TECHNICAL NOTE

Automated Turbidimetry of Serum Lipoprotein(a)

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Summary: We describe a simple immunoturbidimetric method for quantifying lipoprotein(a) in serum based on latex-enhanced particle agglutination technology. Carboxylated latex particles (diameter 240 nm) covalently coated with $F(ab')_2$ fragments of anti-lipoprotein(a) antibodies are incubated with the sample for 5 min at 37 °C, and the resulting agglutination is quantified by measuring the change of turbidity produced at 700 nm. The assay is rapid, precise and fully automated on the Hitachi 911 analyser. The assay range is about $0.03 - 0.9$ g/l. Average analytical recovery was 97.8%. Precision (CV) ranged from 1.9 to 3.1% at different lipoprotein(a) values. There was no interference from bilirubin, Intralipid®, haemoglobin, plasminogen or apolipoprotein B. Comparisons with a latex nephelometric assay carried out on the Behring nephelometer analyser, and with three commercially available methods, a radioimmunoassay and two ELISA assays, gave good correlations ($r > 0.95$), although a large among-method variation in lipoprotein(a) values was found. We conclude that the proposed latex turbidimetric immunoassay method is suitable for routine use in clinical laboratories.

These findings generated the need for a serum lipoprotein(a) assay in the clinical chemistry laboratory. Various quantitative immunological assays for lipoprotein(a) determination, including electrophoretic techniques (10), radial immunodiffusion (11), enzyme immunoassays (12), and radio immunoassays (13) have been developed. However, these procedures generally require specialized reagents and equipment, long reaction times, and multiple washing steps, and they are not easily automated. Recently, in an effort to overcome these problems several automated fluid-phase immunoprecipitation procedures have been described (14, 15).

In this study, we present a turbidimetric method which uses particle-enhanced immunoassay technology and is carried out on a last generation clinical chemistry analyser. This turbidimetric method has the advantage of being precise, rapid, easy to perform, and suffers no interference from apolipoprotein B, plasminogen, or from endogenous lipids, haemoglobin, or bilirubin. Here we describe the characteristics and performance of the method.

Introduction

Lipoprotein(a) [Lp(a)] was initially though to be a genetic variant of low density lipoprotein (LDL) (1). Lp(a) is a low density lipoprotein-like particle containing apolipoprotein B-100 disulphide-linked to one (or two) large glycoprotein called apolipoprotein(a) (M_r 300 000 — 700 000) (2). Apolipoprotein(a) has been shown to have a considerable degree of homology with human plasminogen (3). The characteristic feature of lipoprotein(a) is that it is distinct from all other serum proteins and apolipoproteins. This protein is believed to be inherited as an autosomal dominant trait and appears to be insensitive to either diet, lifestyle or most hypolipidaemic drugs (4, 5).

Since its discovery by *Berg* in 1963 (1), there has been a considerable rise in interest, not only in specialized research centres but also in clinical routine laboratories, in the accurate measurement of lipoprotein(a) in blood. This interest was stimulated by reports indicating that levels above $0.2 - 0.3$ g/l, present in approximately 25% of the population, are associated with an increased risk of coronary heart disease (6, 7). Many investigators have confirmed that a high lipoprotein(a) concentration represents an indicator of risk for cardiovascular disease, especially when other serum lipidic quantities differ significantly from the values for healthy subjects (8, 9).

Materials and Methods

Apparatus

We used an Hitachi 911 analysis system (Boehringer Mannheim, D-68305 Mannheim, Germany) for all the determinations.

Samples

Blood was taken in red-top vacutainer tubes (Becton Dickinson, Rutherford, NJ), allowed to clot, and the serum removed for immediate assay or for storage at -30 °C if the analysis was to be carried out later. Unselected serum specimens used for comparative studies were obtained from the lipid laboratory of our hospital.

Antibody

Rabbit polyclonal antiserum directed against human lipoprotein(a) was manufactured by Dakopatts A/S (Glostrup, Denmark). It showed no cross-reaction with apolipoprotein B or plasminogen and was stored at 4 °C. (Lot No. 012, 3.7 g/l of protein.)

IgG was separated from this antiserum by precipitation with half-saturated ammonium sulphate, followed by treatment with Rivanol®. To obtain F(ab)'$_2$ fragments, the purified IgG was digested with twice-crystallized pepsin (EC 3.4.23.1) as described previously (18). This preparation was tested for purity by immunoelectrophoresis against serum and plasma containing a high lipoprotein(a) concentration; only a single precipitation line was formed.

Calibrator

As calibrator we used N Lp(a) standard (Behringwerke AG, Marburg, Germany) containing 0.827 g lipoprotein(a) per litre, as determined by electroimmunodiffusion with reference to a highly purified lipoprotein(a) preparation. In addition, a pool of serum was used as a secondary standard. Its lipoprotein(a) concentration (0.900 g/l) was determined by a nephelometric method by repeated testing (five days, n = 10 each day) as described elsewhere (16). This calibrator was aliquoted and stored in plastic vials at −30 °C or lyophilized in the presence of 600 mmol/l sucrose (17).

Latex reagent

Ten percent solid carboxylated polystyrene latex particles, 240 nm in diameter (CLM-006), were obtained from Polymer Laboratories Ltd., Church Sireton, England. F(ab')$_2$ fragments were covalently coupled to latex particles as described previously (18). The F(ab')$_2$ fragments of the IgG fraction of the anti-lipoprotein(a) antibodies were added in a protein/latex ratio of 1/10. The resulting 0.5% solid coated particle solution was stored at 4 °C. This stock solution can be frozen or freeze-dried for long term conservation.

Assay procedure

Sample processing, dilution, pipetting steps, and quantification were performed automatically on the Hitachi 911 analyser. A six point calibration curve was obtained by automatic dilution of the calibrator (from 0 to 0.900 g/l; saline solution was used for the zero). The assay procedure was as follows: after incubation of 3 µl of the sample with 300 µl of the glycine buffer (0.1 mol/l glycine, 0.15 mol/l NaCl, 10 g/l bovine serum albumin (Cohn Fraction V, Sigma Chemical Co., St. Louis, MO), 30 g/l polyethylene glycol, 0.6 mmol/l sodium azide, adjusted to pH 8.2) for 3 min, the reaction was started by the addition of 30 µl of latex reagent and monitored at 700 nm for a further 5 min. The test was run as a two point assay method with sample blank correction.

Comparison methods

Correlation studies were carried out on two different groups of 52 and 80 patient serum samples, which had been stored at −30 °C for up to 3 months. We compared the results obtained by the present method with those of a latex nephelometric test carried out on the Behring nephelometer analyser, and with three different commercial assays performed according to the manufacturers' instructions.

Latex nephelometric assay

We measured lipoprotein(a) in serum samples by a latex-enhanced nephelometric immunoassay as previously described (16). This method uses carboxylated latex particles covalently coated with purified F(ab')$_2$ fragments, the latter being derived from a rabbit IgG antibody against lipoprotein(a) obtained from Behring (Behringwerke AG, Marburg, Germany). This assay is rapid (12 min), precise (CVs < 8%), uses a high sample

dilution (1/400) and is fully-automated on the Behring nephelometer analyser. In addition, the method was free of turbid or lipaemic interferences, and no significant differences were observed when fresh and frozen samples were compared.

RIA test

We used the Pharmacia apo (a) RIA 100 (Pharmacia Diagnostics AB, Uppsala, Sweden) for the quantitative determination of human apolipoprotein(a) in serum. This method is a two-site immunoradiometric assay using two different monoclonal antibodies directed against the apolipoprotein(a) part of the lipoprotein(a) molecule. Values of apolipoprotein(a) protein are expressed as U/l.

ELISA assays

We used the Macra™ Lp(a) assay (Terumo Medical Corp., Elkton, MD., USA), and the Biopool TintElize Lp(a) kit (Biopool AB Umea, Sweden) according to the manufacturers' instructions. The MACRA Lp(a) kit is a sandwich ELISA that utilizes a monoclonal antibody for capture and a polyclonal antibody for detection (both directed against lipoprotein(a)).

The Biopool TintElize Lp(a) kit is a sandwich ELISA that utilizes affinity purified polyclonal antibodies raised against lipoprotein(a).

Furthermore, 20 hypertriacylglycerol samples, with triacylglycerol concentrations between 2.26 and 16.98 mmol/l, were assayed with the turbidimetric test and results were compared with those by the Macra™ Lp(a) assay.

Interfering substances

For interference studies we followed the procedure of Glick (19). We assessed the effect of bilirubin, Intralipid^R (Kabi Pharmacia, Stockholm, Sweden) and haemoglobin by adding known amounts of these substances to a baseline serum pool containing 0.40 g/l of lipoprotein(a). Interference from plasminogen was assessed by adding to the same pool various amounts of a purified human plasminogen (Lys) preparation (Sigma Chemical Co., St. Louis, USA). To assess the effect of apolipoprotein B, we added increased amounts of a purified human apolipoprotein B solution (International Enzymes Inc., Fallbrook, USA) to a normolipaemic serum containing 0.09 g/l of lipoprotein(a).

Statistics

The relationships between the turbidimetric method and the four others assays were demonstrated by applying the non-parametric method of Passing & Bablok (20). The correlation coefficients of lipoprotein(a) values between methods were determined by means of linear regression analysis.

Results

Calibration curve

Using a six-point calibration procedure, the turbidimetric lipoprotein(a) assay has an analytical range of 0 to 0.900 g/l (fig. 1). This covers the majority of normal and pathological serum values. If the concentration exceeds 0.900 g/l, a 5-fold dilution of the sample with saline is prepared and reanalysed automatically by the instrument. The calibration curve is stable for up to two weeks (reagents stored at + 2 to + 8 °C). In addition, the system did not show the prozone phenomenon with lipoprotein(a) concentrations of up to 3.500 g/l.

Imprecision

The imprecision of the assay was examined with three serum pool samples with different lipoprotein(a) values (tab. 1). For within-run imprecision, which was determined by assaying 20 replicate samples, coefficients of variation ranged from 1.9 to 2.4%. Between-run imprecision was determined by assaying the same samples on 20 consecutive days, and the coefficients of variation were all < 4%. These data indicate that the precision of the turbidimetric method is good and comparable with or better than those obtained by other lipoprotein(a) methods (10–13, 16).

Interfering substances

Due to the assay measuring principle (wavelength 700 nm), there was no endogenous interference by bilirubin (up to 340 μmol/l), haemoglobin (up to 9 g/l) or Intralipid® (up to 2% or 19.47 mmol/l triacylglycerol equivalent) (fig. 2). In addition, no effects were observed in any sample with spiked levels of up to 1.5 g/l of apolipoprotein B, and 2.0 g/l for plasminogen.

There was good agreement between the results obtained for 20 hypertriacylglycerol samples by the proposed method (y) and those obtained by the Macra™ Lp(a) assay (x); (y = 1.386x + 0.04; r = 0.990; n = 20).

Linearity and sensitivity

Linearity was evaluated by serial dilutions (with saline solution) of four different samples, which contained values of lipoprotein(a) in the range of analysis. Linear regression values of lipoprotein(a) g/l vs. dilution yielded correlation coefficients, r > 0.995, for all samples. Within the assay's measuring range, the deviations of measurement from theoretical values did not exceed the 5% level. Calculation of the mean plus 3 SD of twenty replicates of saline solution (zero signal) resulted in a lower limit of detection of 0.03 g/l.

Recovery

The recovery of known concentrations of human serum standard added to aliquots of five pooled serum samples ranged from 95.6 to 102.9% (mean = 97.8%).

Correlation

We assayed 52 and 80 sera with the turbidimetric latex immunoassay on the Hitachi 911 (y) and with a latex nephelometric test (x), an RIA test (x) and two ELISA methods (x). The results of the method comparison studies are represented in figure 3. Serum lipoprotein(a) values ranged from 0.03 to 1.5

Tab. 1. Precision of the turbidimetric lipoprotein(a) assay

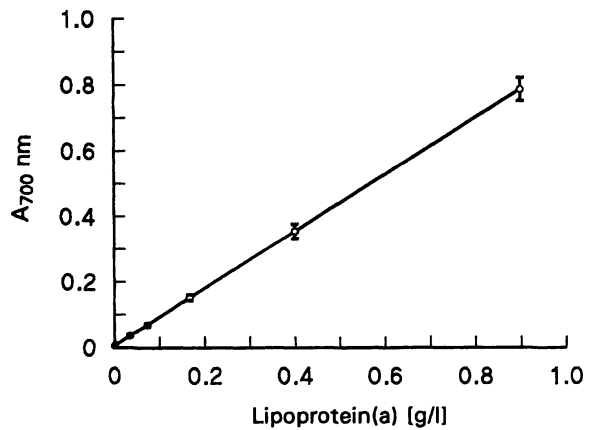|	Lipoprotein(a) (g/l)		
	Mean	SD	CV (%)
Intra-assay	0.158	0.0040	2.4
(n = 20)	0.403	0.0075	1.9
	0.692	0.0140	2.0
Inter-assay	0.160	0.0050	3.1
(n = 20)	0.413	0.0100	2.4
	0.698	0.0160	2.3

Fig. 1. Lipoprotein(a) calibration curve for the latex turbidimetric method. Absorbance (mean ± 2 SD of 10 calibrations) versus calibrator concentrations.
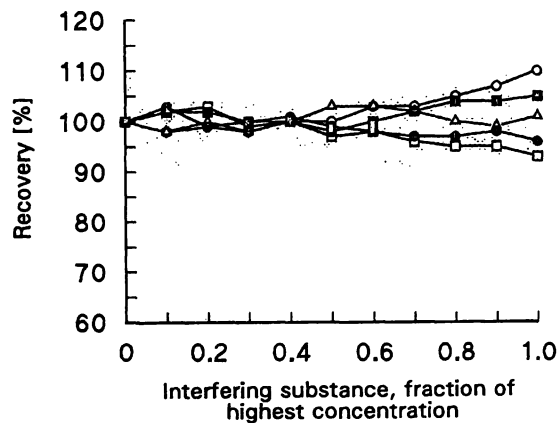

Fig. 2. Interference studies in the latex turbidimetric test for lipoprotein(a). On the abscissa are represented the concentration of interfering substances, expressed as fraction of highest concentration:

o—o Intralipid 2%;
□—□ bilirubin, 340 μmol/l;
●—● haemoglobin, 9 g/l;
△—△ plasminogen, 2 g/l;
▣—▣ Apolipoprotein B, 1.5 g/l.

g/l. Correlation coefficients (r) ranged from 0.95 (p < 0.001) to 0.98 (p < 0.001), indicating a close correlation between all the assays. However, we found slopes significantly different (p > 0.05) from 1.0, indicating that there was no transferability among the different methods. The apparent discrepancy in the accuracy of the present method is symptomatic of the current absence of a international standardization of lipoprotein(a) assays.

Non-specific precipitations

We assayed different turbid specimens, 46 fresh and 79 frozen sera, selected by visual inspection. The lipoprotein(a) values obtained using the original turbidimetric method but replacing the latex reagent by glycine buffer solution, were always less than 0.03 g/l, indicating that the polyethyleneglycol solution does not produce non-specific precipitation in the present turbidimetric test.
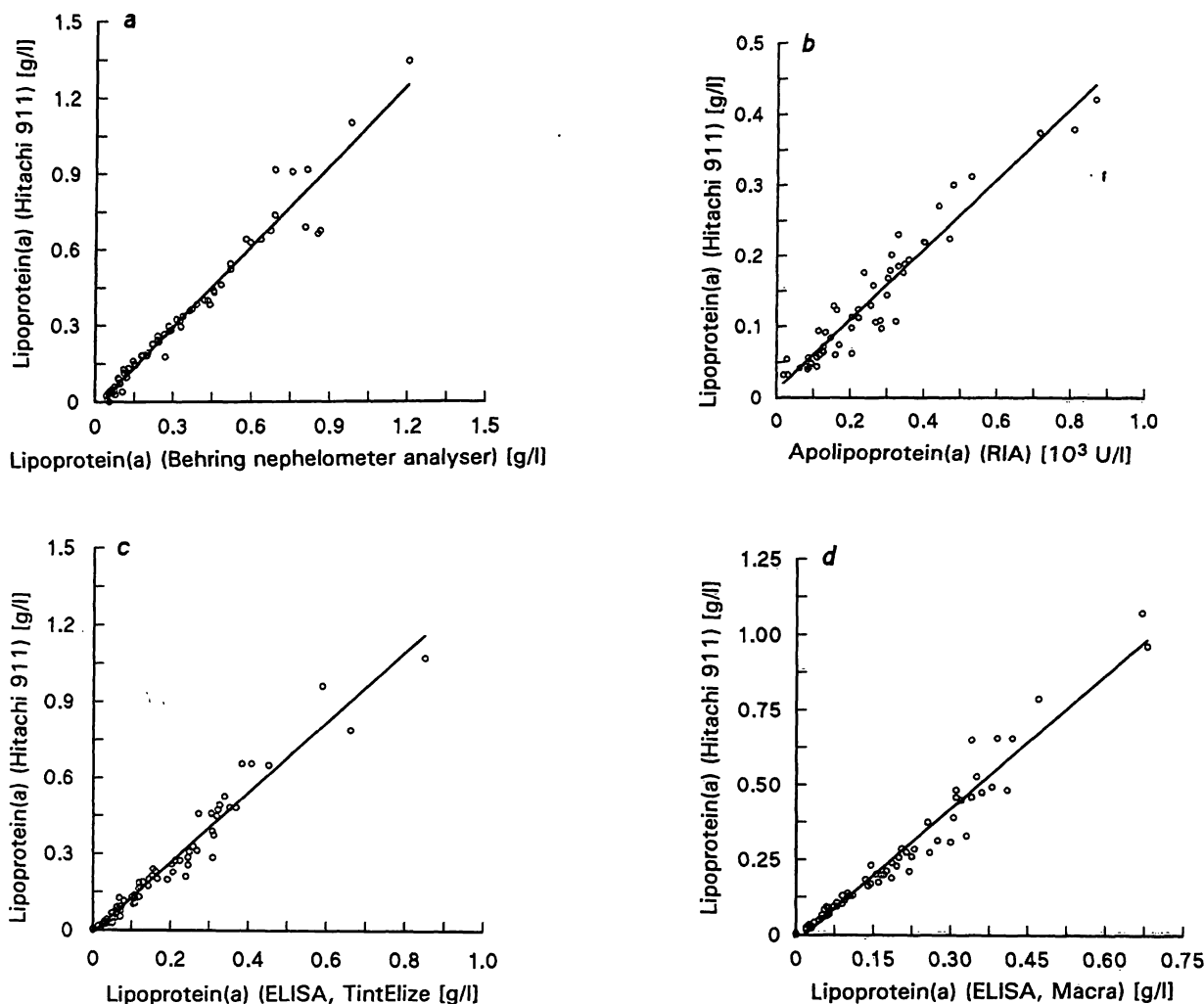
Fig. 3. Comparison of results according to a non-parametric regression analysis (19) for lipoprotein(a) assayed by the turbidimetric method versus:

a) latex nephelometric immunoassay ($y = 1.052\,x - 0.012$; $r = 0.975$; $n = 80$)
b) RIA test ($y = 0.495\,x + 0.011$; $r = 0.951$; $n = 52$)
c) ELISA TintElize method ($y = 1.380\,x - 0.012$; $r = 0.978$; $n = 80$)
d) ELISA, Macra method ($y = 1.483\,x - 0.025$; $r = 0.978$; $n = 80$)

Discussion

Lipoprotein(a) is a quantity of recent interest in atherosclerosis and thrombosis, since it has been demonstrated to be an independent risk factor in both of these afflications (21). At present, however, because of methodology and standardization problems, the clinical application and role of lipoprotein(a) measurements have not been as fully determination as those of lipid and lipoprotein measurements. Conventional methods for lipoprotein(a) measurement generally include tedious manual enzyme or radioimmunoassay techniques. These procedures require laboratory training for the production of reliable results. To overcome this problem, several automated turbidimetric or nephelometric immunoprecipitation assays have been developed recently (14, 15, 22).

A major problem of these assays for serum lipoprotein(a) is the weak signal generated by the presently available commercial antibodies. This leads to misinterpretation of results due to ground noise signal, and/or non-specific precipitation due to the need to use a high concentration of polyethylene glycol in the reaction medium (22, 23).

The latex turbidimetric test presented here allows a fast and convenient lipoprotein(a) determinations of high analytical quality in small amounts of serum. The assay is fully automated

on the Hitachi 911 analysis system. The proposed method offers advantages over non-enhanced turbidimetric assays, since a larger signal is produced with a smaller sample volume. Comparison of the performance of the latex turbidimetric test with those of heterogeneous immunoassays for lipoprotein(a), such as radio- or enzyme-immunoassays, shows that the new method provides a considerable saving in time and labour without sacrificing reliability. In addition, latex reagents have a long shelf life, are easy to prepare, and as many as 300 tests can be performed with 1 ml of antibody.

This simple and rapid method for quantifying lipoprotein(a) in serum involves the use of F(ab')$_2$ fragments of antibody covalently coupled to latex particles of 240 nm diameter. Therefore, this assay measure whole lipoprotein(a) molecules by the signal generated from the immunoagglutination of large latex particles. The heterogeneity caused by genetic isoforms of apolipoprotein(a) contributes only slightly to the overall size heterogeneity.

It is essential to maintain a stable suspension of the reactant particles before use in the assay and to prevent non-specific aggregation caused by other components in the sample during the analysis. It has been recently reported that the use of F(ab')$_2$ fragments of the antibody covalently coated onto latex particles improves reagent stability (24). In our case, the stability of

calibration curve is maintained for at least two months, when the latex reagent is stored appropriately.

Neither haemoglobin, bilirubin nor Intralipid® interfered with the assay. Also, the anti-lipoprotein(a) antiserum used in the assay did not show cross-reactivity with, or interference from, plasminogen or apolipoprotein B. On the other hand, the correlation between the present method and the Macra™ Lp(a) assay was not affected in 20 hypertriacylglycerolaemic samples assayed.

We stored samples at −30 °C because we have previously found no significant differences in the lipoprotein(a) concentratrions of fresh and frozen samples (16).

The sensitivity limit, linearity, and precision of the test are good and comparable to those of other homogeneous and heterogeneous immunoassays for lipoprotein(a) (10−13, 16).

Comparison of the turbidimetric method with a latex nephelometric test and three existing commercial immunoassays for lipoprotein(a) showed good correlations (r > 0.95), although a large among-method variation in lipoprotein(a) values was obtained. In order to reconcile the data obtained by the different methods, the use of suitable reference materials with properly

assigned target values is necessary. Such material is not yet available and our efforts must be directed towards the international standardization of lipoprotein(a) assays similar to that recently performed for apolipoprotein A-I and B (25). It is, however, clear that much more work is needed to achieve an adequate standardization of lipoprotein(a) (26).

In conclusion, we have shown the feasibility of performing a turbidimetric latex immunoassay for quantifying lipoprotein(a) in serum on the Hitachi 911 analyser. The method is rapid, it has a wide measuring range, good linearity and precision and is fully automated. This particle-enhanced method not only represents an interesting alternative to the widely used heterogeneous immunoassays for lipoprotein(a) quantitation, but is also a practical method for routine application.

Acknowledgement

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