the usual apoA-I and A-II peaks, but it was a major peak when the column was heavily overloaded (Fig. 2b, c).

Purified apoA-I was labeled with ^{125}I, and 91.5–98% of the radioactivity was precipitable with 12% trichloracetic acid, i.e., was bound to apoA-I. This labeled apoA-I was mixed with serum, and lipoproteins were separated by density gradient ultracentrifugation. The majority of radioactivity was recovered in the range 1.06–1.15 g/ml with the visible band of HDL (1.10–1.18 g/ml) and a total of 82.5% of the radioactivity floated out of the sample application layer, i.e., was associated with lipoprotein (**Fig. 4a**). Very little (1.5%) radioactivity was found in the VLDL and LDL. When the HDL-associated ^{125}I-labeled apoA-I obtained above was mixed with another sample of serum and recentrifuged, the distribution of radioactivity (Fig. 4b) was similar to that at the previous stage (where ^{125}I-labeled apoA-I was mixed with serum): 89.5% floated out of the application layer, 6.3% was found in VLDL and LDL, but mainly as the end of the HDL peak. HDL was reisolated in the range 1.10–1.17 g/ml. The distribution of radioactivity was the same no matter how long the ^{125}I-labeled apoA-I or ^{125}I-labeled apoA-I-labeled HDL was incubated with serum (0, 1.5, 3, 6, 24 hr) before pouring the density gradients and centrifuging the samples. This showed that the equilibration was very rapid between the ^{125}I-labeled apoA-I and unlabeled apoA-I in the lipoproteins of the serum sample, and was complete within 10 min, the time taken to prepare one density gradient. This refers only to those pools of apoA-I that were able to exchange with the ^{125}I-labeled apoA-I.

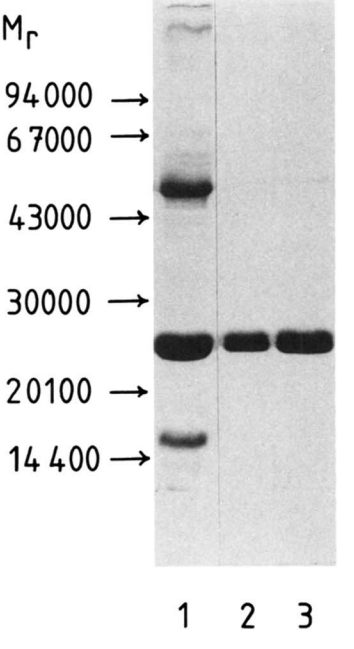

Fig. 3. SDS-polyacrylamide gel electrophoresis of HDL protein (100 μg in lane 1) and apoA-I (25 μg in lane 2, 50 μg in lane 3) purified by HPLC gel filtration. The samples were not reduced; the gel was stained with Coomassie Blue R250. The migration of reduced M_r standards is shown at the right.

TABLE 1. Amino acid composition of apoA-I

Amino Acid	Fraction 15 (a)	Fraction 16 (b)	ApoA-I (c)
Asp	88.4	87.7	89.0
Thr	41.2	43.2	42.4
Ser	55.6	56.2	63.6
Glu	204.6	203.5	194.9
Pro	46.3	43.8	42.4
Gly	52.8	44.9	42.4
Ala	87.0	84.4	80.5
Val	55.9	60.7	55.1
Ile	1.3	2.4	—
Leu	157.4	162.4	156.8
Tyr	15.7	13.7	29.7
Phe	27.8	28.6	25.4
Lys	79.5	81.0	89.0
His	19.8	21.3	21.2
Arg	67.4	66.4	67.8

Values in the table are expressed as mol per 10^3 mol of those amino acids listed, i.e., excluding tryptophan and methionine. Columns (a) and (b) show the composition of HPLC fractions in this study, after 24 hr of hydrolysis with no correction for losses. Column (c) shows data recalculated from the integers in reference 37, in which serine and threonine were corrected for losses.

The reisolated HDL samples containing ^{125}I-labeled apoA-I were denatured with SDS and chromatographed through the G3000SW plus G2000SW columns. The separation of proteins is shown in Fig. 2a by the absorbance at 276 nm and radioactivity. The three 0.25-ml fractions containing the most radioactivity coincided with the apoA-I peak (by absorbance and SDS-PAGE), and the specific radioactivity in these fractions was used to calculate the amount of apoA-I in the serum samples (Tables 2 and 3). The calculation was as follows:

$$Mu = (Ao / SAr) - Mo$$

where Mu is the mass of apoA-I in the unknown serum, Ao is the amount of radioactivity (cpm) added to the unknown serum, SAr is the specific activity (cpm/μg of apoA-I) of the recovered apoA-I, and Mo is the mass of apoA-I (μg) added to the unknown serum. In a typical experiment with 3 ml of serum the values were:

$$Mu = (332{,}112 / 88.3) - 193.9$$
$$= 3566.3\ \mu g \text{ of apoA-I}$$
or 1.19 mg of apoA-I / ml of serum.

Evidence of the complete and rapid equilibration of ^{125}I-labeled apoA-I-labeled HDL with all apoA-I in serum at stage 2 was seen in the similarity of values (means ± SD, 132 ± 4.5 mg/100 ml, 3.5%) for five aliquots of the same serum, incubated with labeled HDL for 0–24 hr before centrifugation (**Table 2**). The small value for the coefficient of variation in this experiment, 3.5%, indicated that the isotope dilution assay was more precise than most immunoassays (1).

We performed the experiment shown in the lower half of Table 2 to be sure that the isotope dilution method would measure the amount of apoA-I in serum, when the amount present was varied keeping the other serum constituents unchanged. A serum sample of 3 ml was found to contain 4.6 mg of apoA-I. The HDL and serum protein fractions were isolated from another aliquot of serum. The mixture of 1.5 ml of serum plus 1.5 ml of serum protein fraction had essentially the same protein composition and volume as serum but only half the lipoprotein content. As expected it was found to contain less apoA-I (2.83 mg) than the 3.0 ml of serum. This was more than the 2.3 mg of apoA-I in 1.5 ml of serum because some apoA-I was contributed by the 1.5-ml serum protein fraction. From the proportion of radioactivity in the serum protein fraction, and from the density gradient ultracentrifugation, we estimated that 10.5% of serum apoA-I could have been in this fraction, i.e., 0.48 mg. A sample of 2.0 ml of HDL alone contained 2.32 mg of apoA-I. The mixture of 3.0 ml of serum plus 2.0 ml of HDL was found to contain 7.41 mg of apoA-I. This was more than the 4.6 mg of apoA-I in 3.0 ml of serum, and was 107% of the expected value,

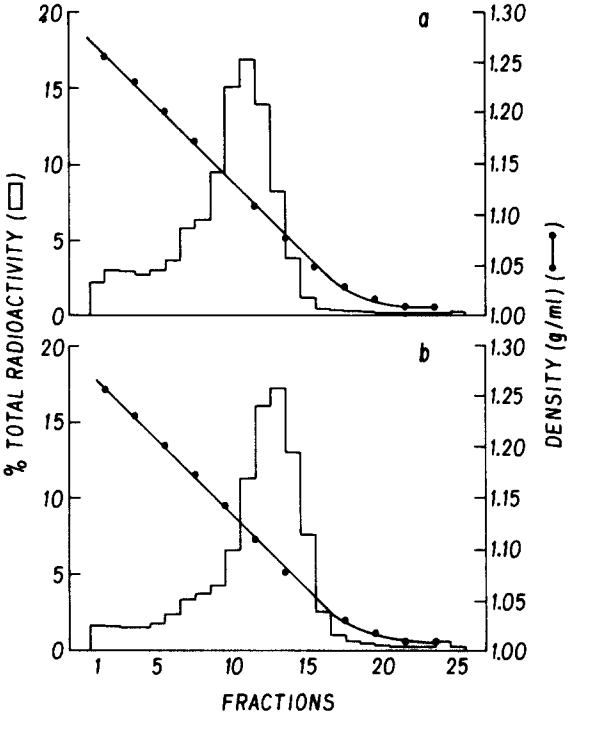

Fig. 4. Distribution of ^{125}I-labeled apoA-I on density gradient ultracentrifugation. The density gradient is shown by the line, and the radioactivity by the histogram. Panel (a) shows the result of mixing ^{125}I-labeled apoA-I with serum. Panel (b) shows the result of mixing HDL, isolated as in (a), with another aliquot of serum. Fractions of 0.5 ml were collected from the bottom of the density gradient, and the measured cpm of radioactivity were corrected for absorption by the KBr density gradient. The major peak of radioactivity in (b) corresponded with the layer of visible HDL.

TABLE 2. Effect of incubation time and sample composition on the apparent amount of apoA-I in serum

Incubation Time	Sample Volume	Mass of ApoA-I	Concentration of ApoA-I
hr	*ml*	*mg*	*mg/100 ml*
Subject 1			
0	3.0	3.89	130
1.5	3.0	4.14	138
3	3.0	3.80	127
6	3.0	4.08	136
24	3.0	3.94	131
Mean ± SD		3.97 ± 0.14	132 ± 5
Subject 2			
Serum	3.0	4.60	153[a]
Serum	1.5	2.83	189[a]
+ Lpds	1.5		94[b]
HDL	2.0	2.32	116[b]
Serum	3.0	7.41	247[a]
+ HDL	2.0		148[b]

Aliquots of serum from normolipidemic subject 1 were incubated with ^{125}I-labeled apoA-I-labeled HDL at 22°C, for the periods indicated in the first column, before ultracentrifugation. In the second experiment, serum from normolipidemic subject 2 was mixed with lipoprotein-deficient serum (Lpds) or HDL. The apparent concentration of apoA-I was calculated using ([a]) only the volume of serum in the sample, or ([b]) the total volume of the sample, i.e., 3.0 ml of serum + Lpds, and 5.0 ml of serum + HDL.

this being within two standard deviations (7%) of the expected amount. These results showed that the isotope dilution method could measure apoA-I concentrations that were either more or less than that in 3 ml of normal serum.

The isotope dilution method was used to measure apoA-I concentration in the sera of six normolipidemic subjects (**Table 3**). The mean value of 1.33 mg/ml (standard deviation 0.177 mg/ml) which we found was similar to the average of mean values reported for men (1.24 mg/ml) and women (1.38 mg/ml) measured by immunoassays calculated from the data in reference (1). Also, the mean value in the present study was almost identical to that obtained (1.34 mg/ml, 18 samples, 7 antisera) using a reference serum as the secondary standard for electroimmunoassay (18). These data show that the six normal volunteers that we studied had serum cholesterol, triacylglycerol, and HDL-cholesterol concentrations, on average, similar to those found in more extensive published studies. Thus, our subjects were, in general, comparable to the normal subjects studied by other investigators, but owing to the small number of subjects we did not attempt to correlate lipid and apoA-I concentrations.

We measured the apoA-I concentration in the serum of three hyperlipidemic patients who had been treated with Probucol and Atromid (Table 3). This treatment usually results in low serum HDL-cholesterol concentrations and we wanted to test, under this condition, whether or not the isotope dilution method would measure an apoA-I concentration different from that in the normal subjects. In each of the patients we found a lower concentration of apoA-I than in any of the normolipidemic subjects. The average value for the three patients (0.95 mg/ml) was 71% of that in the normal subjects. Duplicate assays in two of the patients gave values that differed by only 3.3 and 3.5%.

DISCUSSION

We have described a method for measurement of apoA-I that does not depend on any immunoreaction and, there-

TABLE 3. ApoA-I concentration in sera of normolipidemic and hyperlipidemic human subjects

Subjects	Sex	Cholesterol	Triglycerides	HDL-Chol	Sample Volume	Mass of ApoA-I	Concentration of ApoA-I
		mg/dl			*ml*	*mg*	*mg/dl*
Normal							
1	F	145	62	na	3.0	3.97	132
2	F	180	134	61	3.0	4.60	153
3	F	184	124	40	3.0	4.72	157
4	M	232	89	65	3.0	3.57	119
5	M	242	77	45	3.0	3.55	118
6	M	235	164	44	3.0	3.60	120
Mean ± SD		203 ± 39	108 ± 35	51 ± 11			133 ± 18
Patient							
1	M	204	252	29	2.0	2.13	106
2	F	197	207	34	3.0	3.37[a]	112[a]
					3.0	3.26[a]	109[a]
3	F	261	178	(11)[c]	3.0	2.05[b]	68[b]
					3.0	2.13[b]	71[b]
Mean ± SD		221 ± 35	212 ± 37	32			95 ± 22

[a,b] ApoA-I was assayed in two aliquots of the same serum from patients 2 and 3.
[c] HDL-cholesterol was measured on a previous sample from this patient.

fore, is independent of the heterogeneity of antigenic sites in apoA-I, heterogeneity in their expression, and heterogeneity of antibodies raised against apoA-I. The isotope dilution method is precise, having a coefficient of variation of about 3.5%, and needs only simple measurements such as pipetting, a protein assay (27), radioactivity measurement, and care not to overload the chromatography column. Because the isotope dilution assay of apoA-I can be made on whole serum, other aliquots of this serum could be used as a standard to evaluate the accuracy of an immunoassay, or be used as the primary standard in immunoassay. An advantage of serum as the standard in immunoassay is that the physicochemical state of apoA-I is likely to be the same in both the standard and the unknown serum samples to be assayed (18), while, in addition, frozen serum pools currently constitute the best reference material (1).

To our knowledge, the only other recent instance of isotope dilution being used as an assay for a protein is the measurement of apoA-II in isolated HDL reported by Weech et al. (30). Nevertheless, the method should be equally applicable to other proteins that can be purified easily and in adequate quantity. The isotope dilution assay is analogous to radioimmunoassay in which the antigen is labeled with a radioisotope and mixed in constant amount with each standard or unknown sample. In radioimmunoassay, a constant but limiting amount of antibody, which is specific for the protein in question, is added to each mixture of sample or of standard plus labeled antigen. This antibody serves two purposes: *1*) to reisolate the protein in question for measurement of its radioactivity, and *2*) to reisolate a fixed (but usually unknown) mass of the protein, this being less than the total amount of the protein in the sample, because the amount of antibody is limiting. Therefore, the specific radioactivity (SA) of the protein, reisolated by immunoprecipitation, is directly proportional to the radioactivity in the immunoprecipitate, and is less than the SA of the pure labeled antigen because of dilution by the unlabeled antigen in the sample or standards. In the isotope dilution assay for apoA-I, we reisolated and purified serum apoA-I by a single-step, density gradient ultracentrifugation followed by a single-step HPLC gel filtration. This scheme was sufficient to purify enough apoA-I to measure its specific radioactivity using the protein assay of Lowry et al. (27). Isotope dilution assays have been used more often for chemical compounds simpler than proteins, especially those that can be isolated by gas–liquid chromatography. For example, six publications describe potential reference methods for serum cholesterol assay using gas–liquid chromatography and mass spectrometry to measure the dilution of stable isotope-labeled cholesterol in the samples.

Each of the techniques used in the present study was developed separately in other laboratories. We have verified that they function well together with the amounts of sample needed for this isotope dilution method. The principal advantages that we have exploited in the density gradient centrifugation and HPLC are their rapidity and the small amount of sample required. In addition, we believe that it was important to choose techniques that would give high yields from one step to the next, with good resolution of HDL and apoA-I from other serum proteins, and the least tendency to separate apoA-I into its different charge isoforms.

A major assumption in isotope dilution assays is that the percent yield of the radioactive, labeled proteins is identical to the unlabeled protein in the unknown samples and throughout the reisolation procedure. That is to say, that the specific radioactivity of the reisolated apoA-I is identical to that in the serum before centrifugation and HPLC. In our experiments we determined the distribution of radioactivity in all fractions at each step of the procedure to ensure that this resembled what we know of apoA-I distribution by immunodetection and gel electrophoresis. We performed radioimmunoassay with monoclonal antibody anti-apoA-I 4H1 (16) on all fractions from density gradient ultracentrifugation of plasma, and in two experiments found 11% of the total apoA-I was in the plasma protein fraction d > 1.21 g/ml. This compares favorably with the 10.5% of ^{125}I-labeled apoA-I that we found in that fraction. Using radioimmunoassay or electroimmunoassay, other investigators have found that the 1.21 g/ml fraction contained 8–10% (2), 11.7% (31), 1.2–20% (32, 33), 8% (34), or 46% of plasma apoA-I. Most of these values are close to the 11% that we found; most of the variation among values was explained by Kunitake and Kane (34) to be due to the length of time or repetition of ultracentrifugation—with each repeated centrifugation, more apoA-I was found in the d > 1.21 g/ml fraction. In concordance with this idea, Cheung and Albers (33) reported finding 1.2% apoA-I in that fraction after 2.5 hr of centrifugation in a vertical rotor, but 20% after centrifugation in a CsCl gradient for 72 hr (32). The value of 46% (35) was not qualified by a time for centrifugation and is obviously different from the other available values. Most authors found only trace amounts of apoA-I in the VLDL and LDL (2, 31, 34), and our own studies with the present density gradient centrifugation showed that both immunoreactive apoA-I and ^{125}I-labeled apoA-I in the d < 1.063 g/ml fraction was typically 1.5%. Thus the distribution of ^{125}I-labeled apoA-I among the lipoproteins and the d 1.21 g/ml fraction of serum was the same as the distribution of apoA-I by immunoassay of those fractions. We attempted to isolate apoA-I from the d > 1.21 g/ml fraction and compare its specific activity with that of HDL-apoA-I in an isotope dilution assay. Ninety-seven percent of ^{125}I-labeled apoA-I was removed from the d > 1.21 g/ml fraction by chromatography through phenyl-Sepharose, but on repeated HPLC gel filtration it appeared that another

protein of slightly greater molecular weight than apoA-I was contaminating the apoA-I peak. This was evident from the greater width at half-height of the apoA-I-containing peak from the d > 1.21 g/ml fraction (1.3 ml) compared with that from the HDL (1.0 ml). A more complex series of chromatography must be developed to purify apoA-I from the serum protein fraction in order to measure directly the specific activity of this 10% of plasma apoA-I.

Osborne et al. (36) showed that the ICl technique for labeling apoA-I gave a heterogeneous product. Gel filtration of labeled plus unlabeled apoA-I in nondenaturing buffers separated forms of the protein that had different specific activities. One pool of apoA-I could self-associate like native apoA-I but a second pool remained monomeric. The number of g-atoms of I per mol of apoA-I in these two pools was not quoted in that report (36), although Osborne et al. favored the idea that iodination of apoA-I results in the formation of an "incompetent monomer due to either double labelling of a single protein molecule or the labelling of a specific tyrosine residue." The chloramine-T labeling procedure in our experiments incorporated less than 0.4 g-atoms of I per mol apoA-I, which should have minimized the risk of producing diiodotyrosine or diiodo-apoA-I. To avoid the use of a heterogeneous preparation of labeled apoA-I in our studies, we made two steps of isotope dilution. At the first step ^{125}I-labeled apoA-I was mixed with serum and HDL was isolated from the serum. Thus, only that ^{125}I-labeled apoA-I that retained the property of binding to HDL was isolated and used at the second step of the experiment, when it was mixed with a serum sample of unknown apoA-I content to determine its isotope dilution.

We expect that individual serum samples, even from normal subjects, will differ from the mean we measured (1.33 mg/ml, Table 3), due to normal biological variation, and so will the mean apoA-I concentration of large populations. Normal variation is also to be expected in the relationship of HDL-cholesterol and apoA-I concentrations in individual samples, e.g., in Table 3, subject 3 has low HDL-cholesterol but high apoA-I concentration, although subject 4 has high HDL-cholesterol and low apoA-I concentration. Such inconsistencies are probably due to other differences in the composition of HDL in these subjects. Nevertheless, the results of this study indicate that target values close to 1.33 mg of apoA-I per ml of normolipidemic serum, in immunoassay standardization, are likely to be valid.

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