CLIN. CHEM. 31/2, 247–251 (1985)

Quantification of Human Serum Apolipoprotein AI by Enzyme Immunoassay

J. Bury and M. Rosseneu

We developed a quantitative assay for apolipoprotein AI (apo AI) in human serum, using a "sandwich"-type enzyme-linked immunosorbent assay. Diluted serum samples were pipetted into the wells of polystyrene microtiter plates that had been previously coated with purified rabbit anti-human apo AI antibodies. After incubation for 2 h and washing, antibodies conjugated to horseradish peroxidase (EC 1.11.1.7) were added and incubated for 2 h; after further washing, the bound enzyme was assayed by oxidation of *o*-phenylenediamine. Assay conditions were optimized for the incubation time and the amounts of coating antibodies and conjugate. Assay sensitivity is about 0.5 ng of apo AI, with a working range of 1 to 14 ng, similar to that of radioimmunoassays for human apo AI. The standard curves for apo AI in serum or HDL and for purified apo AI were parallel. Delipidation, heat treatment, or addition of detergents did not affect the amount of immunoassayable apo AI in human serum. The intra- and interassay CVs were 4 and 8%, respectively. Results for 100 serum samples compared well with those by immunonephelometry ($r = 0.94$).

Additional Keyphrases: *lipoprotein subfractions · immunonephelometry compared · myocardial infarction · heart disease · screening*

Epidemiological studies have demonstrated an association between low concentrations of high-density lipoprotein (HDL) cholesterol and an increased risk of coronary artery disease (*1–4*). Moreover, HDL apolipoproteins, especially the major apolipoprotein (apo) AI, may be useful in identifying potential survivors of myocardial infarction (*5, 6*).

Human apo AI has been quantified by radioimmunoassay (*9, 10*), electroimmunoassay (*11*), and immunonephelometry (*12*). Recently an enzyme immunoassay for nonhuman (primate) apo AI has been described (13). We have developed a "sandwich"-type enzyme immunoassay for human apo AI; the advantages of this technique include high sensitivity, small antiserum requirement, absence of radioactive tracers, and the potential to analyze a large number of samples per assay.

This technique is especially suited for turnover studies, where the label does not interfere with the enzyme immunoassay (*13*), and for screening studies, e.g., with newborns, where only small amounts of sample are available (*14*). Because of its high sensitivity, this technique is also especially suited for quantifying apoproteins after lipoprotein fractionation by isopycnic ultracentrifugation (*15*), gel chromatography (*16*), or liquid chromatography (*17*).

Here we report a detailed description of the "sandwich" enzyme immunoassay and its validation by comparison with immunonephelometry.

Department of Clinical Chemistry, A. Z. St. Jan, Ruddershove, B 8000 Brugge, Belgium.

Received December 19, 1983; accepted November 15, 1984.

Materials and Methods

Isolation of apo AI and preparation of anti-apo AI serum. HDLs were isolated from the plasma of normal volunteers by ultracentrifugal flotation at densities between 1.080 and 1.190 kg/L in a Model L5-65 ultracentrifuge (Beckman Instruments, Fullerton, CA 92634), then delipidated with ether/ethanol (3/1 by vol) at 4 °C.

The apo AI was purified by ion-exchange chromatography (*18*) for use as primary standard and immunogen. Its purity was established by electrophoresis in polyacrylamide gel (containing 8 mol of urea per liter) and sodium dodecyl sulfate; by immunodiffusion with antisera against apoproteins AI, AII, B, CII, CIII, and E and albumin; and by amino acid analysis based on the reported composition of purified apo AI (*19*).

Antiserum to apo AI was raised in rabbits as previously described (*12*). We checked the specificity of the antiserum by immunodiffusion against apoproteins AI, AII, B, CII, CIII, and E and human albumin and noted a reaction against apo AI only. The extent of antibody cross reactivity with these antigens in the assay was also checked by constructing displacement curves with purified apo AI, AII, B, CIII, and E and human albumin.

Affinity chromatography. An apo AI-affinity column was prepared by covalently linking 10 mg of apo AI to 1 g of CNBr-activated Sepharose 4 B (Pharmacia, Uppsala, Sweden) and subsequent reaction with glycine (*20*) to block any residual coupling sites. After incubation for 90 min with 2–5 mL of rabbit antiserum, the gel was poured into a small column (1 × 10 cm) and eluted with sodium phosphate buffer (10 mmol/L, pH 7.4) containing 0.15 mol of NaCl per liter. The anti-apo AI immunoglobulins retained on the column were eluted with 25 mL of glycine HCl buffer (0.2 mol/L, pH 2.8) and collected into 5 mL of 1 mol/L K_2HPO_4 buffer, concentrated by ultrafiltration, and dialyzed against either sodium phosphate buffer (10 mmol/L, pH 7.4, containing 0.15 mol of NaCl and 1 g of NaN_3 per liter) for coating, or sodium carbonate buffer (10 mmol/L, pH 9.6) for conjugate preparation. The isolated anti-apo AI antibodies were stored at 4 °C and filtered through a 0.45-μm pore-size filter (Millipore Corp., Bedford, MA 01730) before use.

Preparation of the antibody–enzyme conjugate. The antibody–enzyme conjugate was prepared by a modification of the periodate-coupling procedure described by Nakane (*21*). We dissolved 2.5 mg of horseradish peroxidase (EC 1.11.1.7; Boehringer, grade I, RZ >3.0) in 1.0 mL of 0.3 mol/L $NaHCO_3$ and gently mixed with 50 μL of a 0.1 g/L solution of 2,4-dinitrophenylmonofluorobenzene in ethanol. After a 2 h incubation at room temperature, 0.5 mL of 80 mmol/L $NaIO_4$ solution was added. We stopped the reaction after 1 h by adding 0.1 mL of glycerol, then extensively dialyzed the solution against sodium carbonate buffer (10 mmol/L, pH 9.6).

We incubated 5 mg of anti-apo AI antibody (10 mg/mL) with the peroxidase–aldehyde for 3 h at room temperature,

then dialyzed this at 4 °C against sodium phosphate buffer (100 mmol/L, pH 7.4) and mixed the dialysate with an equal volume of glycerol before storage in aliquots at −20 °C.

Sample treatment. Serum was collected after an overnight fast. Apo AI was quantified either in fresh serum or in serum stored at −20 °C for less than three months (*12*). We compared several sample treatments to expose all of the antigenic sites of apo AI in serum: (*a*) Serum was delipidated either with *n*-butanol/diisopropyl ether (40/60 by vol) (*23*), or with ether/ethanol (3/1 by vol), and the dry protein residues were redissolved in sodium phosphate buffer (10 mmol/L, pH 7.4) containing 0.15 mol of NaCl and 8 mol of urea per liter (*24*). (*b*) Samples were diluted 20 000-fold and heated at 54 °C for 3 h in a water bath (*8*). (*c*) Serum samples were diluted 100-fold with either urea, 8 mol/L (*11*), or guanidine chloride, 6 mol/L (*12*), before their final dilution with the immunoassay buffer, to which was added either 0.1 g of the nonionic detergent Apovax (Ortho Diagnostics, Beerse, Belgium) (*25*) or 3.2 g of Tween 20™ (polyoxyethylene (20) sorbitan monolaurate) (*26*) per liter.

Enzyme immunoassay. Sodium phosphate buffer (10 mmol/L, pH 7.4) containing NaCl, 0.15 mol/L, was used for coating, incubation, and washing. The coating buffer contained NaN_3, 1 g/L, the assay buffer contained bovine serum albumin, 10 g/L, and the wash buffer contained Tween 20, 0.5 g/L, to reduce nonspecific binding (*27, 28*). Pipet 110 μL of isolated apo AI antibodies (15 μg/mL) into each well of a polystyrene microtiter plate (Flat Bottom Micro ELISA plates M 129 B; Dynatech Lab Inc., Alexandria, VA 22234), excluding the outer rows to avoid edge effects (*24*). Seal the plates with sealing tape (Dynatech) and incubate for 3 h at 37 °C, then overnight at 4 °C. Wash the plates with wash buffer three times, then shake them dry. To block any residual binding sites with albumin, further incubate each well for 1 h with 150 μL of assay buffer and wash as described above.

Dilute standard or serum samples 20 000-fold (we used a Microlab 1000 dilutor; Hamilton, Bonaduz, Switzerland), then pipet 100 μL of each into the wells of a precoated microtiter plate. Cover the plates and incubate at 37 °C for 2 h. Aspirate the liquids from the wells and wash the wells five times, then pipet 100 μL of peroxidase–antibody conjugate (diluted 7500-fold with the assay buffer) into each well. Again incubate the plates at 37 °C for 2 h and then aspirate and wash as before.

Freshly prepare *o*-phenylenediamine dihydrochloride substrate (Sigma Chemical Co., St. Louis, MO 63178), at 3 g/L in sodium phosphate citrate buffer (0.1 mol/L, pH 5.6) containing 0.2 g of H_2O_2 per liter. Pipet 100 μL of substrate into each well at timed intervals and let stand at room temperature in the dark. After 30 min stop the reaction by adding 100 μL of 2.5 mol/L sulfuric acid (analytical grade; Merck, Darmstadt, F.R.G.) per well. Read the absorbance of each well at 490 nm within 2 h (we used a Chromo Scan EIA reader; Bio-Tek Instruments, Burlington, VT 05401). Prepare calibration curves from the appropriate dilutions of purified apo AI in the phosphate buffer containing 0.1 g of Apovax per liter and further dilute with the assay buffer. Plot absorbance against log apo AI concentration to generate a standard curve, from which the apo AI in serum is determined. We aliquot and store serum from normolipidemic donors at −20 °C, for use as a suitable secondary standard and control (*22*).

Quantification of apo AI by immunonephelometry. The light scattering produced by the antigen–antibody complex was measured in a manual laser nephelometer (PDQ; Hyland Div. Travenol Labs, Costa Mesa, CA 92626), according to a previous method (*12*), which we modified by diluting the serum samples 150-fold with sodium phosphate buffer (10 mmol/L, pH 7.4) containing Apovax, 0.1 g/L (*25*).

Results

Optimizing Assay Conditions

The optimal coating concentration and conjugate dilution were determined by checker-board titration according to Voller et al. (*28*). The horizontal rows of a microtiter plate were coated with 110 μL of anti-apo AI antibodies at concentrations between 0.5 and 20 μg/mL. After coating, the procedure was carried out at several conjugate dilutions (1000- to 20 000-fold) in the vertical rows of the microtiter plate. The best combination of sensitivity and low zero-dose response was obtained at a coating concentration of 15 μg of apo AI antibodies per milliliter and 7500-fold diluted antibody–peroxidase (Figure 1).

Kinetics of the Antigen–Antibody Reaction

We pipeted serum standards into wells of four precoated plates and incubated these at 37 °C either for 30 min, or 1, 2, or 4 h in a single step, or for 4 h at 37 °C followed by 16 h at 4 °C. The rest of the assay was as described above. As Figure 2*A* shows, stable values were reached after 2 h at 37 °C.

The kinetics of conjugate binding were similarly examined by incubating the plates with 7500-fold diluted conjugate for 30 min, or 1, 2, or 4 h at 37 °C, or for 4 h at 37 °C followed by an overnight incubation at 4 °C. No true equilibrium was reached, but after 2 h at 37 °C a calibration curve with a good assay range and sensitivity and low blank values was obtained (Figure 2*B*).

Effect of Sample Treatment

The calibration curves obtained with untreated serum and with HDL freshly isolated from plasma by sequential ultracentrifugation were parallel to that of purified apo AI (Figure 3).

Serum and HDL were delipidated according to the procedure of Cham and Knowles (*23*), with a good recovery (92.4 ± 3.8%, n = 10), but serum delipidation did not influence

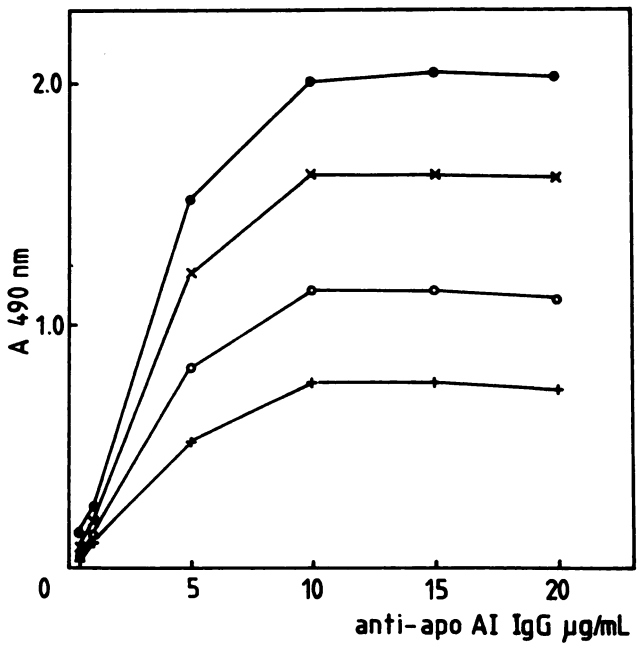

Fig. 1. Influence of the concentration of the anti-apo AI antibody coating on the assay response at various quantities of apo AI: from *bottom* to *top*, 2.5, 5, 10, and 20 ng of apo AI

the apo AI immunoreactivity. This indicates that all apo AI antigenic sites are accessible to titration in native serum.

Heating of the diluted samples, as well as other delipidation procedures, exposure to urea, guanidine hydrochloride or detergents, did not affect the immunological response of apo AI in serum.

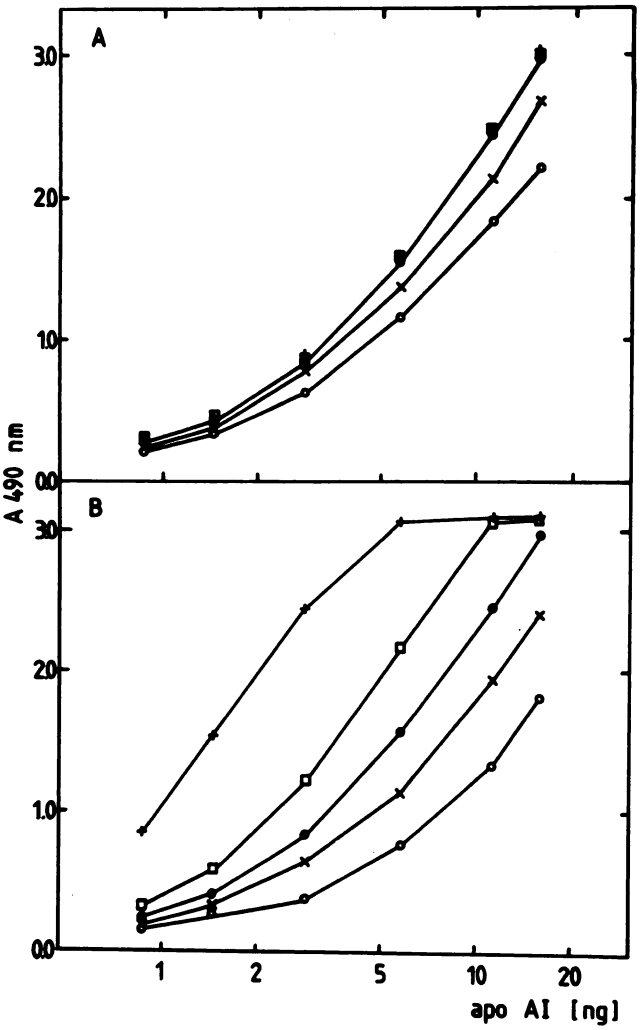

Fig. 2. Effect of incubation conditions on the calibration curves for the first (*A*) and second (*B*) incubation steps

Incubation conditions: 30 min (○), 1 h (X), 2 h (●), 4 h (□) at 37 °C; 4 h at 37 °C + 16 h at 4 °C (+)

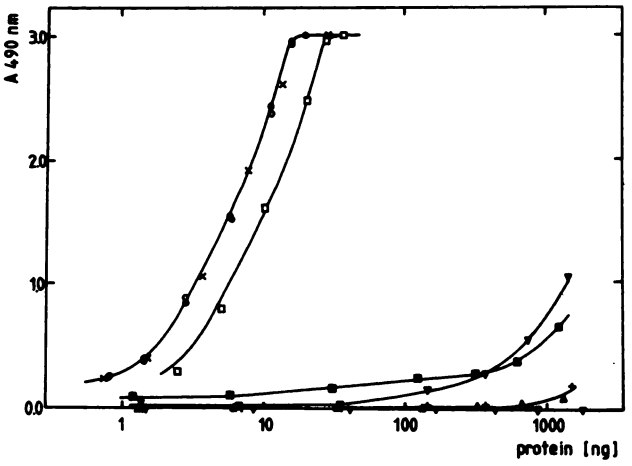

Fig. 3. Calibration curves for purified apo AI (X); human serum before (●) and after delipidation (○); HDL (□); apolipoproteins A-II (■), B (▽), C-III (△), and E (+); and human serum albumin (▼)

Analytical Variables

This enzyme immunoassay can detect as little as 0.5 ng of apo AI, and has a working range of 1 to 14 ng apo AI. For samples diluted 20 000-fold this corresponds to apo AI concentrations in serum between 0.2 and 2.8 g/L. To investigate the specificity of the assay for apo AI, we constructed displacement curves with purified apolipoproteins AI, AII, B, CIII, E, and human albumin. As Figure 3 shows, the extent of cross reactivity between these apoproteins and the anti-apo AI antibodies were 100.00, 0.19, 0.29, 0.05, 0.07, and 0.01%, respectively.

We determined the precision of the assay by assaying six serum samples, with apo AI ranging from 0.62 to 1.96 g/L, 12 times on the same day, and by testing the same six samples on nine separate days. The results, summarized in Table 1, indicate mean intra- and interassay CVs for the whole assay range are 3.9 and 7.8%, respectively.

To determine the accuracy of the assay, we measured apo AI in 100 serum samples, with apo AI concentrations between 0.5 and 2.2 g/L, both by immunonephelometry (x) and by the enzymic assay (y). The regression line between both techniques ($y = 0.913\,x + 0.104$ g/L) has a correlation coefficient (r) of 0.939. To determine whether one of the assays consistently yielded a higher apo AI concentration, we examined the data with a paired t-test; no statistically significant difference was found ($p > 0.05$). We also evaluated the accuracy of the assay by using the admixture technique of Grannis and Miller (*30*): we mixed a serum sample containing 0.49 g of apo AI per liter in various ratios (0.2, 0.4, 0.6, and 0.8) with two serum samples containing 1.46 and 2.23 g of apo AI per liter. The equations of the regression lines between the apo AI concentration and the percentage of each pool were $y = 0.0092\,x + 0.4951$ for the admixture of 0.49 and 1.46 g/L sera and $y = 0.0174\,x + 0.4086$ for the other set of admixtures. Using the technique of equi-disparate specimens, we calculated the percentage differences (±SD) between the observed and expected concentrations as $-2.86 \pm 1.57\%$ and $-6.25 \pm 3.65\%$, respectively. Although Grannis and Miller proposed a ± 3% difference limit for chemical assays (*30*), the values we obtained should be acceptable for an immunological technique.

Discussion

Enzyme immunoassays, most of them competitive assays, have been described for quantifying several human serum apoproteins such as apo AII (*31, 32*), apo B (*33–35*), various apo C's, and apo E (*36*) and for nonhuman primate apo AI (*13*). We have developed a fast and highly sensitive sandwich-type enzyme immunoassay for quantifying human apo AI, in which stable immunoglobulins (IgG) are used both for plate coating and for conjugate preparation. The affinity-purified antibodies were prepared by passage through a human apo AI-affinity column, whereas Koritnik and Rudel (*13*) used an immunosorbent column with glutaraldehyde-fixed normal monkey serum. The use of purified rabbit

Table 1. Precision of the Enzyme Immunoassay for Apo AI

	CV, %	
Apo AI concn, g/L	Intra-assay	Interassay
0.62	3.49	9.58
0.83	4.48	5.84
1.14	3.88	5.76
1.38	3.95	5.53
1.58	3.85	10.94
1.96	3.67	8.88

antibodies to human apo AI in both reaction steps increased the reproducibility of the assay and the comparability with immunonephelometry.

Loss of apoproteins was avoided through the use of bovine serum albumin in the two incubation steps (*26*). The addition of the Tween 20 detergent to the wash buffer, together with a second coating with albumin (*37*), decreased the intra- and interassay variability from 7.5 to 3.9% and from 12.1 to 7.8%, respectively. The precision of this assay is comparable with that of other immunological techniques for human apo AI (*7–12*) and of the enzyme immunoassay for monkey apo AI (*13*).

The working range of 1 to 14 ng of apo AI is similar to that of the most sensitive radioimmunoassays [1 to 20 ng of human apo AI (*7, 8*)]. The stability of the reagents is very good: the enzyme conjugate was kept for more than one year at −20 °C without any loss of activity. Serum apo AI concentrations determined by the enzyme immunoassay compared well with concentrations measured by immunonephelometry. This agreement with an independent method supports our conclusions regarding the method's accuracy.

We tested several methods reported to maximize the exposure of the apo AI antigenic sites in serum (*22*). In contrast to the data from other techniques (*7–12, 22*) and to the results for monkey apo AI (*13*), our assay detected all of apo AI in untreated serum. Neither delipidation, heat treatment, nor addition of denaturants or detergents could increase the response of the apo AI antigenic sites to the antisera we used. Using an apo AI- affinity column to isolate the anti-apo AI antibodies might account for the higher reactivity of serum apo AI in this assay (*26*).

In conclusion, the sandwich enzyme immunoassay for human apo AI offers the advantage of stable reagents and avoids the use of radioactivity. Its sensitivity is similar to that of radioimmunoassay; its selectivity, precision, and accuracy meet the requirements of a good immunological technique; and it provides results within one working day. We therefore propose it as a valuable alternative to radioimmunoassay and expect it to be especially useful for the characterization and quantitation of lipoprotein subfractions. We plan to investigate the heterogeneity of lipoproteins and evaluate their clinical significance with this new assay.

J. Bury was a recipient of an I.W.O.N.L. fellowship.

References

1. Miller GJ. High density lipoproteins and atherosclerosis. *Annu Rev Med* **31**, 97–108 (1980).

2. Gordon T, Castelli WP, Hjortland MC, et al. High density lipoproteins as a protective factor against coronary heart disease: The Framingham Study. *Am J Med* **62**, 707–714 (1977).

3. Miller ME, Førde OM, Thelle DS, et al. The Trømso Heart Study: High-density lipoprotein and coronary heart disease: A prospective case-control study. *Lancet* **i**, 965–968 (1977).

4. Albers JJ, Cheung MC, Hazzard WR. High-density lipoproteins in myocardial infarction survivors. *Metabolism* **27**, 479–485 (1978).

5. Avogaro P, Bittolo Bon G, Cazzolato G, et al. Relationship between apolipoproteins and chemical components of lipoproteins in survivors of myocardial infarction. *Atherosclerosis* **37**, 69–76 (1980).

6. Maciejko JJ, Holmes DR, Kottke BA, et al. Apolipoprotein AI as a marker of angiographically assessed coronary-artery disease. *N Engl J Med* **309**, 385–389 (1983).

7. Schonfeld G, Pfleger B. The structure of human high density lipoproteins and the levels of apo AI in plasma as determined by radioimmunoassay. *J Clin Invest* **54**, 236–246 (1974).

8. Karlin JB, Juhn DJ, Starr JI, et al. Measurements of human high density lipoprotein AI in serum by radioimmunoassay. *J Lipid Res* **17**, 30–37 (1976).

9. Albers J, Wahl P, Cabana V. Quantitation of apolipoprotein AI in human plasma high density lipoprotein. *Metabolism* **25**, 633–644 (1976).

10. Reman FC, Vermond A. The quantitative determination of apolipoprotein AI (apo Lp Gln I) in human serum by radial immunodiffusion assay (RID). *Clin Chim Acta* **87**, 387–394 (1978).

11. Curry MD, Alaupovic P, Suenram CA. Determination of apolipoprotein A and its constitutive AI and AII polypeptides by separate electroimmunoassays. *Clin Chem* **22**, 315–322 (1976).

12. Rosseneu M, Vercaemst R, Vinaimont N, et al. Quantitative determination of human plasma apo AI by laser immunonephelometry. *Clin Chem* **27**, 856–859 (1981).

13. Koritnik DL, Rudel L. Measurement of apolipoprotein A-I concentration in nonhuman primate serum by enzyme-linked immunosorbent assay (ELISA). *J Lipid Res* **24**, 1639–1645 (1983).

14. Van Biervliet JP, Vinaimont N, Caster H, Rosseneu M. A screening procedure for dyslipoproteinemia in newborns. Apoprotein quantitation on dried blood spots. *Clin Chim Acta* **120**, 191–200 (1982).

15. Rosseneu M, Van Biervliet JP, Bury J, Vinaimont N. Isolation and characterization of lipoprotein profiles in newborns by density gradient ultracentrifugation. *Pediatr Res* **17**, 788–794 (1983).

16. Van Tornout P, Caster H, Lievens MJ, et al. "In vitro" interaction of human HDL with human apolipoproteins AII. Synthesis of apolipoprotein A-II-rich HDL. *Biochim Biophys Acta* **663**, 630–636 (1981).

17. Vercaemst R, Rosseneu M, Van Biervliet JP. Separation and quantitation of plasma lipoproteins by high performance liquid chromatography. *J Chromatogr* **276**, 174–181 (1983).

18. Blaton V, Vercaemst R, Rosseneu M, et al. Characterisation of baboon plasma high density lipoproteins and their major apoproteins. *Biochemistry* **16**, 2157–2163 (1977).

19. Brewer HB Jr, Fairwell T, La Rue A, et al. The amino acid sequence of human apo AI, an apolipoprotein isolated from high density lipoproteins. *Biochem Biophys Res Commun* **80**, 623–630 (1978).

20. *Affinity chromatography. Principles and methods.* Pharmacia, Uppsala, Sweden, 1979, 15 pp.

21. Nakane PK. Peroxidase-labeled antibody. A new method of conjugation. *J Histochem Cytochem* **22**, 1084–1091 (1974).

22. Steinberg KK, Cooper GR, Graiser SR, et al. Some considerations of methodology and standardisation of apolipoprotein AI immunoassay. *Clin Chem* **29**, 415–426 (1983).

23. Cham BE, Knowles BR. A solvent system for delipidation of plasma or serum without protein precipitation. *J Lipid Res* **17**, 176–181 (1976).

24. Shepherd J, Packard CJ, Patch JR, et al. Metabolism of apolipoproteins AI and AII and its influence on the high density lipoprotein subfraction distribution in males and females. *Eur J Clin Invest* **8**, 115–120 (1978).

25. Rosseneu M, Vinaimont N, Vercaemst R, et al. Standardisation of immunoassays for the quantitation of plasma apo B protein. *Anal Biochem* **116**, 204–210 (1981).

26. Mao SJT, Kottke BC. Tween 20 increases the immunoreactivity of apo lipoprotein AI in plasma. *Biochim Biophys Acta* **620**, 447–453 (1980).

27. Samaké H, Rajkowski K, Cittanova N. The choice of buffer protein in steroid (enzyme-) immunoassay. *Clin Chim Acta* **130**, 129–135 (1983).

28. Voller A, Bidwell D, Barlett A. The enzyme linked immunosorbent assay. Dynatech, Alexandria, VA, 1979.

29. Kricka L, Carter J, Burt S, et al. Variability in the adsorption properties of microtiter plates used as solid phase supports in enzyme immunoassay. *Clin Chem* **26**, 741–744 (1980).

30. Grannis GF, Miller WG. On the design of clinical chemistry quality-control sera. *Clin Chem* **22**, 500–512 (1976).

31. Stein EA, Pesce AJ. Enzyme linked immunoassays for apolipoproteins: Advantages, problems, and prototype assay. Proc. of the workshop on apolipoprotein quantitation. NIH publ. no. 83-1266, NIH Bethesda, MD, 1983, pp 319–331.

32. Dufaux B, Ilseman K, Assmann G. Competitive enzyme immu-

noassay for apolipoprotein AII. *J Clin Chem Clin Biochem* 21, 34–43 (1983).

33. Fruchart JC, Desreumaux C, Dewailly P, et al. Enzyme immunoassay for human apolipoprotein B, the major protein moiety in low-density and very-low-density lipoproteins. *Clin Chem* 24, 455–459 (1978).

34. Fruchart JC, Fievet C, Moschetto Y, et al. Enzyme immunoassays for human apolipoprotein B. Ref. *31*, pp 314–318.

35. Holmquist L. Quantitation of human serum apolipoprotein B by enzyme immunoassay. *Clin Chim Acta* 121, 327–336 (1982).

36. Holmquist L. Quantitation of human serum very low density apoproteins CI, CII, CIII and apo E by enzyme immunoassay. *J Immunol Methods* 34, 243–251 (1980).

37. Johnstone A, Thorpe R. *Immunoassays. Immunochemistry in Practice,* Blackwell Scientific Publications, Oxford, U.K., 1982, pp 233–255.