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Nonimmunochemical quantitation of mammalian apolipoprotein A-I in whole serum or plasma by nonreducing gel electrophoresis

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Summary A rapid and convenient method for the quantitation of mammalian apoA-I has been developed. The method involves nonreducing sodium dodecyl sulfate gel electrophoresis and Coomassie blue staining, and takes advantage of the relative abundance of apoA-I in whole serum or plasma. ApoA-I was sufficiently resolved to allow quantitation by laser densitometry or spectrophotometry. The assay was linear from 0.25 to 4.0 μg of apoA-I. Analytic recovery was 98%. Within-assay variability was 3.1% and between-asaay variability was 7.5%. A high degree of positive correlative (r = 0.98) was observed with a human apoA-I radioimmunoassay. For several species investigated, the apoA-I values obtained correlated strongly and positively with high density lipoprotein cholesterol values. When applied to a study of nutritional perturbation in the Mongolian gerbil, the method detected sensible and significant changes in serum apoA-I that paralleled changes in HDL cholesterol. — **France, D. S., T. E. Hughes, R. Miserendino, J. A. Spirito, J. Babiak, J. B. Eskesen, C. Tapparelli, and J. R. Paterniti, Jr.** Nonimmunochemical quantitation of mammalian apolipoprotein A-I in whole serum or plasma by nonreducing gel electrophoresis. *J. Lipid Res.* 1989. **30:** 1997–2004.

Supplementary key words sodium dodecyl sulfate gel electrophoresis • laser densitometry

Apolipoprotein A-I (apoA-I) is the major protein constituent of high-density lipoproteins (HDL), comprising 60–70% of the total protein mass of this class of particles (1). In humans, plasma levels of apoA-I are inversely correlated with relative risk for coronary artery disease (2, 3). ApoA-I is thought to play an important role in reverse cholesterol transport from peripheral cells to the liver, largely through its activation of lecithin:cholesterol acyltransferase and HDL removal by the liver (4).

Various isotopic and nonisotopic immunoassays for quantitation of apoA-I have been reported for man, nonhuman primates, and the rat (5–8). Although conservation of apoA-I amino acid sequences has been demonstrated in a number of mammalian and avian species (9), antibodies raised against one species may cross-react to varying degrees with apoAI derived from other species. Thus, several different assays would likely be required to quantitate apoA-I from several species. In addition, a drawback of some immunoassays is the need to pre-heat in detergent or to delipidate serum or plasma samples to fully expose the antigenic sites of apoA-I (10, 11). Furthermore, apoA-I immunoreactivity with both polyclonal and monoclonal antibodies can be altered by conditions of sample storage (12).

Electrophoresis followed by densitometric analysis of HDL apolipoproteins isolated by ultracentrifugation has been used frequently to quantitate HDL apoA-I content (13–16). Ultracentrifugation of even a moderate number of samples is laborious. In addition, a significant fraction of apoA-I disassociates from HDL during ultracentrifugation (17) in a variable manner, dependent on such factors as the ionic strength of the centrifugation medium (18). A method for the direct quantitation of apoA-I by densitometric scanning of electrophoresed whole serum or plasma has not been reported previously.

We have exploited the relative abundance of apoA-I in serum (approximately 2.0% of total serum protein in humans) combined with sodium dodecyl sulfate polyacrylamide gel electrophoresis under nonreducing conditions (SDS-PAGE-NR) to develop a simple robust method to resolve, identify, and quantitate apoA-I in whole serum or plasma from a variety of species. Since there are no disulfide linkages in the secondary structure of apoA-I, there is no need to include a reducing agent (i.e., dithiothreitol or 2-mercaptoethanol) in the gel sample buffer. In the absence of reducing agent, the quaternary structure of immunoglobulin G (IgG; migrating at 150 kDa) is preserved. The IgG light chains, therefore, do not migrate at 22.5 kDa and interfere with the migration, resolution, or visualization of the apoA-I band.

The method described in this study will allow investigators to evaluate dietary, hormonal, or pharmacologic alterations of apoA-I levels in clinical or animal model studies without the need for either delipidation of samples, specific antibodies, or ultracentrifugation. Furthermore, this technique can be successfully applied to microliter quantities of frozen serum or plasma.

MATERIALS AND METHODS

Human subjects

Fifteen healthy consenting volunteers were fasted for 18 h and venous blood was collected in the presence of 2 mM EDTA. Plasma was separated by centrifugation at 2000 g for 20 min at 4°C. Blood was collected from one human volunteer either in the presence of 2 mM EDTA, 200 units/ml sodium heparin, or with no anticoagulant. The plasma and serum were separated as described above. EDTA was added

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Abbreviations: SDS-PAGE-NR, nonreducing sodium dodecyl sulfate gel electrophoresis; SB-NR, nonreducing sample buffer; apoA-I, apolipoprotein A-I; HDL, high density lipoproteins; EDTA, disodium ethylenediamine tetraacetate; RIA, radioimmunoassay; BSA, bovine serum albumin; TBS, Tris-buffered saline; NC, nitrocellulose; TEMED, N,N,N',N'-tetramethylethylenediamine.

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to some aliquots of serum to a concentration of 2 mM. Sera and plasma were aliquoted and assayed immediately and after 2 weeks at −70°C for apoA-I content SDS-PAGE-NR. Serum from an apoA-I/C-III-deficient patient previously described (19) and a rabbit anti-human apoA-I antiserum were kindly provided by Dr. Henry Ginsberg (Columbia University College of Physicians & Surgeons). Post-heparin plasma was obtained from a volunteer 15 min after the intravenous injection of 60 U/kg of sodium heparin.

Animals

Male Sprague-Dawley rats, weighing 175 g to 200 g, were housed two per cage and fed Purina rodent chow #5002 (Purina Laboratories, St. Louis MO) and water ad libitum. Male Mongolian gerbils, weighing 50 to 70 g, were housed two per cage and fed for 1 week with rodent chow #5002. In some cases, the chow was supplemented with 15% coconut oil and various amounts of cholesterol (0.05%, 0.10%, and 0.20% (w/w)). Blood was collected from the orbital sinus and the serum was separated by centrifugation at 2000 *g* for 20 min at 4°C. Male beagles were housed individually and fed Purina dog chow #5007 and water ad libitum. Dogs were bled from the cephalic vein after an overnight fast and the serum was separated as described above. Aliquots of serum were frozen at −70°C and thawed immediately prior to assessment of apoA-I content by SDS-PAGE-NR. Sera from three nonhuman primate species were obtained from the laboratory of Dr. Lars Ostberg (Sandoz Research Institute). Normal rabbit and goat serum were obtained from Pocono Rabbit Farms (Canadensis, PA). Twenty-seven male Cebus monkeys used in other studies (Hennessey, L. K., J. S. Millar, E. J. Schaefer, and R. J. Nicolosi, unpublished results) were maintained for 2 years on diets supplemented with either 0.0% or 0.1% cholesterol in the presence of either 10% coconut oil or 10% corn oil. Freshly isolated serum was frozen and shipped overnight on dry ice to Sandoz Research Institute for analysis.

Determination of HDL cholesterol

Fresh 175-μl aliquots of human, gerbil, and canine sera were adjusted with NaCl to densities of 1.060, 1.055, and 1.045 g/ml, respectively, and ultracentrifuged at 42,000 rpm for 2.5 h in a 42.2 Ti fixed-angle rotor (Beckman Instruments, Irvine, CA) by established methods (20). The samples were fractionated into a top fraction containing β-lipoproteins and a bottom fraction containing HDL. The purity of HDL fractions (90.5 ± 2.4%, mean percent ± SD) was monitored by agarose electrophoresis (Corning) followed by enzymatic staining for cholesterol with the use of a commercial kit (Nippon Chemiphar, Tokyo). Cholesterol content of HDL fractions, determined using the Technicon Auto Analyzer industrial method #339-09, was expressed as mg HDL cholesterol/dl serum.

Gel electrophoresis

Electrophoresis was performed on 16 cm × 16cm × 1.5 mm gels prepared according to a modification of the method of Laemmli (21), using the Bio-Rad Protean II apparatus (Bio-Rad Laboratories, Richmond, CA). All electrophoresis reagents were also purchased from Bio-Rad. Both separating and stacking gels were prepared from the same 30% acrylamide stock (29.2% acrylamide and 0.8% bis-acrylamide). The separating gel consisted of 11.5% acrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% sodium dodecyl sulfate (SDS). Ammonium persulfate and N,N,N',N'-tetramethylethylenediamine (TEMED) were added to polymerize the separating gel at final concentrations of 0.05% and the gels were poured to a height of 11 cm. The stacking gel consisted of 4.0% acrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.05% ammonium persulfate, and 0.1% TEMED. A 1.5-mm-thick 15-well comb was used in all experiments. Resultant wells were 6.5 mm wide and 2.5 cm high, allowing up to 100 μl of sample to be applied per lane.

Sample preparation

Fifteen μl of serum or plasma was diluted with 210 μl of nonreducing sample buffer (SB-NR; 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.005% bromphenol blue) in a 96-well flat-bottom microtiter plate (Corning). The sealed plate was placed into a 90°C incubator for 20 min. After cooling to room temperature, 15–45 μl, equivalent to 1–3 μl of undiluted serum or plasma was loaded with a Hamilton syringe into the gel wells. Standards of purified human apoA-I (Sigma Chemical Co, St. Louis, MO) were diluted appropriately in SB-NR buffer from a stock solution of 0.5 mg/ml in 100 mM Tris-HCl (pH 8.0). Electrophoresis was carried out at 75-80 volts (constant voltage) for 15–18 h with cooling of the apparatus to 4°C. The electrophoresis buffer consisted of 25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3. Determination of protein concentration was performed according to the method of Lowry et al. (22).

Analytic recovery

Human apoA-I standards were diluted in SB-NR so that 0.5–4.0 μg was added to 2 μl of serum from a human A-I/C-III-deficient patient. These samples were subjected, in quadruplicate, to SDS-PAGE-NR and the recovery of human apoA-I was calculated.

Gel staining and destaining

After electrophoresis, gels were fixed for 10 min in 10% acetic acid and stained in 500 ml of freshly prepared staining solution (0.25% Coomassie Blue R-250 in 10% acetic acid and 40% methanol) for 2 h with gentle agitation. Gels were destained in 10% acetic acid and 20% methanol for 4–6 h with several changes. Use of methanol

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at concentrations greater than 20% resulted in excessive destaining of protein bands, as has been reported elsewhere (23).

Laser densitometry

After destaining, gels were equilibrated for 5 min in distilled water, and both standards and unknown apoA-I bands were scanned with a laser densitometer (LKB, Bromma, Sweden). One- and two-dimensional scans were evaluated using the LKB 2400 GelScan XL software package installed on an IBM PC-AT computer.

Analysis of data

Linear regression analysis was performed using the least squares method. Student's *t*-tests, coefficients of correlation, and two-way analysis of variance were calculated using the RS/3 software package (BBN Research Systems, Cambridge, MA).

Elution of Coomassie Blue from stained apoA-I bands

As an alternative to laser densitometry apoA-I was quantitated by spectrophotometric measurement of the Coomassie Blue eluted from the apoA-I bands. Bands were excised and placed into 3.0-ml glass screw-top vials containing 1.0 ml distilled deionized water and 1.5 ml water-saturated n-butanol. Vials were heated at 90°C in a heating block for 2–4 h with periodic mixing. One ml of the upper butanolic phase was transferred to a disposable cuvette and the absorbance was measured at 590 nm with a Beckman DU8 spectrophotometer blanked against water-saturated n-butanol. Baseline corrections were performed by excising a gel slice corresponding to mol wt of 28,300 from lanes containing the electrophoresed serum of the A-I/C-III-deficient human subject, and from lanes to which no sample was added.

Western blotting

Proteins from SDS-PAGE-NR gels were transferred to nitrocellulose sheets (NC) essentially as described by Towbin, Staehelin, and Gordon (24). The transfer buffer for SDS-PAGE-NR gels consisted of 25 mM Tris, 192 mM glycine, and 20% methanol. Transfer was conducted at 50 volts for 4 h using a Bio-Rad Transblot apparatus. To block nonspecific sites, NC sheets were then placed in a solution of Tris-buffered saline (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl (TBS)) containing 5% bovine serum albumin (BSA) (fraction V, Sigma Chemical Co., St. Louis, MO) and incubated overnight. Primary incubation consisted of a 1:200 dilution of rabbit anti-human apoA-I in TBS containing 5% BSA for 2–6 h. Following four successive 15-min washes in TBS with 0.05% Tween-20, NC sheets were incubated in a 1:3000 dilution of an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Bio-Rad) for 1 h. After washing with TBS–Tween-20, NC sheets were briefly washed with TBS, and immunoreactive bands were visualized after the addition of a chromogenic substrate mixture consisting of 150 μg/ml 5-bromo-4-chloro-3-indolyl phosphate and 300 μg/ml nitro blue tetrazolium in a carbonate buffer containing 0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8. The color reaction was stopped by washing the NC sheets with distilled water.

Radioimmunoassay of apoA-I

To validate the SDS-PAGE-NR method, the quantitation of apoA-I by an established radioimmunoassay (RIA) (25) was performed on 15 human plasma samples in parallel.

RESULTS

SDS-PAGE-NR of whole serum and Western blotting

As shown in **Fig. 1a**, electrophoresis of sera from several species under nonreducing conditions revealed a major protein band of appropriate molecular weight for apoA-I of human, dog, goat, gerbil, babbon, and the rat (26). Parallel immunostaining with a rabbit anti-human apoA-I antibody showed strong reactivity with human, canine, goat, and baboon apoA-I (Fig. 1b). By contrast, this antibody failed to react with gerbil or rat apoA-I (Fig. 1b), which were identified by their comigration with purified human apoA-I (Fig. 1a) or with rat apoA-I (data not shown). Serum from an apoA-I/C-III-deficient patient lacked Coomassie Blue staining at the expected molecular weight for human apoA-I (28,300), and was negative for immunostaining with the anti-apoA-I antibody. These data demonstrate that under these conditions there is no other human plasma protein that comigrates with apoA-I of sufficient abundance to be detected by Coomassie Blue R-250 staining.

Linearity of response: comparison of laser densitometer and band elution

We found linearity of the standard curve of purified human apoA-I from 0.25 to 4.0 micrograms. **Fig. 2** depicts the standard curves derived from four gels quantitated both by laser densitometry and the butanol extraction technique. Since the curves exhibit comparable linearity and sensitivity, the butanol extraction method is applicable in laboratories without access to a densitometer.

Reproducibility

The conditions of sample preparation, electrophoresis, staining and scanning are subject to variabilities in reagents, temperatures, and other factors. It is important,

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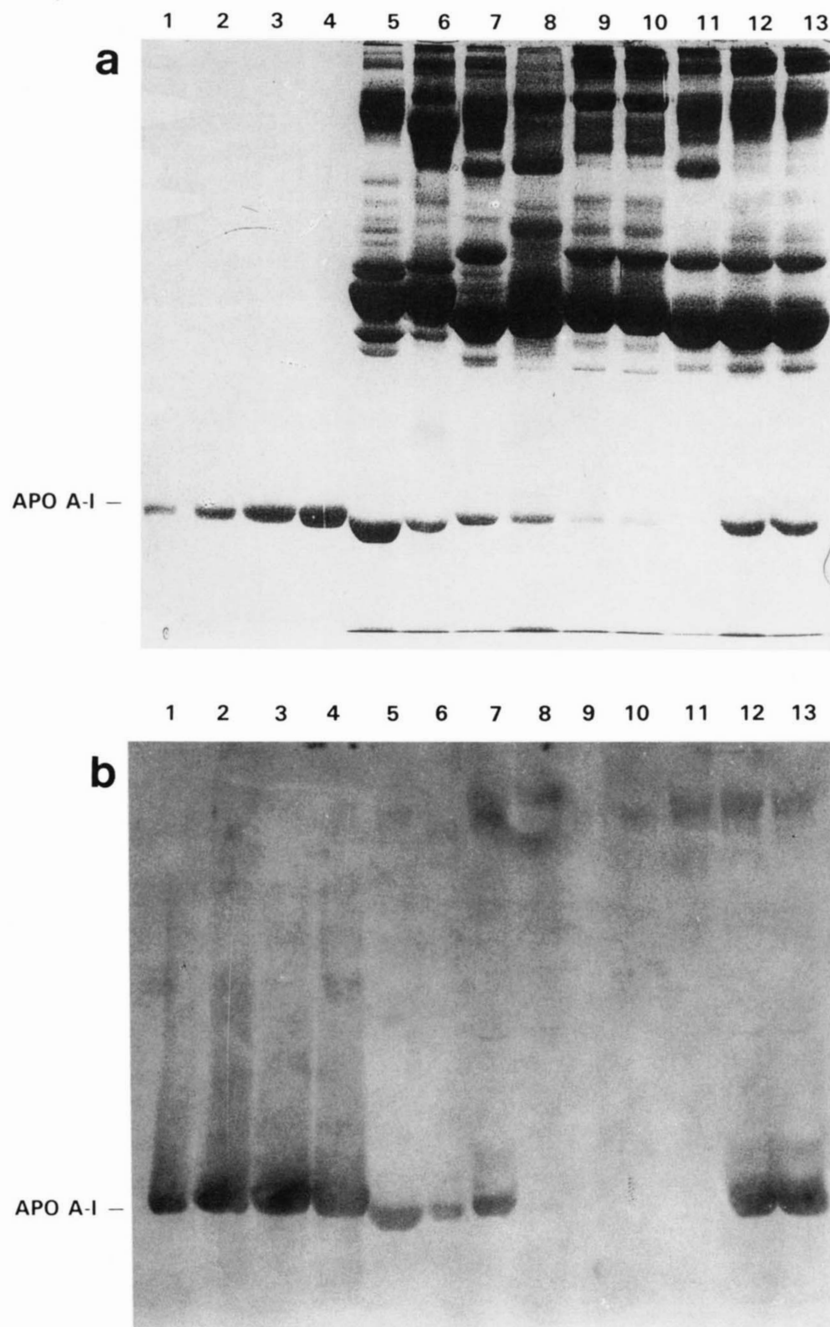

Fig. 1. a: Nonreducing SDS-PAGE of 2 μl of whole serum or plasma from various species and purified human apoA-I. Sera or plasma were diluted 1:15 in SB-NR. Standards were diluted appropriately to yield the indicated mass of apoA-I/lane. Thirty μl of sample was applied per lane. Following staining in Coomassie Blue R-250, the gel was destained in 20% methanol–10% acetic acid. 1) 0.5 μg apoA-I; 2) 1.0 μg apoA-I; 3) 2.0 μg apoA-I; 4) 4.0 μg apoA-I; 5) canine serum; 6) baboon serum; 7) goat serum; 8) gerbil serum; 9) rat plasma; 10) rat serum; 11) human A-I/C-III-deficient serum; 12) human plasma; 13) human serum. b: Western immunoblot of apoA-I in whole serum from various species using a polyclonal rabbit anti-human apoA-I antiserum. Lanes are as shown in Fig. 1a.

therefore, to include apoA-I standards on each gel. Eleven rat sera were evaluated on 16 separate gels run on 4 consecutive days. By one-dimensional laser densitometry the between-assay coefficient of variability obtained was 7.5 ± 4.0% (mean ± SD). Within-assay variability was assessed by triplicate determinations of sera samples on the same gel derived from a human subject, a rat, a dog, a gerbil, and a rabbit. The within-assay coefficient of variability from this experiment was calculated to be 3.1 ± 1.8%.

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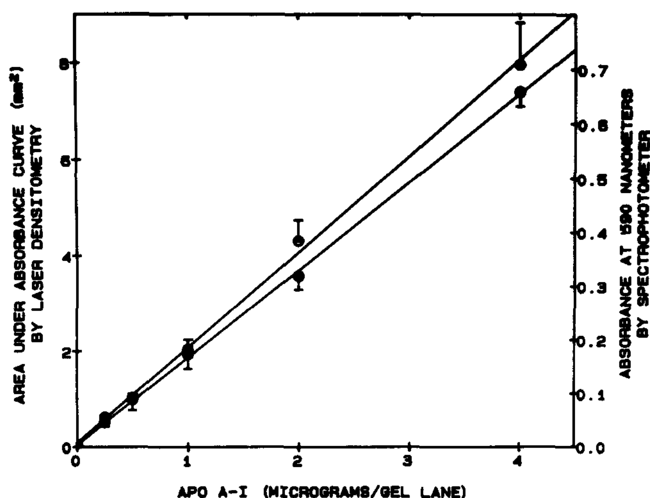

Fig. 2. Standard curves of human apoA-I quantitated by SDS-PAGE-NR. Data represent mean ± SD of four experiments. After scanning of Coomassie Blue-stained apoA-I bands by laser densitometry (○), bands were excised, the stain was eluted, and the absorbance was measured at 590 nm (●).

TABLE 1. Effect of sample collection and storage for apoA-I quantitation by SDS-PAGE-NR

Source of ApoA-I	ApoA-I Fresh	ApoA-I Frozen
	mg/dl	
Serum	155 ± 8	166 ± 9
Serum and EDTA	163 ± 7	162 ± 11
EDTA plasma	165 ± 10	153 ± 9
Heparinized plasma	155 ± 6	150 ± 7
Post-heparin plasma	N.D.	166 ± 8

Blood from one human volunteer was collected under various conditions and serum and plasma were separated as described in Materials and Methods. Aliquots of serum or plasma were assessed for apoA-I content immediately by SDS-PAGE-NR and after 2 weeks at −70°C. ApoA-I content of fresh post-heparin plasma was not determined (N.D.). Data represent the mean ± SD of three separate apoA-I determinations.

Analytic recovery

Purified human apoA-I was added to A-I/C-III-deficient serum at 0.25, 0.5, 1.0, 2.0, and 4.0 μg/μl serum and subjected to SDS-PAGE-NR. Analytic recoveries were calculated to be: 97 ± 2.4 (mean % ± SD), 98.5 ± 1.6, 99.0 ± 1.4, 98.7 ± 1.2, 97.5 ± 2.2, respectively, for the human apoA-I.

Effect of sample collection and storage

We sought to compare the results of apoA-I analysis using samples from the same human subject obtained and stored by different methods. Serum was collected with or without subsequent addition of EDTA, and plasma was collected in either EDTA or heparin. The effect of freezing and thawing such samples was also assessed. Post-heparin plasma, from this subject, which had been stored at −70°C for 2 weeks was also compared. As shown in **Table 1**, all modes of collection yielded apoA-I values differing by less than 5%. Furthermore, freezing and thawing did not adversely change the mass of apoA-I detected regardless of the mode of collection.

Correlation of SDS-PAGE-NR with apoA-I RIA

We compared the serum apoA-I values obtained by SDS-PAGE-NR and RIA from 15 human subjects (**Fig. 3**). Linear regression analysis of the values showed a highly significant correlation (r = 0.98) at a high degree of confidence (P = 0.0001). The regression equation was 0.85x + 28.15.

Application of the method to various species

In addition to human sera, we evaluated sera from rats, gerbils, dogs, rabbits, goats, a baboon, a chimpanzee, and a rhesus monkey. All sera subjected to SDS-PAGE-NR exhibited sufficient resolution of the apoA-I band to allow densitometric scanning. As expected, considerable heterogeneity in serum apoA-I levels was observed among species. The lowest levels were found among rats (73 ± 8.4 mg/dl: n = 24) and the highest among dogs (270 ± 40 mg/dl: n = 12). Due to the low levels of apoA-I observed in the rat, 3 μl of serum/gel lane was applied to produce an apoA-I band suitable for scanning.

Correlation of apoA-I by SDS-PAGE-NR and HDL-cholesterol levels

The apoA-I values generated by SDS-PAGE-NR derived from this method were strongly correlated with HDL cholesterol within the four species studied: human r = 0.78, P = 0.0009, n = 16; dog r = 0.94, P = 0.000004, n = 12; gerbil r = 0.79, P = 0.0001, n = 47; Cebus monkey r = 0.68,

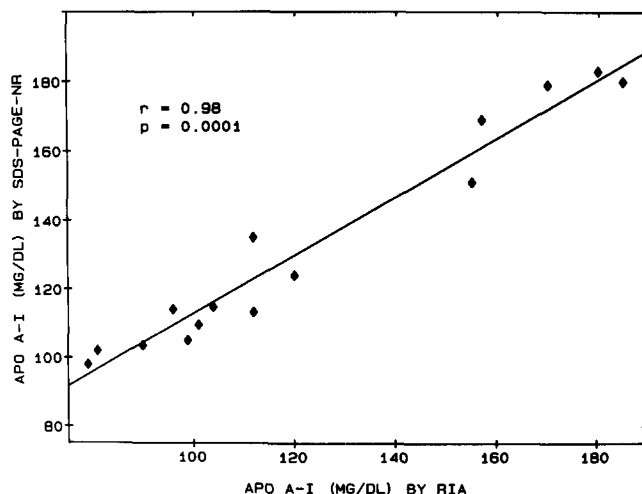

Fig. 3. Correlation of human plasma apoA-I values determined by SDS-PAGE-NR and radioimmunoassay.

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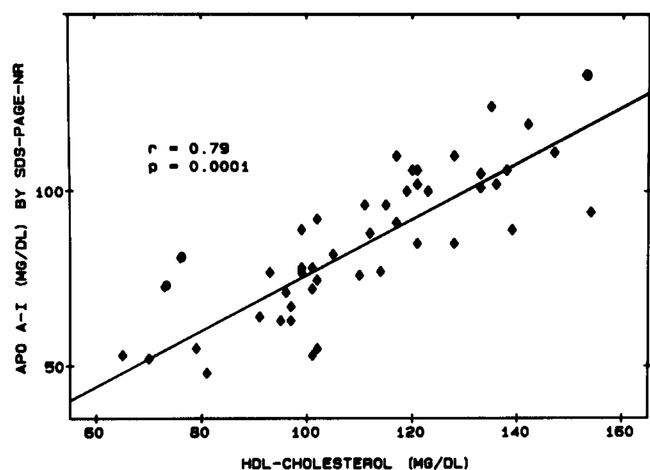
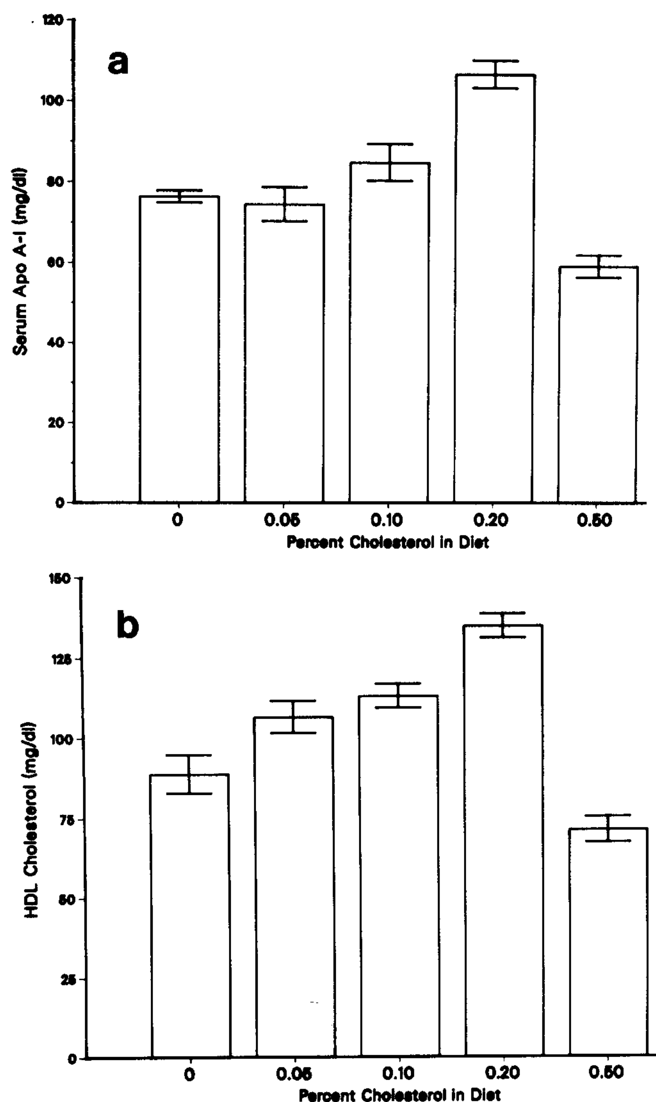


Fig. 4. Correlation of serum apoA-I levels determined by SDS-PAGE-NR and high density lipoprotein cholesterol among gerbils fed various amounts of dietary cholesterol.

P = 0.0001, n = 27). The data for gerbils are depicted in **Fig. 4**.

Application of the method to studies of nutritional perturbation of serum apoA-I

The SDS-PAGE-NR method was used to assess the effects of dietary fat and/or cholesterol on the levels of serum apoA-I in the gerbil. **Fig. 5** shows the effect of increasing amounts of dietary cholesterol on the concentrations of apoA-I (Fig. 5a) and HDL cholesterol (Fig. 5b) in the gerbil. Low amounts of dietary cholesterol (up to 0.2% in diet) which induce moderate increases in total cholesterol in these animals (Fig. 5c) increased the concentrations of both apoA-I and HDL cholesterol. In contrast, high amounts of dietary cholesterol (0.5% in diet) induced greater increases in total cholesterol (Fig. 5c) but a significant decrease in both apoA-I and HDL cholesterol concentrations.



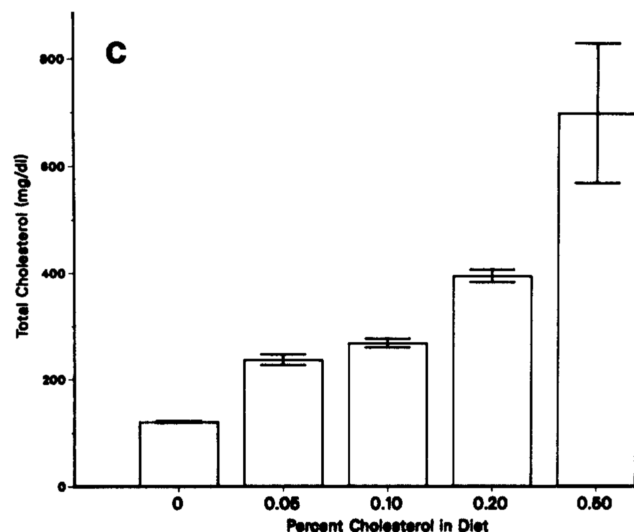


Fig. 5. a: Effect of dietary cholesterol on serum apoA-I in the Monogolian gerbil. Animals were maintained on various diets as described in Materials and Methods. Data represent mean ± SD of 5 to 14 animals/group. By Student's t-test $P < 0.0001$ for the 0.2% and 0.5% cholesterol groups versus control animals. b: Effect of dietary cholesterol on HDL cholesterol in the Monogolian gerbil. All groups are significantly different from controls (P = 0.045, 0.10, 0.0001, 0.05 by Student's t-test, respectively). c: Effect of dietary cholesterol on total serum cholesterol in the gerbil. All groups significantly greater than controls ($P < 0.0001$ for all groups).

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DISCUSSION

The use of animal model and human systems to study the pathophysiology of atherosclerosis has led to a high demand for reliable apoA-I quantitation across several species. Although human apoA-I is highly immunogenic in rabbits, anti-human antisera may not recognize apoA-I of other species (e.g., rat and gerbil; Fig. 1b). With the SDS-PAGE-NR system described in this study, we have circumvented the need for antibodies, ultracentrifugation, or delipidation of samples for apoA-I quantitation. A highly positive, statistically significant correlation was noted between human apoA-I values derived from SDS-PAGE-NR and RIA (r = 0.98, P = 0.0001, n = 15). The slope of the resultant regression line was 0.85 ± 0.05. The SDS-PAGE-NR method gave higher values for the apoA-I content of human sera samples compared to RIA by 9.5 ± 2.4%. However, for sera with greater than 100 mg/dl apoA-I by RIA the between-method % C. V. improved to 5.3% ± 2.1 (n = 10), indicating that within the range of 100 to 190 mg/dl, the two methods yielded comparable mass values.

In a recent study (27), the between-method coefficient of variability for human apoA-I determination was reported to be 13% among 28 laboratories. It has been suggested that lab-to-lab discrepancies in apoA-I determination may be ascribed in part to differences in Lowry protein methods, including both the source of the albumin standard and whether detergents are employed (28).

Under the conditions of electrophoresis used here, we clearly demonstrated no protein band staining at 28.3 kDa in the sample of A-I/C-III-deficient serum (Fig. 1a). However, we cannot rule out the presence of contaminants comigrating with apoA-I in other nonhuman species. In this study we have used purified human apoA-I obtained commercially as standards for this gel system. We cannot assess from the data in this study whether apoA-I from humans and from other species possess identical chromogenicity with Coomassie Blue R-250. If absolute apoA-I values are critical, purified apoA-I from the species of interest should be used as standards in this gel system. Pure apoA-I can be readily obtained preparatively from plasma using gel filtration column chromatography of delipidated HDL (29).

We applied SDS-PAGE-NR to quantitate serum apoA-I levels in response to dietary manipulations in the gerbil. The changes in serum apoA-I observed in this study correlated highly with changes in HDL cholesterol. We observed an increase in serum apoA-I using diets that result in a moderate increase in serum cholesterol. When serum cholesterol was greatly elevated by dietary cholesterol feeding, apoA-I levels decreased. This phenomenon has also been observed in the African green monkey (30, 31).

We have described a rapid, simple, and reliable method for measuring apoA-I among a variety of mammals using commercially available equipment and reagents. It requires neither radioisotopes nor specific antibodies and the method possesses intrinsically high recovery, due to minimal sample manipulation. This method can be readily applied to the study of this important apoprotein in cholesterol metabolism.

Note Added in Proof: The SDS-PAGE-NR method has been applied to the quantitation of mouse and human apoA-I in sera of mice transgenic for the human apoA-I gene both within our laboratory (M. E. Swanson, T. E. Hughes, I. M. St. Denny, D. S. France, M. D. Mone, C. Isaacson, R. Miserendino, J. R. Paterniti, Jr., and K. Burki., submitted for publication) and elsewhere (32).

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