Effect of storage on the measurement of apolipoproteins A-I and A-II by radial immunodiffusion

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Abstract We studied the effect of storage time and conditions on the measurement of apolipoprotein A-I and A-II by radial immunodiffusion. Purified A-I and A-II standards were stable for at least 6 months before any change in immunoreactivity was detected if stored at 4°C at concentrations of 0.06–0.24 mg/ml for A-1 and 0.016–0.064 mg/dl for A-II in 0.84 M tetramethylurea, 6.4 M urea, and 8 mM Tris-hydrochloride, pH 8.0. Purified A-1 (0.8–1.6 mg/ml) and A-II (0.5–1.0 mg/ml) were stable for 1 year if stored at −60°C in 5 mM NH_4HCO_3 with or without 4.2 M tetramethylurea. Serum or plasma could be stored at 4°C (under conditions where evaporation and bacterial growth were minimized) for at least 46 days or at −20°C for up to 3 years without any change in A-I or A-II levels. For four serum samples stored at −20°C for 2 to 3 years, the coefficient of variation of measurement ranged from 6.3 to 9.8% for A-I and from 6.7 to10.6% for A-II. Samples stored at 4°C had comparable apolipoprotein levels to those stored at −20°C. However, apolipoprotein levels in serum samples were 3–5% higher than those obtained on plasma samples. We conclude that purified A-I or A-II and serum and plasma can be stored for long periods without any change in the measurement of the A-I or A-II by radial immunodiffusion.—**Albers, J. J., M. C. Cheung, and P. W. Wahl.** Effect of storage on the measurement of apolipoproteins A-I and A-II by radial immunodiffusion. *J. Lipid Res.* 1980. **21:** 874–878.

Supplementary key words high density lipoprotein

Immunochemical assays of the high density apolipoproteins A-I and A-II have been applied to many epidemiological and physiological studies using frozen and unfrozen samples which have been stored for various lengths of time. The effects of the conditions and duration of storage on A-I and A-II levels, however, have never been established. For the past few years our laboratory has been performing A-I and A-II analyses using the radial immunodiffusion (RID) assays we developed (1, 2). We wish to report here our observations on the effect of storage on serum and plasma A-I and A-II levels analyzed by radial immunodiffusion.

METHODS

Plasma and serum samples

Venous blood was obtained from subjects, after a 12–14 hr overnight fast, in Vacutainer tubes according to standardized conditions (3). To obtain plasma, the Vacutainer tubes contained disodium EDTA 1 mg/dl. Upon separation of serum or plasma at 4°C by low speed centrifugation, 0.5 g/l sodium azide, 0.01 g/l chloramphenicol, and 0.005 g/l gentamycin were promptly added.

Short-term stability study

For the short-term stability study, portions of each of the twenty serum and plasma samples from ten men and ten women were stored at either 4°C (unfrozen) or −20°C (frozen) in sealed Wheaton vials. Separate portions stored at −20°C were used on each of the 5 days of analysis. However, for the samples stored at 4°C, 50 μl was removed from each vial on each day of analysis and the vials were promptly resealed. This study covered a period of 1½ months (46 days).

Long-term stability study

Four plasma samples with different A-I and A-II levels were stored in 0.5-ml portions in sealed Wheaton vials at −20°C. A portion of each plasma

Abbreviations: RID, radial immunodiffusion; TMU, tetramethylurea.

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sample was analyzed in each A-I and A-II assay over a span of 2 to 3 years.

A-I and A-II standards

Apolipoproteins A-I and A-II were prepared from guanidine hydrochloride-treated HDL as described (4). Purified A-I and A-II were dialyzed against 5 mM NH_4HCO_3, pH 8.0. The mass of the apolipoproteins was routinely determined by the method of Lowry et al. (5), and occasionally by carbon, hydrogen, nitrogen analysis, and amino acid analysis (2). A number of different preparations of A-I and A-II were used in the course of the long-term stability study, but only one set of A-I and A-II preparations was used in the 1½ month short-term stability study. Purified A-I and A-II were diluted with 8.4 M 1,1,3,3-tetramethylurea (TMU) from Burdick and Jackson (Muskegon, MI), and then 10 mM Tris-HCl buffer, pH 8, in 8 M ultra-pure urea from Schwarz and Mann (Orangeburg, NY) (Tris-urea) was added to give six different A-I and A-II protein concentrations ranging from 6–24 mg/dl for A-I and 1.6–6.4 mg/dl for A-II. These standard solutions were stored at 4°C and could normally be used for a period of 6 months before changes in immunoreactivity could be detected. When this occurred, as indicated by a significant change in the slope of the standard curve or decreased "intensity" of the precipitate ring or lack of demarcation of the edge of the precipitate ring, new standards were prepared from aliquots of A-I and A-II that were frozen in 5 mM NH_4HCO_3, pH 8.0.

Stability of purified A-I and A-II

Purified A-I and A-II that had been dialyzed against 5 mM NH_4HCO_3, pH 8.0, were stored under two different conditions and their immunoreactivity was tested 12 months after purification. The two storage conditions were: a) at −60°C in the presence of 4.2 M TMU, and b) at −60°C without TMU. A-I was stored at 1.6 mg/ml in the absence of TMU and 0.8 mg/ml in the presence of TMU. A-II was stored at 1.0 mg/ml in the absence of TMU and 0.5 mg/ml in the presence of TMU.

A-I and A-II antisera

Anti-A-I and anti-A-II sera were prepared as described (1, 2). Throughout the course of the long-term stability study, five different pools of rabbit anti-A-I sera, three different pools of rabbit anti-A-II antisera, and one pool of goat anti-A-II antiserum were used. However, all analyses during the short-term study were done with one pool of anti-A-I and one pool of anti-A-II sera.

Immunoassay procedure

Plasma levels of A-I and A-II were determined by the radial immunodiffusion (RID) assays as described (1, 2, 4). Plasma samples of 50 μl were diluted with an equal volume of TMU and mixed. The TMU-plasma mixtures were further diluted with 400 μl of Tris-urea, and 4 μl of each sample was applied into wells of the RID plates. The RID plates were incubated in humid chambers until the precipitate rings attained their final size (48–72 hr). The diameters of the precipitate rings were measured in 0.1 mm units using a calibrating RID viewer from Transidyne General Corporation (Ann Arbor, MI).

Statistical analyses

A-I and A-II values from the twenty samples in the short-term study were analyzed, using the analysis of variance technique (6) for repeated measures on three factors: temperature (4°C versus −20°C), nature of the sample (plasma versus serum), and the age effect (days 0, 4, 11, 25, and 46). The Newman-Keuls multiple comparison procedure (6) was used to compare A-I and A-II averages among the various days. A-I and A-II values of the four plasma samples in the long-term study were plotted against the age of the plasma sample (number of days at which they were stored at −20°C) to detect overall trends in the data. Pearson's correlation coefficient and associated linear regression coefficients (7) were used to describe the relationship between long-term storage and the A-I and A-II levels.

RESULTS

The relationship between the square of the diameter of the precipitate ring (D^2) and the concentration of the A-I or A-II standards was linear over the range of 6–24 mg/dl for A-I and 1.6–6.4 mg/dl for A-II. Stability of the standard solutions was therefore monitored by precipitate ring sizes or the slope of the standard curve D^2 versus concentration of apolipoprotein standard for each RID assay. Using these criteria, standards diluted in TMU and Tris-urea were stable for at least 6 months, since the slope of the standard curve did not change with storage (**Fig. 1**). The line describing the relationships between the slope of the standard curve and storage time had a slope that was not significantly different from zero (slope = 0.0012 for A-I and slope = −0.0003 for A-II).

Purified A-I and A-II that were stored at −60°C for 12 months in the presence or absence of 4.2 M TMU did not show any change of immunoreactivity. The

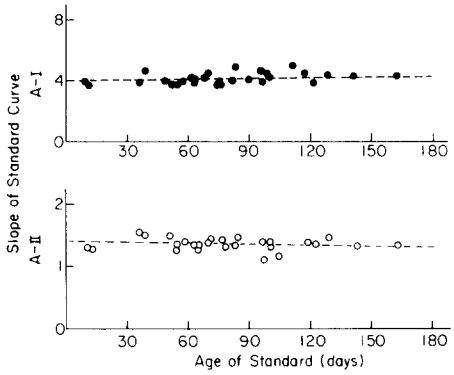

Fig. 1. Scatterplot of slope of standard curve of A-I and A-II as a function of standard storage time at 4°C at A-I concentrations of 0.240–0.056 mg/ml and A-II concentration of 0.065–0.016 mg/ml.

stored A-I and A-II preparations were diluted to concentrations similar to the standard solutions and applied on RID plates. The square of the diameters obtained from the dilutions of the stored preparations were superimposable upon the standard curve (**Fig. 2**).

Since it was necessary to use more than one pool of anti-A-I and anti-A-II antisera in the course of the long-term stability study, variation in A-I and A-II due to changes of antisera was assessed. Twenty-six plasma samples were asayed on the same day using

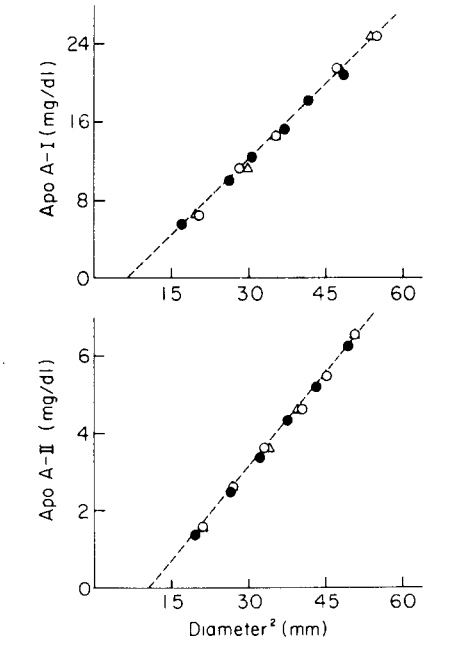

Fig. 2. Relationship of precipitate ring size to A-I and A-II concentration. The dotted line (– – –) is the linear regression of A-I or A-II standards (●) versus diameter² of the precipitate rings. Diameter² of the precipitate rings formed by dilutions of purified A-I or A-II that was stored at −60°C for 1 year in 5 mM NH_4HCO_3, pH 8.0, in the absence and the presence of 4.2 M TMU are represented by (○) and (△), respectively.

TABLE 1. A-I and A-II levels in serum and plasma stored at 4°C and −20°C

	Temperature	Serum[a]	Plasma[a]
A-I	4°C	121.6 ± 9.06	116.2 ± 5.79
	−20°C	120.9 ± 7.68	115.3 ± 8.89
	4°C and −20°C combined	121.2 ± 8.31	115.7 ± 7.34
A-II	4°C	30.3 ± 1.99	29.3 ± 1.88
	−20°C	29.9 ± 2.08	28.9 ± 2.08
	4°C and −20°C combined	30.1 ± 2.04	29.1 ± 1.98

[a] Mean ± standard deviation in mg/dl. They were calculated as follows: for each of the 20 samples, the standard deviation across the 5 days (0, 4, 11, 25, 46) was found; then the average standard deviation over the 20 samples was computed separately for serum and plasma at 4°C and −20°C temperature.

three anti-A-I (A, B, C) and three anti-A-II (D, E, F) sera. Antisera A, B, C, and E, respectively, were obtained from different rabbits and antisera D and F from different goats. None of these antisera was used in the long-term storage study. The mean A-I levels of the 26 samples obtained by using the three different antisera (A, B, and C) were 125, 121, and 128 mg/dl, respectively. The A-I values obtained by antisera B were significantly lower, 3.2% ($P < 0.005$) and 5.5% ($P < 0.001$), respectively, than those obtained with antisera A and C. However, the values obtained by antisera A and B and by B and C were highly correlated, $r = 0.951$ and 0.965, respectively. The mean A-II levels of the 26 samples obtained by using the three different antisera (D, E, and F) were 32, 20, and 31 mg/dl, respectively. The values obtained by antisera D and F were not significantly different whereas the values obtained by antisera E were 36–37% lower ($P < 0.001$) than those obtained with antisera D and F. Yet the values obtained by D and E and by E and F were highly correlated, $r = 0.892$ and 0.887, respectively.

The means and standard deviations of the A-I and A-II levels of the twenty serum and plasma samples stored at 4°C and −20°C over a period of 46 days are shown in **Table 1.** Serum A-I and A-II levels were significantly higher than plasma A-I and A-II levels by 5.5 mg/dl (4.75%) ($F_{1,19} = 46.1$, $P < 0.001$) and 1.0 mg/dl (3.44%) ($F_{1,19} = 46.8$, $P < 0.001$), respectively. Although the average A-II levels in serum and plasma stored at 4°C were slightly higher than the average levels in serum and plasma stored at −20°C, by 0.4 mg/dl, this difference did not reach significance ($F_{1,19} = 3.25$, $P = 0.087$) (Table 1).

The twenty serum and plasma samples stored at 4°C and −20°C were analyzed for A-I and A-II on days 0, 4, 11, 25, and 46. Since the average A-I and A-II values of plasma and serum stored at 4°C did not

differ significantly from those stored at −20°C, the A-I and A-II values for samples stored at these two temperatures were combined to assess short-term age effect on these apoprotein analysis. **Table 2** shows the mean serum and plasma A-I, A-II values on the 5 days of analysis. On day 25, the mean A-I and A-II levels were significantly higher than those on the other 4 days (A-I: $F_{4,76} = 11.04$, $P < 0.001$; A-II: $F_{4,76} = 17.22$, $P < 0.001$). However, values on each of these days (0, 4, 11, and 46) did not differ significantly among themselves.

The A-I and A-II levels of the four plasma samples analyzed over a period of 2–3 years were plotted against the number of days at which they were stored at −20°C. A representative example of such plots is shown in **Fig. 3.** Regression analysis was used to describe the effect of long-term storage on plasma A-I and A-II levels. Analyses of these data indicate that within a period of 2–3 years there was no change in apolipoprotein levels with storage time, since the slope of the line describing the relationship between apolipoprotein level and storage time was not significantly different from zero for any of the plasma pools. The coefficient of variation for the four pools ranged from 6.3 to 9.8% for A-I and from 6.7 to 10.6% for A-II during this 2–3 year period.

DISCUSSION

Our data clearly show that when serum and plasma samples were kept under conditions whereby bacterial growth and evaporation were eliminated or minimized, the samples could be stored for at least 1½ months (46 days) at 4°C and 2–3 years at −20°C without any change in the A-I and A-II level when measured by RID. If storage time of greater than 3 years is anticipated, a storage temperature of −60°C or lower would be recommended.

In RID assay, several factors contribute to interassay and intrassay variation. These include variation

TABLE 2. Short-term age effect on A-I and A-II levels for serum and plasma

Sample Age	Serum[a]		Plasma[a]	
	A-I	A-II	A-I	A-II
(Days)				
0	115.8	30.0	110.4	28.8
4	119.6	30.2	116.4	28.8
11	120.8	29.4	115.4	30.8
25	127.1	31.9	120.4	30.8
46	123.0	29.1	116.2	28.4

[a] Mean A-I and A-II values in mg/dl of the 20 serum and 20 plasma samples stored at 4°C and −20°C.

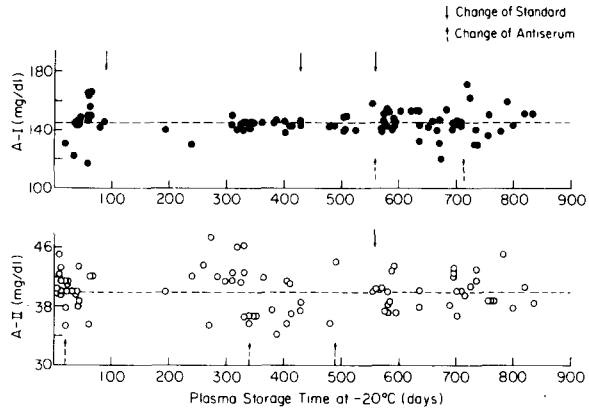

Fig. 3. Scatterplot of plasma A-I and A-II levels as a function of plasma storage time.

in preparation of reagents, standards, antibodies, and finally, technical skill. The factors that are the most critical to our assay are antibody and standards. We have shown that purified A-I and A-II standards are reasonably stable. When stored in dilute solutions (A-I, 0.056–0.240 mg/ml, A-II, 0.016–0.065 mg/ml) in 0.84 M TMU, and Tris-urea at 4°C, they could be used for at least 6 months before changes in immunoreactivity were detected, as judged by changes in the size of precipitate ring. A-I (0.8–1.6 mg/ml) and A-II (0.5–1.0 mg/ml) stored at −60°C either in the presence or the absence of 4.2 M TMU were stable for 1 year. The reactivity of antibodies has been known to be stable for years when stored frozen at −20°C or at −60°C. However, since the concentration and binding characteristics (avidity and affinity) of antibody do vary from one bleeding to another, it is reasonable to expect slight differences in antigen–antibody reaction when different bleedings of antibodies are used. For this reason we combined serum from numerous bleedings with similar binding characteristics in order to make a large antiserum pool. This procedure minimizes the assay variations because of the use of different antisera. We have not detected different apolipoprotein values on our frozen serum pools when we switched antisera pools (see Fig. 3). However, it is clear that significant systematic differences in apolipoprotein values can occur because of a difference in antisera. Variations in standard and antiserum preparations may contribute significantly to measurement variation within a laboratory. Even greater variations between laboratories would be expected because of different methods for preparation of the antigens and antisera.

Since numerous factors can contribute to RID assay variations, quality control samples are routinely used in our A-I and A-II assays. These samples are stored in 0.3 ml portions in sealed Wheaton vials at

−20°C until the day of immunochemical analysis. The A-I and A-II levels of each quality control sample are established by repeatedly assaying the sample twenty times within a month after the pool is prepared. Four quality control samples over a range of A-I and A-II levels were included in each RID assay. On days when the bias from the target values (mean assay value for first 20 runs) averaged more than 5%, the results were considered questionable and repeated or adjusted. Now that it is known that stable quality control pools can be prepared, a more rigorous quality control system should be established for the measurement of apolipoproteins similar to that used for cholesterol and triglyceride in the LRC Program (3).

Radioimmunoassay, electroimmunoassay, and radial immunodiffusion assay are all quantitative immunochemical techniques based on the antigen–antibody reaction. However, each is governed by somewhat different principles and assumptions. We wish to emphasize that the present data may only apply to the RID technique. It is possible that other apoprotein measurement techniques may be more sensitive to the potential changes that apoproteins undergo during long term storage.

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