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# A Rapid Apolipoprotein E Radioimmunoassay Using Solid-Phase Staphylococcus Protein. Use of pooled plasma as a secondary standard

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A rapid apolipoprotein E (apo E) radioimmunoassay, which requires a total of 24 hour incubation as compared to the usual 3-5 days, has been developed in our laboratory. Solid phase staphylococcus protein A was used to separate bound and unbound labeled antigen. Use of a pooled plasma (quality control sample) as a secondary standard to reduce interassay variation was also described. 

Measurement of apolipoprotein E (apo E) in plasma by the current radioimmunoassays (RIA) (1-5), which utilizes a double antibody immunoprecipitation technique, generally requires a 3-5 day incubation. The long incubation poses problems when measuring apo E in plasma routinely such as in a clinical laboratory.

We now report that a rapid apo E RIA, which utilizes staphylococcus protein A as a solid phase separator and only one day incubation, has been developed in our laboratory.

Our apo E RIA combined the use of purified apo E as a primary standard for quantifying apo E in plasma, and a pooled plasma (quality control sample) as a secondary standard to reduce interassay variation.

## MATERIAL AND METHODS

Isolation of Apo E: VLDL was prepared from plasma of hypertriglyceridemic subjects as described by Weisgraber et. al (6) with a few modifications. The lyophilized VLDL was delipidated with ether-ethanol (3:1). Apo E was isolated from delipidated VLDL by column chromatography using Sephacryl S-300. The pooled fractions were dialyzed, lyophilized, redissolved (4 M Guanidine hydrochloride, 0.1 M Tris.Cl, 1 mM EDTA), and then stored at -20 C. The purity of the isolated apo E was examined by polyacrylamide gel electrophoresis in the presence of SDS, a major band of m.w. 33,000 was observed. The mass of the purified apo E was quantitated

0006-291X/86 $1.50

by amino acid analysis. Double immunodiffusion experiments showed no cross reactivity between the isolated apo E with apo C-II, C-III, A-I, or A-II antisera.

Preparation of Antisera: Apo E (100 ug) was emulsified with 1 ml of Freund's complete adjuvant and injected subcutaneously into New Zealand White female rabbits at 2 week intervals. The rabbits were bled regularly starting after the second injection.

Apo E RIA: An aliquot of purified apo E was used as the standard and the labeled antigen (stored in assay buffer: 8 mM sodium decyl sulphate, 50 mM sodium phosphate, 100 mM sodium chloride 1 % BSA, pH 7.5). Purified apo E was labeled with $^{125}I$ using the iodine monochloride procedure (4). The labeled $^{125}I$ apo E was purified by column chromatogrphy using Sephadex G-25 (equilibrated in 100 mM sodium decyl sulphate, 50 mM sodium phosphate, 100 mM sodium chloride, 1 % BSA, pH 7.5).

Disposable glass tubes (12 x 75 mm) were used. The reaction mixture consisted of : 100 ul plasma (1:100 in RIA buffer (0.1 % BSA, 0.01 % $NaN_3$, 0.1 M Borate, pH 8.5), 100 ul antiserum (1:800), and 100 ul $^{125}I$ apo E (20000 cpm in the assay buffer). The reaction mixture was incubated for 24 hours at room temperature, 100 ul of staphylococcus protein A (diluted 1:3 in 0.1 M Tris, pH 8.0) added, and the reaction mixture incubated for another 30 min. Two ml of washing buffer (RIA buffer diluted 1:5 in saline) was added and the bound antigen separated from the unbound by centrifuging at 2700 rpm for 30 min. The supernate was aspirated and the precipitate counted by a gamma-spectrometer. The percentage of the bound antigen is B/Bo; where B is the total radioactivity (cpm) in the presence of apo E standard or plasma sample minus the nonspecific binding, and Bo is the total radioactivity (cpm) in the absence of standard or plasma sample minus the nonspecific binding. The non-specific binding generally was 700-900 cpm.

Experimental Design: For determining the intra- and interassay variation, the plasma apo E levels of 16 healthy subjects and the quality control (QC) sample were measured 13 times during 6 months. Approximately 40 ml of fasting blood drawn into EDTA vacutainers was obtained from 16 subjects (8 females and 8 males, age 25-55). The plasma was separated from blood cells immediately and stored at -70° C in 0.5 ml aliquots. The QC plasma sample was a pooled fasting plasma from 8-10 healthy subjects. An aliquot of the frozen samples was thawed only once and used for measurement. For each experiment: three separate dilutions were made from each plasma sample, triplicate assays were measured from each diluted plasma giving a total of 9 replicates. A total of 27 replicates (27 assays/9 dilutions) were measured for the QC sample.

## RESULTS

A typical displacement curve of $^{125}I$ apo E from rabbit antibody to human apo E by unlabeled apo E is shown in Figure 1. A parallel displacement curve was also observed using human plasma (Figure 1).

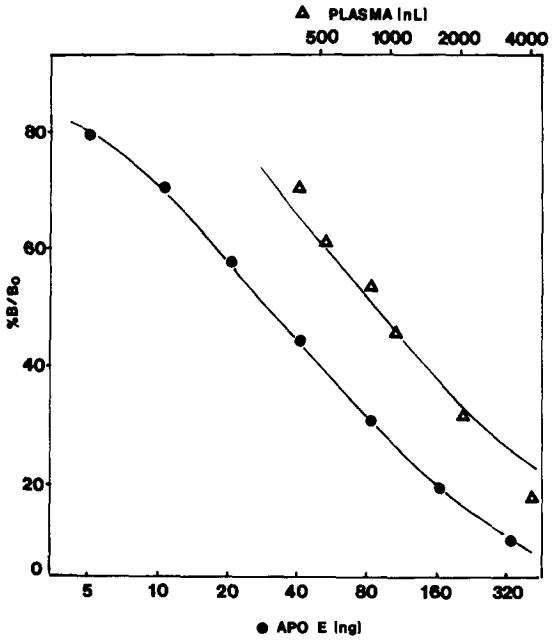


Figure 1: A typical displacement curve of $^{125}I$ apo E from rabbit antibody to human apo E by unlabeled apo E. A parallel displacement curve was also observed using human plasma. Each point represents the mean of triplicates.

Apo E in plasma of 11 subjects determined after a 24 hour and after a 48 hour incubation were similar, as shown in Table 1, thus, the reaction reached equilibrium after a 24 hour incubation.

The intra- and interassay variation were determined by measuring the plasma apo E levels of 16 subjects 13 times during 6 months. A total of 6 different preparations of $^{125}I$ apo E were used. The average intra- and interassay variation for the 16 subjects were 8.9 % and 19.9 %. Recently, we have reported that a large part of the interassay variation in our apo A-I RIA was due to degradation of $^{125}I$ apo A-I (7). The measured plasma apo A-I levels increased as $^{125}I$ apo A-I degraded over time. However, the interassay variation in the apo A-I RIA could be reduced substantially by using a QC sample as a secondary standard. The

TABLE 1 Apo E in Plasma Determined After 24 Hour and 48 Hour Incubation

| | Plasma apo E (mg/L) | |
|---|---|---|
| Sample # | 24 hour | 48 hour |
| 1 | 54.8 ± 2.4 [1] | 54.6 ± 2.7 |
| 2 | 37.2 ± 2.1 | 37.6 ± 1.8 |
| 3 | 67.5 ± 3.5 | 68.8 ± 4.9 |
| 4 | 49.6 ± 3.8 | 46.5 ± 2.7 |
| 5 | 52.7 ± 3.5 | 49.3 ± 2.4 |
| 6 | 74.9 ± 8.0 | 73.6 ± 5.1 |
| 7 | 68.3 ± 6.9 | 63.4 ± 3.7 |
| 8 | 57.9 ± 3.7 | 50.7 ± 2.1 |
| 9 | 36.2 ± 2.2 | 38.6 ± 0.9 |
| 10 | 43.2 ± 2.3 | 43.9 ± 2.3 |
| 11 [2] | 609.4 ± 51.1 | 628.6 ± 148.9 |

1. mean + S.D. (n=9)
2. High triglyceride plasma sample.

QC sample used as a secondary standard was verified in our apo E RIA by adjusting the levels of 16 subjects:

$$\text{plasma apo E}_{(\text{assay i})} \times \frac{\text{QC plasma apo E}_{(\text{assay 1})}}{\text{QC plasma apo E}_{(\text{assay i})}}$$

The interassay variation decreased from a range of 13.7% - 24.9% (the mean was 19.9 %) before the adjustment to a range of 4.8% - 15.6% (the mean was 11.4%) after the adjustment (Table 2). The mean plasma apo E levels of 16 subjects measured 13 times were compared before and after adjusting by the ratio (Figure 2); the adjustment reduced the changes observed in the measured plasma apo E levels.

A reference QC plasma apo E level was established so that results from different experiments could be compared. In our apo A-I RIA, the measured QC plasma apo A-I levels correlated with

TABLE 2 The Interassay Variation of The Measured Plasma Apo E Levels Before and After Adjustment Using The Ratio [1]

| Subject# | Plasma Apo-E Before Adjustment Mean [2] + S.D. (Interassay Variation %) | Plasma Apo-E After Adjustment Mean + S.D (Interassay Variation %) |
|---|---|---|
| 1 | 37.8 ± 7.6 (20.1) | 39.9 ± 4.7 (11.8) |
| 2 | 82.6 ± 22.3 (27.0) | 86.1 ± 12.8 (14.9) |
| 3 | 48.7 ± 11.4 (23.4) | 51.2 ± 6.9 (13.5) |
| 4 | 51.7 ± 7.1 (13.7) | 54.5 ± 2.6 (4.8) |
| 5 | 74.7 ± 14.5 (19.4) | 78.6 ± 7.6 (9.7) |
| 6 | 65.6 ± 12.8 (19.5) | 68.9 ± 7.4 (10.7) |
| 7 | 32.3 ± 6.1 (18.9) | 34.1 ± 4.4 (12.9) |
| 8 | 27.7 ± 5.7 (20.6) | 29.3 ± 4.1 (14.0) |
| 9 | 55.4 ± 10.4 (18.8) | 58.3 ± 5.5 (9.4) |
| 10 | 63.1 ± 11.5 (18.2) | 66.4 ± 5.6 (8.4) |
| 11 | 36.9 ± 5.1 (13.8) | 39.1 ± 4.1 (10.5) |
| 12 | 40.6 ± 7.2 (17.7) | 42.8 ± 5.2 (12.1) |
| 13 | 35.4 ± 6.6 (18.6) | 37.3 ± 4.0 (10.7) |
| 14 | 49.0 ± 9.2 (18.8) | 51.6 ± 4.8 (9.3) |
| 15 | 76.0 ± 19.0 (25.0) | 79.6 ± 12.4 (15.6) |
| 16 | 94.4 ± 23.1 (24.5) | 98.8 ± 14.1 (14.3) |

1. Ratio: QC plasma apo $E_{(assay\ 1)}$/QC plasma Apo $E_{(assay\ i)}$.
2. mean plasma apo E level (mg/L) measured 13 times during 6 months.

the age of $^{125}I$ apo A-I (7). Thus, the reference level was obtained by linear regression of the measured value upon the age of $^{125}I$ apo A-I. However, a similar correlation was not observed in the apo E RIA. The reference QC plasma apo E level was 43.2 mg/L, the mean value estimated from 648 replicates in a total of 24 experiments measured during 6 months. A total of 7 different preparations of $^{125}I$ apo E were used.

With the use of a QC sample as a secondary standard, plasma apo E levels of a group of normolipidemic subjects (43 females and 95 males, age 20-70) were determined. These subjects are

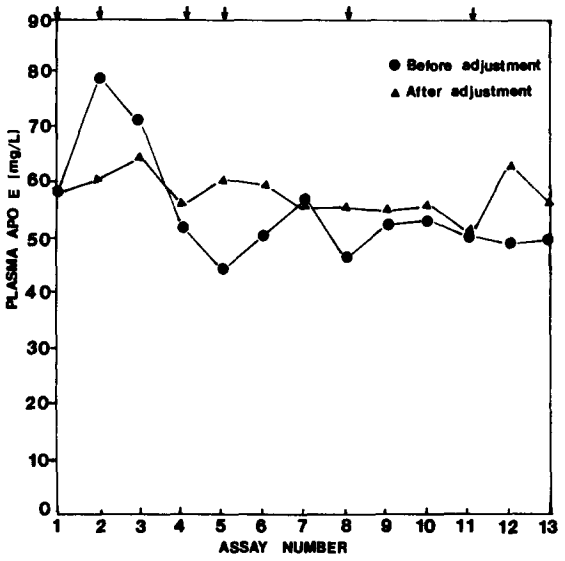


Figure 2: The measured plasma E levels before and after adjustment for the ratio QC plasma apo E $_{(assay\ 1)}$/ QC plasma apo E $_{(assay\ i)}$ as a function of time. Each point represents the mean of 16 subjects.

nonsmokers, not on any medication (the selected females were not on oral contraceptives), and free of disease. The medical charts of these healthy subjects have been carefully reviewed and showed the absence of: cardiovascular disease, diabetes, hypertension, thyroid disease, liver disease, or any known diagnosed disease. Fasting plasma was obtained from these subjects and stored in

TABLE 3 Plasma Triglycerides, Cholesterol, HDL-Cholesterol and Apo E Levels in Healthy Females and Males

| | Age | N | Triglycerides (g/L) | Cholesterol (g/L) | HDL-Cholesterol (g/L) | Apo E (mg/L) |
|---|---|---|---|---|---|---|
| F | 50-70 | 16 | 0.99 ± 0.19[1] | 2.05 ± 2.68 | 0.61 ± 0.10 | 52.6 ± 13.5 |
| | 30-50 | 11 | 0.76 ± 0.28 | 1.79 ± 2.81 | 0.61 ± 0.11 | 52.6 ± 16.1 |
| | 20-30 | 16 | 0.61 ± 0.17 | 1.56 ± 2.08 | 0.56 ± 0.15 | 47.3 ± 14.2 |
| M | 50-70 | 12 | 1.26 ± 0.60 | 1.95 ± 0.31 | 0.43 ± 0.09 | 56.8 ± 14.4 |
| | 40-50 | 17 | 1.10 ± 0.33 | 1.78 ± 0.35 | 0.41 ± 0.10 | 51.1 ± 16.2 |
| | 30-40 | 28 | 1.01 ± 0.35 | 1.79 ± 0.21 | 0.42 ± 0.09 | 49.1 ± 11.9 |
| | 20-30 | 38 | 0.86 ± 0.54 | 1.57 ± 0.21 | 0.44 ± 0.08 | 47.1 ± 13.1 |

1. mean ± S.D.

TABLE 4 Incubation Time and Plasma Apo E Levels Measured By RIA

| | Incubation Time (days) | Plasma Apo E (mg/L) |
|---|---|---|
| Our Laboratory | 1 | 50.6 ± 14.3[1] (females, n=43)<br>49.6 ± 13.7 (males, n=95) |
| Falko et. al (1) | 3 | 53.0 ± 8.0 (n=4) |
| Blum et. al (2) | 3-4 | 36.0 ± 13.0 (n=26) |
| Havel et. al (3) | 3 | 58.9 ± 23.7 (females, n=86)<br>49.7 ± 17.1 (males, n=86) |
| Gregg et. al (4) | 3-4 | 57.0 ± 14.0 (n=58) |
| Karlin et. al (5) | 5 | 38.3 (n=3) |

1. mean ± S.D.

small aliquots at -70 C. Plasma triglycerides, cholesterol, HDL-cholesterol and apo E levels of these subjects are summarized in Table 3. The plasma apo E levels ranged from 47.3 to 52.6 mg/L in females, and 47.1 to 56.8 mg/L in males. These values are comparable to those reported by others (Table 4). Moreover, the plasma apo E levels of the normolipidemic subjects were compared to those of the hyperlipidemic subjects. We have measured plasma apo E levels of a group of hyperlipidemic subjects (46 females and 60 males, age 30-80, plasma triglycerides 2.97 ±1.12 g/L, plasma cholesterol 2.36 ±.49 g/L) and found that the plasma apo E levels of the hyperlipidemic subjects (82.0 ± 33.4 mg/L in females and 84.9± 48.5 mg/L in males) were significantly higher than those of the normolipidemic subjects (50.6 ± 14.3 mg/L in females and 49.6 ±13.7 mg/L in males, $p < 0.001$) (8).

## DISCUSSION

The apo E RIA established in our laboratory is an improved assay. It involves a simpler procedure, and more importantly, it is a time saving technique. Only a 24 hour incubation is required instead of the usual 3-5 days as reported by others.

The source of the interassay variation in the apo E RIA has yet to be determined. A large part of the interassay variation in

our apo A-I RIA was due to degradation of the label such that the measured levels increased with the age of the label. However, a similar correlation was not observed in apo E RIA.

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