Standardization of Apolipoprotein B and A-I Measurements

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Apolipoprotein B, the major protein of low-density lipoprotein, and apolipoprotein A-I, the major protein of high-density lipoprotein, can serve as important predictors of atherosclerotic cardiovascular disease. However, these apolipoprotein measurements have not realized their full potential because of inadequate standardization. Purified apolipoprotein A-I of known absolute mass, in lyophilized form, can serve as primary standard for apolipoprotein A-I, and freshly isolated low-density lipoprotein of narrow density range can serve as primary standard for apolipoprotein B. These primary standards can be used to assign target values to secondary reference material and calibrators by reference laboratories using standardized, validated immunoassay procedures. Lyophilized serum can serve as secondary reference material for apolipoprotein A-I. Freshly frozen serum pools should be used as reference material for apolipoprotein B until more practicable materials that do not exhibit matrix interactions are developed. Implementation of proper standardization procedures can lead to significant improvements in apolipoprotein measurements.

Additional Keyphrases: sample handling · monoclonal antibodies · enzyme-linked immunosorbent assay · reference methods · reference materials

Identification and treatment of hypercholesterolemic subjects have become public-health priorities. In the last few years, significant efforts have been directed at improvements in the accuracy, precision, and standardization measurements of cholesterol and lipoprotein cholesterol measurements. Much less effort has been directed at improvements in the measurement of apolipoproteins, the protein components of the lipoproteins, although researchers, clinical chemists, and clinicians are showing a growing interest in their measurements. Apolipoprotein measurements help in monitoring the progress of dietary and drug intervention, and aid in the diagnosis of dyslipoproteinemic states. Furthermore, there is increasing evidence that apolipoprotein B (apo B), the major protein of low-density lipoprotein (LDL), and apolipoprotein A-I (apo A-I), the major protein of high-density lipoprotein (HDL), can serve as important predictors of atherosclerotic cardiovascular disease (1). However, we have not realized the full potential for using these apolipoprotein measurements because of inadequate standardization and problems in methodology. In turn, our ability to establish adequate reference intervals for clinical use has also been limited. On the basis of our long-term experience with immunoassay development and application, we outline here the considerations and steps needed for proper standardization of the measurements of apo B and apo A-I.

Properties of Apo B and Apo A-I

Human apo B is heterogeneous, but exists primarily in two forms: apo B-100 and apo B-48. Apo B-100, with an approximate molecular mass of 513 kDa, is the major protein of LDL and VLDL. The second form of apo B, apo B-48, about 214 kDa, is homologous with the amino-terminal portion of B-100. Apo A-I, the major protein of HDL, has a molecular mass of about 28 kDa. Both apo B and apo A-I have physical and chemical properties that differ substantially from other serum proteins. In serum or tissue fluids, these apolipoproteins are found in association with lipids and form lipid–protein macromolecular complexes referred to as lipoproteins. The conformation of the purified apolipoprotein in the absence of lipids differs significantly from the conformation of the apolipoprotein in a lipoprotein particle. Furthermore, both apolipoproteins are found in heterogeneous populations of particles that differ not only in size and hydrated density, but also in lipid and apolipoprotein composition. The immunoreactivity or epitope expression of these apolipoproteins may vary with the particular lipoprotein species with which they are associated. Lipids have an important influence on the apolipoprotein conformation and can alter the epitope expression of an apolipoprotein. They can also block or mask an antigenic determinant or epitope, making it unavailable to react with antibodies. To minimize this effect, some investigators have dissociated the lipid from apo A-I before immunoassay. Because the molecular conformation and epitope expression of apolipoproteins can change as a function of environmental conditions and lipoprotein lipid composition, investigators face a particularly challenging problem in obtaining accurate and precise apolipoprotein measurements. These unique properties of apolipoproteins make it more difficult to standardize immunoassays of them than those of most other serum proteins.

Variations in Apolipoprotein Measurements

Apolipoproteins have been measured by various methodological approaches, including radial immunodiffusion, electroimmunoassay, radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunonephelometric assay, and immunoturbidimetric assay. Between-laboratory coefficients of variation are large: 10–15% for apo A-I and nearly 30% for apo B (2). Although some of these
differences can be attributed to the values assigned to the calibrators, some method-dependent differences remain (3, 4).

Variations in measurements of apolipoproteins A-I and B can be attributed to both pre-analytical and analytical variation. Pre-analytical sources of variation can be minimized by standardization of sample collection, handling, and storage (5, 6). Serum or plasma samples can be stored for as long as seven days at 4°C without affecting their content of apo A-I or B. For longer-term storage, most samples can be validly stored at −70°C in securely sealed vials or tubes for at least one year. If ultra-low-temperature freezers are not available, samples should be stored below freezing at a constant temperature. Our experience suggests that nonlipemic samples are stable for up to two years at −70°C, as determined by RIA of apo B (7) and by RIA and radial immunodiffusion assay of apo A-I (3, 8); our experience with long-term storage of hyperlipemic samples is limited. We must emphasize that stability or lack of change of measurable apo B or apo A-I not only is sample-dependent but also depends on the method used to measure these apolipoproteins.

Preparation of Primary Standards

A key step in standardization is the preparation of stable primary standards for apo A-I and apo B, to be used to assign accurate values to the reference materials and secondary standards. Apo A-I is usually isolated from delipidated HDL by chromatography. Care must be taken to ensure that apo A-I does not undergo chemical alteration such as deamidation, oxidation, or proteolytic degradation during isolation and storage. Differences in apo A-I preparation, handling, and storage may alter apo A-I immuno-reactivity. Purified apo A-I can be stored in aqueous solution of low ionic strength at −70 to −80°C or stored lyophilized for at least a year.

The European Bureau of Standards has funded a study on the suitability of purified apo A-I in lyophilized form as a primary standard. The results of this study indicate that purified apo A-I in lyophilized form does not have matrix effects with different methods. The protein values of the different batches of this primary standard, as determined by the method of Lowry et al. (9), were nearly identical to the absolute mass determined by amino acid analysis. A stability study, performed under accelerated degradation conditions, confirmed the stability of the material.

The immunoreactivity or epitope expression of purified apo A-I does not reflect the epitope expression of apo A-I in native lipoprotein particles, as demonstrated with monoclonal antibodies. When purified apo A-I is used as primary standard, the samples in the immunoassay need to be pretreated with delipidating agents to be in the same conformational or physical state as the purified protein. To avoid the use of delipidating agents, particularly in monoclonal antibody-based immunoassays, one can reconstitute the immunoreactivity of the purified apo A-I back to that of the native protein by recombination with phospholipids. Liposomes containing apo A-I have immunological properties similar to native apo A-I-containing particles and can readily be prepared by the cholate dialysis technique (10). The absolute mass of apo A-I in the liposomes can be determined by amino acid analysis or by the method of Lowry et al. (9) and the preparation used as primary standard.

Purified lipid-free apo B undergoes a high degree of self-association and irreversible aggregation and is not suitable as a primary standard. Freshly prepared LDL of the narrow density range of 1030 to 1050 g/L, obtained from donors with normal blood content of cholesterol and triglyceride, is recommended as a primary standard for apo B to ensure a minimal degree of contamination with non-apo B proteins (5). Although the conventional LDL density range of 1019 to 1063 g/L has been used by some investigators as a primary apo B standard, we do not recommend this broader density range of LDL, because it is usually contaminated with apolipoproteins other than apo B. Purity of the LDL apo B should be established by apolipoprotein gel electrophoresis and immunoassay of the potential contaminating proteins such as human serum albumin and apolipoproteins A-I, A-II, C, D, E, and apo(a) from the Lp(a) lipoprotein. Purified LDL at concentrations from 0.5 to 5 g/L can be stored without detriment in a buffered aqueous solution at pH 8.0 at 4°C for no longer than three weeks; that is, the LDL apo B standard is best prepared freshly. Because the determination of the absolute mass of LDL by amino acid analysis is very expensive and time-consuming, the method of choice may be to use a standardized Lowry-based protein procedure (11, 12). The addition of detergent is necessary to minimize the potential interference by lipid and self-association and aggregation of LDL. Determination of the absolute protein mass of eight LDL preparations by amino acid analysis indicated that the absolute mass was 93 ± 3% of the protein value measured by the Lowry procedure (11). Because a universally accepted chromogenicity factor for LDL apo B is lacking, it has been recommended that apo B be expressed in terms of Lowry protein (5). However, the bias between the Lowry-based protein procedure and amino acid analysis should ultimately be taken into account with any standardization efforts. A standard source of bovine serum albumin, such as that obtained from the United States Institute of Standards and Technology (Standard Reference Material No. 927), is recommended in all Lowry-measured values for protein in apolipoproteins, because the Lowry-measured protein can vary with the source of albumin (5).

Preparation of Reference Materials

The use of common reference materials that reflect the patient’s serum in apolipoprotein quantification would further minimize variation among laboratories and between measurement techniques. Currently, lyophilized apolipoprotein reference materials are generally used as secondary reference materials and as calibration material for commercial instruments and kits because of the ease of storage and economy of shipping. The most commonly used reference material in the United States is the International Union of Immunological Societies (IUIS) Matrix apo A-I and B Reference Material (Pool 1989), prepared by the Centers for Disease Control (2). However, this and other lyophilized reference materials have, thus far, not been found to be suitable for use as reference materials or calibrators because of matrix interactions with certain methods. That is to say, the apolipoprotein results for these pools do not reflect results obtained for actual patients' specimens. We recently evaluated five different lyophilized materials, including the IUIS reference pool, for matrix interactions. Apo B was measured in the lyophilized pools and in four frozen serum pools by RIA (7), the candidate reference ELISA (11), two immunonephelometric methods, two immunoturbidimetric methods, and two radial-immu-
nodiffusion methods. Similar apo B values were observed among the different methods for the frozen serum pools, but the lyophilized materials exhibited a large range of variation for the apo B values obtained by the different methods.

To estimate the effect of the lyophilization process on the determination of the apo B values, a detailed study has been conducted in our laboratories by immunochemical methods differing in principle and sensitivity. The study was carried out on nine different serum pools with triglyceride values ranging from 0.79 to 1.35 g/L and a cholesterol content ranging from 1.65 to 2.81 g/L. Fifty aliquots of each pool were immediately frozen at −80 °C and 50 aliquots were lyophilized. The apo B concentration on the nine pools in frozen and lyophilized states was measured by RIA (7), a fixed-time nephelometric assay, and by radial-immunodiffusion (13), calibrated with the same preparation of freshly isolated LDL of narrow density range. A mean decrease of 12% was observed in the apo B values determined by nephelometric assay on the lyophilized material as compared with values obtained for the frozen samples, whereas a mean decrease of 18% and 94%, respectively, was found in the apo B values determined by RIA and radial immunodiffusion. In the same experiment, the apo B concentration in the IUIS material was found to be 0.56 g/L by radial immunodiffusion, and 0.84 g/L by nephelometric assay and RIA. It is clear that lyophilized materials exhibit matrix interactions with different apo B methods, and the use of lyophilized material for standardization or calibration can result in substantial biases with these methods. Therefore, we do not recommend use of lyophilized materials as reference material or calibrators for apo B measurements.

Because reference materials should be selected that reflect the characteristics of actual patients' specimens, use of fresh-frozen serum reference pools has been recommended (5). However, shipping frozen reference material is expensive, so the development of alternative reference materials that mimic patients' serum is strongly encouraged. A liquid reference serum with appropriate additives could meet these requirements, but further evaluation is needed before such a material can be recommended for use as calibrators. The target values assigned to reference serum pools and calibrators should be determined by validated, accurate immunoassay procedures, with the use of standardized purified preparations of apo A-I or LDL for which the absolute mass has been established.

Reference Procedures for Apolipoprotein Measurements

For an apolipoprotein immunoassay to be accurate—that is, for correct measurement of the mass of the analyte—the immunoreactivity per unit mass of the purified apolipoprotein standard must be comparable with the immunoreactivity of the apolipoprotein in the native lipoprotein in serum or plasma. This assumption is difficult to demonstrate. A necessary but insufficient criterion for a valid immunoassay is that the dose–response of the standard should parallel that of the serum specimen.

Because it is not possible to validate every apolipoprotein procedure, reference apolipoprotein procedures are needed for comparisons, so one can assign target values to secondary reference pools and calibrators and set guidelines for acceptability of new methods. A Reference Method must be accurate and precise and have little, if any, susceptibility to interferences. Normally, the accuracy and precision are demonstrated by direct comparison with a Definitive Method.

Development of a definitive reference procedure in which the accuracy of the assay has been unambiguously documented is not feasible for the measurement of apo B and apo A-I in serum. Although independent chemical procedures are available for determining the mass of apo A-I in HDL or that of apo B in LDL and VLDL, it is highly questionable whether these procedures represent a serious approach to validating the accuracy of candidate Reference Methods.

The Antibody and Apoprotein Standardization Program Planning Committee has recommended monoclonal antibody-based ELISA procedures as candidate Reference Methods for apo B and apo A-I (5). Monoclonal antibodies are preferred to polyclonal antibodies in reference assays, because they are chemically uniform and highly specific and can be readily purified and produced in large amounts for distribution to reference laboratories. Furthermore, ELISA procedures require no radioisotopes, have long-term reagent stability, and can be semi-automated.

Before monoclonal antibodies can be used for immunossay, they must be characterized as to identification of heavy-chain type, documentation of antibody specificity, demonstration that the antibody binds all forms of the apolipoprotein with the same affinity (necessary for some assay formats), and verification that the epitope identified by the antibody is expressed on all particles containing the apolipoprotein (Marcovina S, Curtiss LK, Milne R, Albers JJ. Selection and characterization of monoclonal antibodies for measuring plasma levels of apolipoproteins A-I and B. Submitted as an International Federation of Clinical Chemistry document). Monoclonal antibodies used in reference assays should be chosen for their ability to recognize epitopes that are not sensitive to change during sample preparation, handling, and storage, and that are equally expressed in purified apo A-I or LDL and all native apo A-I or apo B-containing particles.

A direct-binding ELISA procedure has been developed as a candidate Reference Method for measuring apo B-100 in human plasma or serum (11). The monoclonal antibody MB47 is a capture antibody and MB24 conjugated to horseradish peroxidase (EC 1.11.1.7) is the detecting antibody (14). These antibodies bind to distinct apo B epitopes expressed by all LDL particles and they can detect apo B in VLDL as well. Because MB47 binds an epitope in the carboxyl-terminal portion of apo B-100, it does not recognize apo B-48 and is therefore specific for apo B-100. In the assay, MB47 bound about 97% of the apo B in all LDL preparations (11).

Apo B-100 was chosen as the analyte because the clinical significance of apo B-48—a constituent of intestine-derived, large, triglyceride-rich chylomicrons and chylomicron remnants—remains to be established. Moreover, the large amount of lipid in chylomicrons is likely to mask or block some of the epitopes and make it difficult to measure apo B-48 accurately by immunossay. Finally, accurate calculation of total apo B mass in serum is not possible, because LDL is not an appropriate primary standard for measuring of particles containing apo B-48.

The direct-binding double-antibody "sandwich" format was chosen rather than the competitive single-antibody format, because allowing LDL to bind to plastic containers may alter the conformation and epitope expression of LDL, and such binding of different preparations of LDL can give rise to greater assay variability than binding a chemically uniform monoclonal antibody to plastic.
If the direct-binding ELISA of apo B is carried out exactly as described (11), apo B-100 can be accurately and precisely measured in most serum specimens. The intra-assay and interassay coefficients of variation average 2.5% and 6.0%, respectively. Small, dense LDL from subjects with familial combined hyperlipidemia and large, buoyant LDL from subjects with familial hypercholesterolemia exhibit binding properties similar to LDL from healthy normolipidemic subjects when tested in the candidate reference ELISA (11). Furthermore, values obtained by this assay for fasting subjects whose triglyceride value is <3.0 g/L are nearly identical to values obtained by a highly standardized RIA procedure for total apo B (7, 11). These results suggest that concentrations of apo B-48 are negligible in fasting subjects with normal triglyceride concentrations. We recommend that this candidate reference ELISA be used to assign apo B values to appropriate reference materials (5). Adopting this apo B reference method and transferring this method to reference laboratories should help decrease the large interlaboratory variability currently seen with apo B measurements and permit the establishment of apo B reference intervals for clinical use. A Reference Method for apo A-I is not yet available.

Reference Values for Apolipoproteins

Recently, there has been a proliferation of commercial kits for apo B and apo A-I. Such methods should be used only if they have been demonstrated to be accurate and precise and if appropriate reference intervals for clinical use have been established. Because the concentrations of apo B and apo A-I can serve as predictors of atherosclerotic cardiovascular disease and provide information not available from measurement of blood lipid and lipoprotein concentrations (1), it is essential that specialists in apolipoprotein measurements reach a general consensus on apolipoprotein standardization and that credible primary and secondary reference materials be made readily available.

Reference intervals for apolipoproteins B and A-I have yet to be established. For most plasma proteins, the 95% confidence limits, or the range into which 95% of an apparently normal population will fall, is considered clinically normal. However, for some serum constituents, what is considered normal may be quite different from what is considered desirable (i.e., what concentrations increase one's risk for disease?). The National Cholesterol Education Program has recommended that a desirable cholesterol concentration is a value that falls below the 75th percentile value, whereas values within the 75th and 90th percentiles increase the risk for coronary heart disease, and those at and above the 90th percentile are at high risk (15).

Because concentrations of apo B are highly correlated with those of total cholesterol and LDL cholesterol, and because an increase of apo B as well as cholesterol predicts the risk of premature coronary disease (1), it is reasonable to postulate the desirability of having apo B concentrations below the 75th percentile and to conclude that individuals with values exceeding the 90th percentile are at increased risk for coronary heart disease.

Similarly, because apo A-I concentrations are highly correlated with those of HDL cholesterol (8), and because HDL cholesterol content below the 10th percentile is considered a risk factor for coronary heart disease (15), we can propose that apo A-I concentrations below the 10th percentile are a risk factor for coronary heart disease.

With proper standardization, all laboratories can adopt these uniform apolipoprotein cutoffs for identifying subjects at increased risk for coronary disease. This will make it necessary for all laboratories to use appropriate methods and calibration procedures and common reference materials to minimize method-specific biases. In addition, population-based reference values are needed for uniform apolipoprotein cutoffs for identifying subjects at high risk for coronary heart disease.

Relatively few population-based values have been reported for apo B and apo A-I. The sex- and age-specific apo B values in white persons reported in five independent studies were quite similar (1). For example, the mean apo B values for white men ages 40-49 from each study ranged from 0.96 to 1.13 g/L (overall mean 1.06 g/L). Age- and sex-specific population-based apo B percentile values have also been estimated from these studies (1) and can serve as a useful guide. The 90th percentile value for apo B in a 45-year-old man is about 1.40 g/L. Apo B concentrations at or above this value can be considered a risk factor for atherosclerotic vascular disease. Examination of apo A-I concentrations by different methods in different populations has yielded similar apo A-I values. However, detailed age- and sex-specific population-based percentile values have not yet been reported.

Conclusion

Only if proper apolipoprotein standardization steps are implemented can all laboratories adopt uniform apolipoprotein cutoffs for identification of subjects at risk for atherosclerotic cardiovascular disease. Apolipoprotein measurements can be improved by implementation of proper standardization procedures as recommended.

In summary, for apo A-I, a stable lyophilized primary standard for which the absolute mass has been determined can be readily distributed. This primary standard can then be used to assign target values to secondary reference material and calibrators by reference laboratories using consensus-standardized, validated immunoassay procedures until a true Reference Method for apo A-I becomes available. The secondary reference material should be as similar as possible to typical patients' specimens with regard to analyte and matrix properties. Thus far, lyophilized serum does not appear to exhibit matrix interactions with most A-I methods and therefore would be practical for distribution.

For apo B, freshly prepared LDL of narrow density range can be prepared by reference laboratories, using the same standardized protocol and Lowry procedure. This LDL primary standard will then be used by the reference laboratories to assign apo B values to secondary reference pools and calibrators through the application of standardized, validated apo B immunoassays along with the candidate reference ELISA apo B procedure. Freshly frozen serum pools should be used as reference material for apo B. Reference materials in liquid-stabilized form that do not exhibit matrix interactions need to be developed. The Northwest Lipid Research Center has made available freshly frozen serum reference pools containing high, medium, and low concentrations of apo B and apo A-I established by a standardized RIA (7) and the candidate Reference Method for apo B (11) and standardized RIA and radial-immunodiffusion procedures for apo A-I (3, 8). It is hoped that manufacturers of instruments and reagents used in apolipoprotein measurement will be able, through the assistance of reference laboratories, to provide accu-
Successful apolipoprotein standardization can be achieved only if the reference laboratories, government, and industry cooperate in their apolipoprotein standardization efforts. We will then be able to measure apolipoproteins accurately, establish appropriate reference values for clinical use, facilitate detection and treatment of dyslipoproteinemias, and identify subjects at risk for atherosclerotic cardiovascular disease.

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References