Preparation of a stable fresh frozen primary lipoprotein[a] (Lp[a]) standard

Gert M. Kostner,[1,*] Anton Ibovnik,[*] Herwig Holzer,[†] and Harald Grillhofer[*]

Institute of Medical Biochemistry,[*] University of Graz, Harrachgasse 21, 8010 Graz, Austria, and Department of Nephrology and Hemodialyses,[†] Auenbruggerplatz 15, 8036 Graz, Austria

Abstract LP[a] is one of the most atherogenic lipoproteins consisting of an LDL-like core particle and a covalently linked glycoprotein of variable size. Due to its structural features, its heterogeneity and instability, there are great difficulties in standardizing quantitative immunochemical Lp[a] assays. One particular problem is the preparation of a pure primary standard, which is sufficiently stable to be used for value assignment of secondary reference material. Here we describe a method to purify Lp[a] to virtual homogeneity. When mixed with glycerol at a ratio of 1:1, the preparation is stable in the deep frozen state for more than 12 months. This latter material gave dose–response curves in several immunochemical assays that were parallel to fresh or frozen sera, freshly prepared Lp[a], and other proposed reference materials. After determination of the protein content by amino acid analysis, it was possible to assign concentrations in molar and mass units to these preparations considering the theoretical molecular weights of the particular apo[a] isoform. Thus we propose to use this procedure for preparation of a "gold standard" for Lp[a] assays.—Kostner, G. M., A. Ibovnik, H. Holzer, and H. Grillhofer. **Preparation of a stable fresh frozen primary lipoprotein[a] (LP[a]) standard.** *J. Lipid Res.* **1999.** 40: **2255–2263.**

Supplementary key words DELFIA • LDL • ELISA • glycerol

Lipoprotein[a] Lp[a] belongs to the class of apoB-containing lipoproteins that is highly associated with atherosclerotic diseases, stroke, and myocardial infarction (1–5). The biological function of Lp[a] is unknown. Lp[a] is composed of one copy of LDL linked via a disulfide bridge with one apo[a] glycoprotein (6). Apo[a] exists in a large number of genetic polymorphic forms that vary in molecular mass including sugars between approximately 230 and 850 kD. The unique features of apo[a] structure explain this polymorphism. The smallest isoform of apo[a] consists of 11 kringle-IVs (K-IV), one copy of K-V, and a pseudo-protease domain. Nine of these K-IVs are unique kringles, which vary slightly in their primary structure. The two others, called repetitive K-IVs, are identical (7). Larger isoforms have a higher number of repetitive K-IVs ranging up to approx. 40 (8). All the structural elements of apo[a] exhibit a great homology with the corresponding structures in plasminogen (Plg). The molecular weight of one K-IV excluding carbohydrates is approx. 12,500.

Because of the high atherogenicity of Lp[a] there is great clinical interest to measure plasma Lp[a] levels accurately. This can be achieved by a variety of methods, the majority of them immunochemical in nature. For routine measurements in the clinical chemical laboratory, nephelometric and turbidimetric assays prevail followed by ELISA, DELFIA, RIA, and rocket electrophoresis.

There are currently commercial Lp[a] kits available from more than 10 companies and most of them have their own in-house prepared reference material. These reference materials are neither officially approved nor do they correspond to each other (9). In order to overcome this problem, the IFCC established a working group (WG) aimed at preparing stable reference material with an accurately assigned value (10). The IFCC-WG evaluated 8 different commercial secondary standards and finally chose one of them as "proposed reference material" (PRM) which best fulfilled the requirements of stability, linearity, and reproducibility (10). This secondary standard consists of pooled lyophilized plasma containing roughly 100 nmol/l of Lp[a] with a major band corresponding to 21 K-IV repeats. Not only has an ultimate value assignment to this secondary standard not been achieved so far, but lyophilized sera proved in the past to be sensitive to distinct assay procedures and thus this PRM may not be generally applicable for calibration of all commercial kits.

In order to overcome these problems, we aimed at preparing a purified primary standard of Lp[a] that should be stable and linear and also should give parallel response curves with fresh plasma or serum using the most common assay procedures. This, in fact, could be achieved by applying a 3-step isolation procedure followed by kryopreservation in 50% glycerol. Our primary standard is stable

Abbreviations: apo[a], designates only the specific apo[a] antigen; apoLp[a], lipid free Lp[a] consisting of apo[a] and apoB-100; BSA, bovine serum albumin; K-IV, kringle-IV; Plg, plasminogen; PRM, proposed reference material; PAGE, polyacrylamide gel electrophoresis.

[1] To whom correspondence should be addressed.

for at least 1 year and as it consists of virtually 100% pure Lp[a], accurate value assignment to this standard is achieved by simple amino acid analysis.

MATERIAL AND METHODS

Preparation of lipoproteins

Plasma or serum was obtained from healthy volunteers on several occasions. In all cases where molar concentrations of pure Lp[a] preparations were calculated, blood from two donors, one with 21 and the other with 23 K-IV repeats exhibiting one single apo[a] isoform only, was used. For serum, 100–300 ml blood was allowed to clot for 30 min at room temperature and immediately centrifuged. Most Lp[a] preparations, however, particularly those for stability testing, were performed with 300–500 ml citrate plasma obtained after plasmapheresis. In some preliminary experiments heparin was used as an anticoagulant. In two cases, the heparin precipitate of VLDL, LDL and Lp[a] obtained from patients who underwent extracorporal LDL-elimination (HELP-system) was used as starting material for Lp[a] purification. The precipitate was solubilized in 0.15 mol/L of NaCl and the addition of drop by drop of 5% Na_2CO_3 until a homogenous solution was obtained. This latter material was then treated like serum or plasma for Lp[a] purification.

EDTA, BHT, and thiomersal at final concentrations of 0.1%, 0.005%, and 0.05%, respectively in addition to 1 mmol/L of phenylmethyl sulfonyl fluoride (PMSF) and *p*-Br-phenacyl bromide were immediately added and plasma or serum was subjected to lipoprotein fractionation. Lp[a] was prepared essentially as described in detail previously (11). In a typical experiment, the density of 500 ml plasma was adjusted to 1.060 g/ml by adding solid NaCl and the mixture was subjected to ultracentrifugation for 24 h at 48,000 rpm using a Beckman Optima XL 70 equipped with a 50.2 Ti rotor. For the purification of LDL, floating lipoproteins were dialyzed against a NaCl solution of d 1.030 g/ml and subjected to ultracentrifugation (45,000 rpm, 24 h). The bottom fraction was finally subjected to density gradient ultracentrifugation as described (12) and LDL was recovered as a narrow band in the middle of the centrifuge tube.

For isolation of Lp[a], the d 1.060 g/ml bottom fraction was adjusted to d 1.125 g/ml with solid NaBr and subjected to ultracentrifugation (48,000 rpm, 24 h). Floating lipoproteins consisted of dense LDL, HDL_2, and Lp[a]. The fraction was concentrated by pressure dialysis to a volume of 10 ml; proline was added to a final concentration of 0.1 mol/L followed by chromatography over Biogel A-15m (200–400 mesh) in a 0.05 m Tris-HCl, 0.15 m NaCl buffer, pH 8.2, containing 0.1% EDTA and NaN_3, and 0.1 mol/L of proline. The fractions containing pure Lp[a] (Fig.1) were pooled and concentrated by pressure dialysis against the Tris-buffer mentioned above but without Pro, to a final concentration of 5–10 mg/ml.

All runs in the ultracentrifuge were performed at 15°C and all further preparations at room temperature (approx. 22°C). Care was taken to perform all purification procedures in the quickest possible time, i.e., within 1 week.

Stabilization and storage of lipoproteins

Although the major goal of this study was the preparation of a stable Lp[a] reference material, all experiments were also done in parallel with LDL. For simplicity we will refer in this manuscript only to those experiments that are relevant for Lp[a]. To obtain a rough idea of lipoprotein concentrations in each step, the cholesterol content was measured and multiplied by the appropriate factor.

Part of the freshly concentrated Lp[a] was immediately mixed at an exact ratio of 1:1 (v/v) with glycerol and stored frozen at −20°C or −70°C. The remaining portion was used for exact chemical characterization and for further standardization purposes.

Chemical characterization of Lp[a]

The exact analysis of Lp[a] protein for each individual preparation was done by amino acid analysis using cation exchange column chromatography. Lp[a] was mixed with the internal standard Nor-Leu and hydrolyzed for 24 h at 110°C in 6 N HCl followed by chromatography on the automated amino acid analyzer Biochrom 20 from Pharmacia according to the manual provided by the manufacturer. The automated amino acid analysis works with a day to day C.V. of <1%. Only the stable amino acids Ala, Asp, Glu, Lys, Leu, and Phe were quantified. In order to calculate molar concentrations of Lp[a], the individual molecular weights and apo[a] amino acid composition tables for different apo[a] isoforms published by Marcovina et al. (13) were used. As will be discussed later, the concentration of Lp[a] in the primary standard is given in molar concentration of apoLp[a]. For this it is not necessary to measure Lp[a] lipids or the carbohydrates of the glycoprotein. In order to get an estimation of the Lp[a] concentration in mass units, the lipid contents (free and esterified cholesterol, phospholipids, and triglycerides) were measured enzymatically using commercial kits from Boehringer-LaRoche. A rough estimate of the protein content before amino acid analysis was done also by the procedure of Lowry et al. (14). As apo[a] is a glycoprotein with variable carbohydrate content, we assumed that 15% (w/w) of apoLp[a] consists of sugars. All assays were performed in duplicate or triplicate.

Electrophoretic techniques

Purified Lp[a] preparations were analyzed by polyacrylamide gel electrophoresis (PAGE) in 3.75% or 5–20% SDS gels with or without Western blotting, or in 1% agarose gels as detailed previously (8, 15–17). Staining was performed for lipids with Sudan black and for proteins with Coomassie blue. The determination of the apo[a] isoforms was performed as described (15).

Immuno quantitation of Lp[a]

Most of the immunochemical assays were performed by DELFIA or ELISA as described in detail in previous publications (16, 17). In short, polyclonal antibodies against apo[a] were prepared in rabbits and sheep in our laboratory. The immunoglobulin fraction of these antisera was affinity-purified by passing over plasminogen (Plg) and apoB-containing adsorbers followed by affinity chromatography on apo[a] linked to Biogel 6CL. These antibodies reacted by immunoblotting only with Lp[a], apo[a], or apo[a] fragments, but not with Plg, LDL, or any other plasma protein or lipoprotein.

The antibodies against apo[a] or apoB were labeled with europium (Eu) or horseradish peroxidase and stored at −20°C in 50% glycerol. Unlabeled affinity-purified anti-apo[a] was used for coating of plates and for detection of the labeled anti-apo[a] or anti-apoB. As there was little difference whether coating and detection antibodies were from the same species, we always used these two antibodies from one species only (sheep or rabbit). When apo[a] was used for detection, we refer to this assay as a:a DELFIA or a:a ELISA and when anti-apoB was used for detection we refer to a:B DELFIA or a:B ELISA. The DELFIA or ELISA assays were usually performed in triplicate or quadruplicate as described (16, 17). DELFIA worked with a within-assay precision of ±1.2% and ELISA with 3.5%.

In some cases, Laurell electrophoresis, latex enhanced immunonephelometry (LINA) and immunoturbidimetry using a COBAS Mira were performed as described (18).

Determination of precision, linearity and parallelism

These experiments were performed essentially as described (10). For precision studies, the purified Lp[a] preparations were treated as analyte and their concentrations were determined in

duplicate on 5 days within 2 weeks. On each day an individual standard curve was constructed using our usual in-house reference material.

Linearity was tested by linear regression at the dilutions of 20, 40, 60, 80, and 100% in triplicate. At each stage of dilution the term "mean of observed values divided by expected values × 100" was plotted against the dilution in percent. Sample linearity was acceptable if the regression slope was between 0.90 and 1.10.

Parallelism studies were performed exactly as described previously with the primary reference material (purified Lp[a] in fresh and frozen form), with fresh frozen plasma from the corresponding blood donors plus or minus 15% sucrose, fresh plasma, or serum samples from various donors, in addition to lyophilized PRM-1 and PRM-2 (10). In order to avoid biases caused by marked differences in the apo[a] isoform, all plasma samples were taken from donors containing one predominant apo[a] band with 22 ± 2 K-IVs. The PRMs were the "proposed reference materials" provided by Baxter-Immuno, Vienna (PRM-1) and Dade-Behring, Marburg (PRM-2) which are characterized in detail in the report of the IFCC-Lp[a] working group. PRM-2 was recently selected for further characterization and is due to be certified as secondary reference material by IFCC, WHO, and/or CDC (10).

As the immunoassays used in this study were designed in a way to give reproducible response curves in the range of 5–100 mg/dl of Lp[a] (approx. 15–300 nmol/L), using the in-house reference material of previous studies (16, 17), all purified Lp[a] samples were pre-diluted with "DELFIA-buffer" immediately before use to a concentration of roughly 300 nmol/L. The exact content of these solutions then was determined by amino acid analysis.

Stability testing

The primary Lp[a] standard was kept frozen at −70°C, −20°C, 4°C, room temperature, or 37°C in the presence or absence of stabilizers (see Results) for prolonged periods of time and then investigated by PAGE and agarose gel electrophoresis and by DELFIA and ELISA.

RESULTS

Characterization of purified Lp[a] and pre-evaluation of different stabilizers

All experiments described here in detail were performed with plasma from two donors, one homozygous for 21 K-IV repeats and the other for 23 K-IV repeats. The reasons for choosing these samples were *a*) the homozygosity, *b*) the sufficient plasma concentration for Lp[a], and *c*) the number of K-IV repeats which corresponds to the average number found in individuals of most populations.

The density fraction 1.050–1.125 g/ml of the fresh plasma of these two donors obtained by ultracentrifugation was chromatographed over Biogel A-15m in the presence of 0.1 mol/L of Pro. **Figure 1** shows a characteristic elution profile. As pointed out previously, Pro in the elution buffer prevents the aggregation of Lp[a] with LDL and yields a significantly better resolution of the two fractions (11). In the absence of Pro, the first two peaks are not well separated and almost all Lp[a] fractions are contaminated with LDL (**Fig. 2A**, lane 4). During the course of this study, five purified Lp[a] fractions each from the two individual donors were prepared and the chemical compositions of these preparations were determined

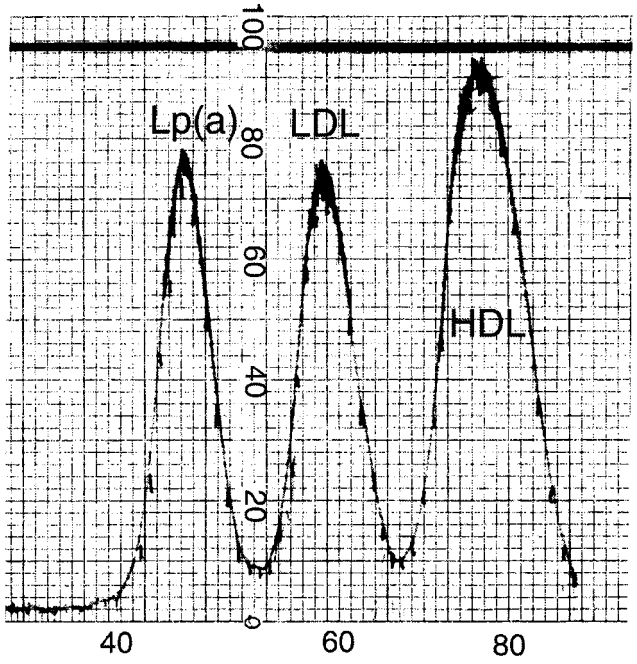

Fig. 1. Purification of Lp[a]. The d 1.050–1.125 g/ml ultracentrifugation fraction obtained from 500 ml plasma of one of the two donors in 10 ml TRIS buffer containing 0.1 mol/L of Pro was chromatographed over a column, 100 × 2.5 cm, containing Biogel A-15m. For experimental details see Material and Methods. The x-axis indicates the fraction number, 3.5 ml each, and the y-axis indicates the optical density, whole range = Extinction 1.

(**Table 1**). The compositions of the two preparations were very similar and varied mostly within the range of the experimental error of the assays. Lp[a] had a calculated molecular weight of approximately 3.2 million and the molecular weight of the core LDL particles (=Lpa−) were close to 2.9 million.

The Lp[a] preparations were virtually homogenous as judged by SDS-PAGE and agarose gel electrophoresis. To fulfill the criteria for a primary Lp[a] standard, the absence of free LDL is of particular importance. This was verified by agarose gel electrophoresis and by 3.75% SDS-PAGE in the absence of reducing agents (Fig. 2, A and B).

In previous experiments, heparin plasma or the heparin precipitate obtained from a patient treated by "HELP" was used as starting material for Lp[a] purification. In these cases we noticed *i*) that the Lp[a] peak was hardly separated from LDL by Biogel A-15m column chromatography; *ii*) the Lp[a]-containing fractions of the Biogel A-15m column eluate were contaminated with LDL and other apolipoproteins which could not be removed even after re-chromatography; and *iii*) the Lp[a]-containing fractions from heparin plasma were unstable and easily precipitated upon concentration. All these results are not shown. These facts were proven at several occasions and thus we decided to strictly avoid heparin in any subsequent experiment.

When such pure Lp[a] preparations were used as primary standards, it was noticed that they had a shelf life of only a few days at room temperature. As Lp[a], on the

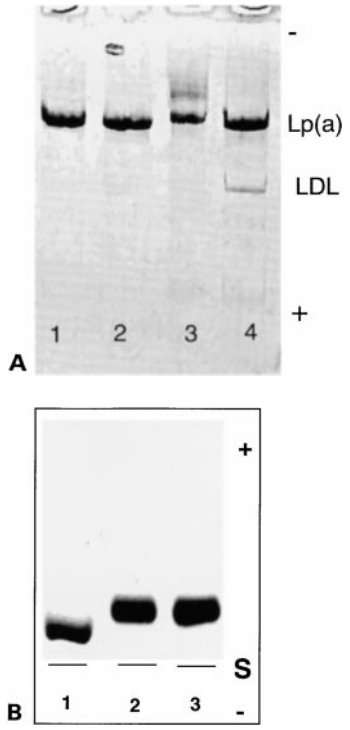

Fig. 2. A: SDS polyacrylamide gel electrophoresis in 3.75% gels in the absence of reducing agents. 1: Freshly prepared Lp[a]; 2: Lp[a] stored for 6 months at −20°C in 50% glycerol. 3: Lp[a] stored for 1 week at −20°C without preservatives; 4: Lp[a] prepared from Biogel A-15m in the absence of Pro. Approx. 20 μg of protein was applied per lane; staining: Coomassie blue. B: Agarose gel electrophoresis of 1: LDL; 2: freshly prepared Lp[a]; 3: Lp[a] stored for 6 months at −20°C. S = start. Approx. 50 μg of lipoproteins was applied. Staining: Coomassie blue.

other hand, belongs to the cryoyprecipitable proteins, pure Lp[a] could not be stored below 10°C without precipitation. During storage at room temperature of pure Lp[a], the apparent concentration when measured by DELFIA or ELISA using the lyophilized PRMs as calibrators decreased steadily and significantly; after 1 week 30–50% lower apparent concentrations for the purified Lp[a] were obtained.

In order to overcome this problem, preservatives, which proved useful in the past, were added to pure Lp[a] and the shelf life at room temperature and at −20°C was followed. The following preservatives were investigated: sucrose, mannitol, bovine serum albumin (BSA), glycerol, ethylene glycol, or mixtures thereof at various concentrations. After long cumbersome experiments, we concluded that only 4% BSA and 50% glycerol performed adequately well. Detailed results of these experiments, are not presented here for the sake of clarity of the manuscript. BSA was finally sorted out as our intention was that Lp[a] preparations free of other proteins are preferred as primary reference material. All subsequent experiments were thus only carried out with 50% glycerol.

Linearity of purified Lp[a] ± 50% glycerol in DELFIA and ELISA

Before proceeding further we ascertained that purified Lp[a] preparations would yield linear response curves in

TABLE 1. Chemical composition of Lp[a]

Variable	Lp[a]-1 n = 21	Lp[a]-2 n = 23
	%	
Free cholesterol	8.4 ± 0.3	8.3 ± 0.4
Cholesteryl esters	38.5 ± 0.7	36.4 ± 1.1
Triglycerides	3.7 ± 0.5	6.5 ± 0.7
Phospholipids	19.6 ± 0.7	18.9 ± 0.6
Carbohydrates	3.9 ± 0.1	3.9 ± 0.1
Protein	25.8 ± 0.9	26.0 ± 1.0
	mol wt	
Lowry factor[a]		
Apo[a][b]	299,358.1	324,339.5
ApoB[b]	512,889.3	512,889.3
Lp[a] lipoprotein[c]	3,148,000	3,220,000
Lp[a]-(parent LDL)[d]	2,849,000	2,896,000

Lp[a] was prepared from two individual donors on 5 occasions within 3 years. The protein content was determined by amino acid analysis; lipids were measured enzymatically. The carbohydrate content was calculated on the assumption that 15% w/w of the protein moiety (apo[a] + apoB) consists of sugars (19–21), n = total number of kringle-IV repeats.

[a] By Lowry et al. (14), an approximate 15% higher value for the protein content was obtained; Lowry protein, however, was not used for any calculations.

[b] Values from Marcovin et al. (13).

[c] Calculated from protein in %/100 × mol wt (apo[a] + apoB).

[d] Calculated from mol wt Lp[a] lipoprotein − mol wt apo[a].

our assays using conventional secondary reference material. **Figure 3** shows some representative results. The linearities of freshly prepared Lp[a], Lp[a] in 50% glycerol stored at room temperature for 24 h, or stored at −20°C or at −70°C for 2 weeks, in the a:a and the a:B DELFIA were excellent with slopes between 0.96 and 1.07. Also with the a:a or a:B ELISA we obtained satisfactory linearities (slopes 0.92–1.09) yet the triplicate values had more variations than that of DELFIA (results not shown).

Stability of Lp[a] in 50% glycerol

Because a valuable primary reference material should be stable for months, Lp[a] was prepared from the two homozygous donors, mixed 1:1 with glycerol, and stored at −20°C and −70°C. It should be emphasized here that at −20°C a 50% glycerol solution is still liquid, but at −70°C it is not. The frozen fractions were then investigated before freezing, 24 h after freezing, and in timely intervals of weeks and months. There was virtually no change in the electrophoretic mobility or turbidity during a storage period of 6 months (Fig. 2). Storage in the absence of glycerol, on the other hand, led to irreversible aggregation and changes in mobility already after 1 week (Fig. 2A, lane 3). Even after 12 months there was virtually no change in the electrophoretic pattern of Lp[a] (figure not shown).

We then measured in timely intervals the apparent Lp[a] concentration of preparations stored at −20°C or −70°C in 50% glycerol using our fresh frozen plasma (in-house reference material) as calibrator. There was no change over a period of 15 months in the apparent concentration using a:a or a:B DELFIA (**Table 2**). From these results we concluded that our Lp[a] preparation might be stable for at least 15 months and potentially useful as a primary standard.

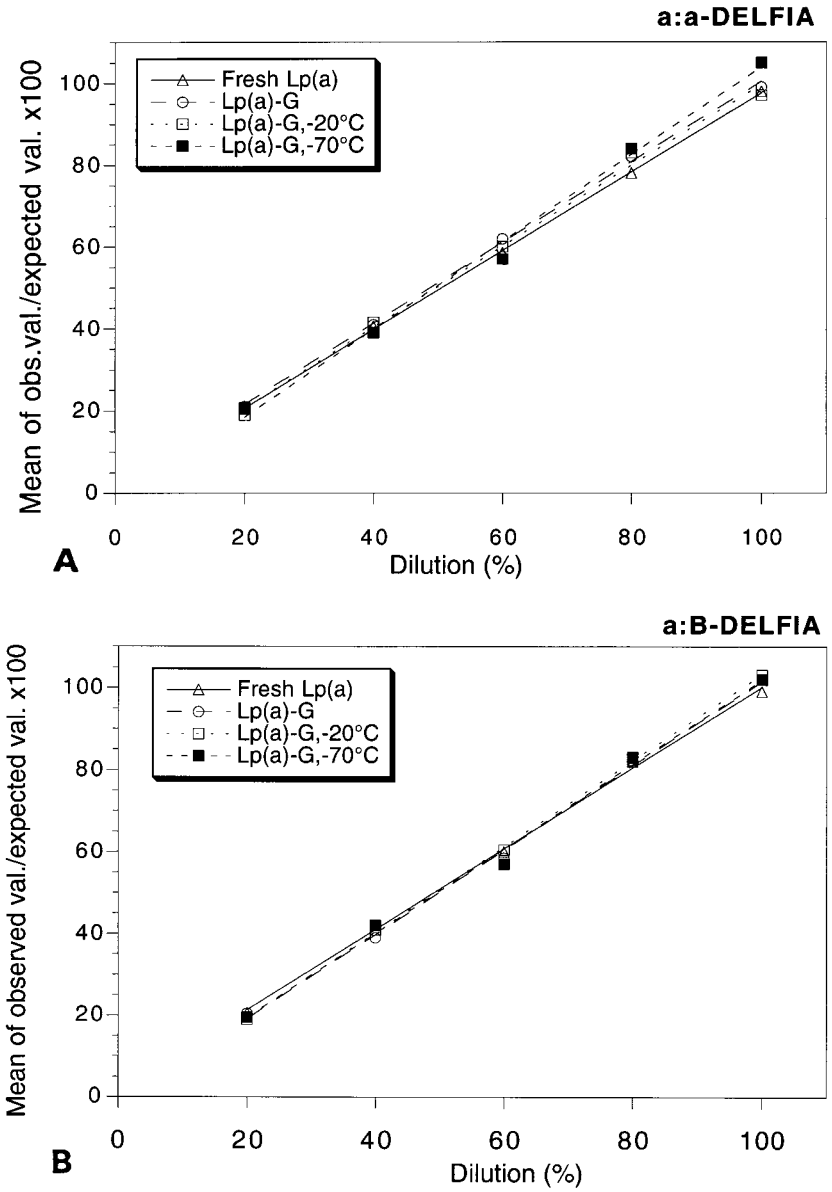

Fig. 3. Linearity of the DELFIA assay using in-house reference material (fresh frozen plasma). The following samples were measured as analyte: freshly prepared Lp[a]; fresh Lp[a] mixed 1:1 with glycerol (Lp[a]-G); Lp[a]-G stored at −20°C or −70°C for 2 weeks. The means of the observed values divided by the expected value × 100 were plotted against the dilution of the samples. Within each assay, measurements were made in triplicate and mean values were plotted. A: a:a-DELFIA. Linear regression lines: fresh Lp[a] : y = 1.35× + 0.963; Lp[a]-G : y = 1.90 + 0.997×; Lp[a]-G, −20°C: y = 0.85 + 0.993×; Lp[a]-G, −70°C: y = 2.94 + 1.05×. B: a: B-DELFIA. Linear regression lines: Fresh Lp[a] : y = 1.60× + 0.985; Lp[a]-G: y = 1.40 + 0.998×; Lp[a]-G, −20°C: y = 1.65 + 0.994×; Lp[a]-G, −70°C: y = 1.10 + 0.990×.

Parallelism

To test for parallelism properties, in-house reference material, fresh plasma, and Lp[a] preparations stored for 6 months in 50% glycerol at −20°C or −70°C were compared. **Figure 4** shows characteristic experiments using a:a or a:B DELFIA. All of the curves were parallel based on the ANOVA test (probability < 0.02). In a similar way, parallelism was tested by the a:a ELISA and the results obtained were comparable ($P < 0.02$, data not shown). We also tested for parallelism of Lp[a] stored in 50% glycerol at −20°C or −70°C with fresh frozen plasma, PRM-1, or PRM-2 and freshly drawn plasma in timely intervals of up to 15 months and found that all the curves were parallel based on the ANOVA test. From these data we concluded that Lp[a] stored for up to 15 months at −20°C or −70°C in 50% glycerol might be used as primary reference material in DELFIA and ELISA.

Next we tested the suitability of the purified Lp[a] stored for 6 months at −20°C to be used as primary standard in other immunoassays. These included Laurell electrophoresis, LINA, and turbidimetry. In all cases, dose–response curves met the criteria of parallelism (contrasts

TABLE 2. Apparent Lp[a] concentrations of purified Lp[a] stored deep-frozen in 50% glycerol up to 15 months

Storage in Months	a:a DELFIA		a:B DELFIA	
	−20°C	−70°C	−20°C	−70°C
	nmol/L		*nmol/L*	
0[a]	327.4	327.4	327.4	327.4
3	335.2	341.2	330.2	335.5
6	315.7	312.5	321.1	315.5
9	339.3	333.6	328.8	333.7
12	319.6	315.5	320.0	316.6
15	316.6	317.7	317.1	319.3
Mean ± SD	325.6 ± 9.1	324.7 ± 10.3	324.1 ± 4.9	324.7 ± 8.0

Freshly prepared Lp[a] was mixed 1:1 with glycerol and stored in vials of 250 μl each at −20°C or at −70°C. The concentration of these preparations was measured by the a:a and the a:B DELFIA using fresh-frozen plasma (in-house reference material) as calibrator. The values are means of triplicate analysis and are given in nmoles/L.

[a] Reference Lp[a] in 50% glycerol before freezing.

giving a probability of <0.02 by one-way ANOVA were regarded as nonparallel).

Determination of the precision in DELFIA and ELISA

Table 3 exhibits the precision data obtained in five separate runs performed in duplicate. Purified Lp[a] stored for 6 months at −20°C was measured as analyte at 100% and 50% dilutions. In DELFIA the C.V. in all cases was <5% and in ELISA <6/%. It should be noted that the within-run precision values for DELFIA and ELISA were lower (<2% and <5%, respectively).

DISCUSSION

There is no doubt that the standardization of Lp[a] measurements in the different routine laboratories over the world is subject to improvement. There is no approved calibrator available yet and therefore it is impossible to compare Lp[a] levels obtained by different methods, with different antibodies and different reference material. This fact is clearly demonstrated in the report of the evaluation of the analytical performance of lipoprotein[a] assays of the IFCC–WG on Lp[a] standardization (10). Eight different proposed secondary reference samples were measured in 33 participating laboratories using eight different types of assays. The mean values obtained for these PRMs varied by a factor of >2 with a large standard deviation. One major reason for this great variation was due to the fact that no common calibrator was used in individual laboratories. After harmonization of the data on the basis of fresh frozen plasma included in all assays, it was possible to obtain significantly better accordance. In another study using ELISA, LINA, and INA and applying identical reference materials in three different commercial immunoassays, it has been demonstrated that there is a tremendous reduction in the variability of data (9). Thus a major goal for still ongoing work of the IFS working group and of other research projects in this field is to prepare a secondary standard that is stable for years and may be used as calibrator for as many different assay systems as possible. To assign an accurate value to such approved secondary standards, a "gold" primary standard is required.

In that respect it must be emphasized that there are several problems inherent to the purification of Lp[a]. Pure Lp[a] not only self-aggregates, particularly in the cold, but it also forms complexes with LDL and with many other proteins. In fact, we intended to purify larger quantities of Lp[a] from plasma of patients undergoing LDL-apheresis. No matter what procedure for the extracorporal LDL elimination was applied, including the HELP system, the Kaneka column, cascade filtration, or the DALI system, the Lp[a] in all cases was packed so tightly together with LDL and other serum proteins that an acceptable purity of Lp[a] could not be achieved. Heparin was particularly disadvantageous in this respect in that it appeared to form complexes with lipoproteins that dissociated only partially during purification.

Another severe problem is the instability of pure Lp[a]. Cooling down below 10°C yielded cryoprecipitation. Freezing below 0°C causes aggregation and precipitation. We therefore stored pure Lp[a] at room temperature under nitrogen and in the dark in the presence of antioxidants and protease inhibitors. Irrespective of these preservatives it was noticed that virtually 100% of pure Lp[a] preparations undergo serious changes in their antigenic structure at room temperature which are already measurable after 24 h. These changes were easily detectable by DELFIA and ELISA techniques as the apparent concentration of Lp[a] solutions stored at room temperature dropped. In order to overcome this problem, we searched for kryo-preservatives; sucrose and mannitol, which proved useful in the past for preserving lipoproteins in whole plasma, had little effect. Ethylene glycol was less effective than glycerol. A 4% albumin solution (BSA) was able to prevent Lp[a] aggregation and changes in the antigenic structure during freezing. BSA, however, was not followed further as glycerol performed equally well.

Glycerol at concentrations between 35–50% when added to LDL or Lp[a] not only prevented precipitation and aggregation by freezing but also preserved the antigenic properties of these lipoproteins as tested by several immunochemical assays. For practical reasons we finally decided to adopt 50% glycerol for all subsequent investigations.

Most of the experiments were performed with pure Lp[a] stored at −20°C, yet there are also some data with material stored at −70°C. At −70°C the sample freezes (gets solid) but at −20°C it does not. The pure Lp[a] stored for 6 months gave parallel dose–response curves as compared to freshly prepared Lp[a], fresh plasma, lyophilized secondary reference material, and Lp[a] stored at −70°C in all immunochemical assays we applied. Even at 12 and 15 months of storage there was no indication of degradation or change in the antigenic behavior. This was concluded from electrophoretic patterns on one hand and from results of DELFIA on the other hand. The reproducibility of data obtained over 1 year by DELFIA was excellent but ELISA also performed quite well. As Lp[a] is linked to LDL by one disulfide bridge, any breakdown or

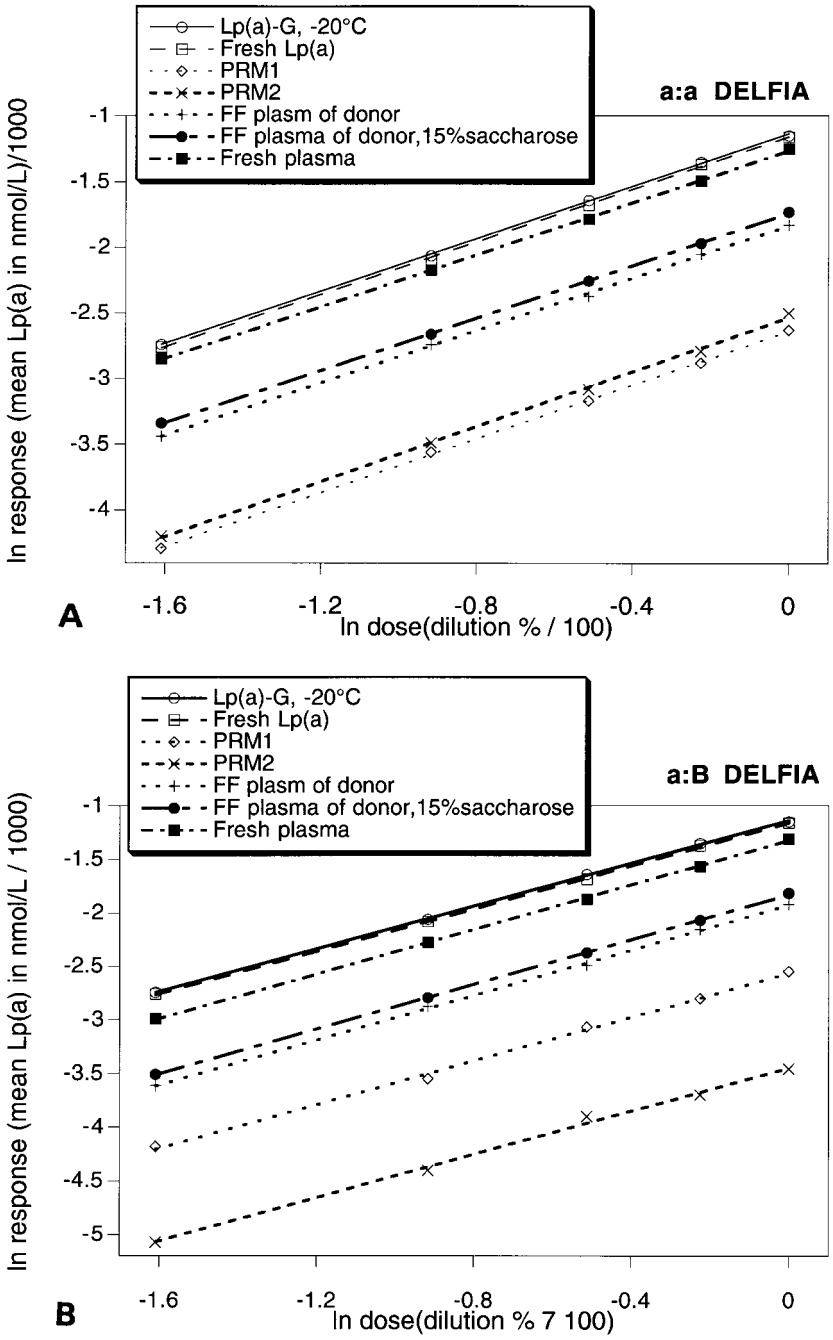

Fig. 4. Parallelism of response curves in DELFIA of various materials: Lp[a]-G, −20°C = purified Lp[a] mixed 1:1 with glycerol and stored at −20°C for 6 months. PRM-1 and PRM-2, proposed reference material from the IFCC working group, described in detail in ref. 10; FF, fresh frozen plasma of the donor for the particular Lp[a] preparation; FF plasma of the donor, 15% sucrose, the same as before, but frozen in the presence of 15% sucrose. The latter two samples were stored for approx. 2 weeks at −20°C. The response, i.e., mean Lp[a] in nmol/l divided by 1000 was plotted against in dilution in % divided by 100. All samples were measured in triplicate. A: a:a.DELFIA; linear regression lines: Lp[a]-G, −20°C: $y = -1.139 + 0.990\times$; fresh Lp[a]: $y = -1.162 + 0.991\times$; PRM1: $y = -2.639 + 1.022\times$; PRM2: $y = -2.533 + 1.041\times$; FF plasma of donor: $y = -1.835 + 0.997\times$; FF plasma of donor, 15% sucrose: $y = -1.741 + 0.982\times$; fresh plasma: $y = -1.2648 + 0.988\times$. B: a:B-DELFIA; linear regression lines: Lp[a]-G, −20°C: $y = -1.137 + 0.993\times$; fresh Lp[a]: $y = -1.163 + 0.995\times$; PRM1: $y = -2.566 + 1.018\times$; PRM2: $y = -3.4482 + 1.030\times$; FF plasma of donor: $y = -1.927 + 1.017\times$; FF plasma of donor, 15% sucrose: $y = -1.8284 + 1.016\times$; fresh plasma: $y = -1.3283 + 0.993\times$.

TABLE 3. Precision of the DELFIA and ELISA assays using purified Lp[a] stored for 6 months at −20°C in 50% glycerol

	DELFIA		ELISA	
Dilution	a:a	a:B	a:a	a:B
	%		%	
50%	4.1	2.8	5.1	4.7
100%	3.7	3.6	5.9	5.5

Standard curves for each assay were obtained from in-house reference material and frozen Lp[a] was measured as analyte. The C.V.s were calculated from assays performed in duplicates at 5 days within 2 weeks.

fragmentation of the Lp[a] complex would have resulted in diverging results using either the a:a DELFIA or the a:B DELFIA; this was, in fact, not the case (Table 2).

Another point that we want to emphasize is that LDL stored under the same conditions (50% glycerol) was equally stable. In the case of LDL we have not done any control experiments using none or other kryopreservatives so we cannot answer the question whether simpler ways exist for LDL preservation. We can, however, stress the fact that pure LDL stored in 50% glycerol for up to 15 months behaves immunochemically identical to freshly prepared LDL.

Because our primary standard was virtually pure and the size of the apo[a] isoform was known, we could measure its molar concentration by determining the amino acid composition assuming the following: (adopted from refs. 6, 13): *1*) Lp[a] contains only one molecule of apo[a] and one molecule of apoB; *2*) the molecular weight of apoB is 512,889.3; *3*) the molecular weight of the kringle-IV Type-2 free apo[a] is 149,487.7 and the molecular weight of one K-IV Type-2 is 12,508.8. As outlined in Table 1, the lipid composition of Lp[a] from different healthy donors appears to be rather stable which is also reflected by the calculated molecular weight of the core LDL particle (2.849 and 2.896 million) and a molecular weight of roughly 3.2 million for Lp[a] with about 22 K-IV repeats. This enables one to convert molar concentrations of standards or analyte into mass values, provided the isoform of apo[a] is known. For calculating exact mass values, an exact knowledge of the apo[a] carbohydrate content is required, which by itself depends on the apo[a] isoform; literature data vary between 240 and 540 μg/mg protein (19–21). Assuming that apoB has a carbohydrate content of roughly 5%, we adopted a mean value for the carbohydrate content in apo-Lp[a] of 15% of the protein mass obtained from amino acid analysis. This is without doubt a simplification, yet a reasonable figure based on literature data.

In summary, we propose here a method for preparing a "gold standard" of Lp[a] which is essentially 100% pure and stable for >1 year in the deep freezer. This Lp[a] seems to be well suited for assigning values to secondary reference material using a variety of immunochemical methods. Of course this material may also be kept in a frozen state and may be used directly as a primary reference material. There is no doubt that the use of a common calibrator such as our primary reference material will greatly improve the harmonization of data obtained in various laboratories over the world. Yet for standardization of Lp[a] assays, the use of appropriate antibodies remains critical.

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