

Immunoturbidimetric assays for serum apolipoproteins A1 and B using Cobas Bio centrifugal analyser

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SUMMARY Immunoturbidimetric assays for measuring the apolipoproteins A1 and B using the Cobas Bio centrifugal analyser are described. The methods were specific, offered good sensitivity (< 0.05 g/l) and intrabatch variability, with coefficients of variation between 2.4% and 3.5%, and were cost effective. Reference ranges were calculated for a group of civil servants, aged 35 to 55 years.

The routine laboratory assessment of the risk and severity of coronary heart disease (CHD) depends on the measurement of total cholesterol, high density lipoprotein cholesterol, and serum triglycerides. Retrospective studies have shown that patients with angiographically defined CHD have significantly higher values of apolipoprotein B (apoB) and lower values of apolipoprotein A1 (apoA1) than patients without the disease.¹ These observations have encouraged a recent interest in the measurement of apoA1 and apoB and their potential use with conventional lipid assays as predictors of risk of CHD.

Material and methods

The assays were performed using the Cobas Bio centrifugal analyser from Roche Products Limited, Welwyn Garden City, Hertfordshire.

Reference ranges for the two apolipoproteins were calculated using non-parametric procedures for a group of civil servants ($n = 421$), aged 35-55 years.

Phosphate buffered saline (PBS) at pH 7.4, 0.34 g of potassium dihydrogen phosphate dihydrate and 1.33 g of disodium hydrogen phosphate, 9 g sodium chloride, and 1 g sodium azide were dissolved in distilled water. The pH was adjusted to 7.4 and made to 1 litre with distilled water. Buffered polyethylene glycol (PEG) and PBS (5% w/v) 5 g PEG 6000, and 200 μ l Tween 20 were dissolved and made up to 100 ml with PBS.

Anti-human apolipoproteins A1 (150 μ l) and B (250 μ l) antisera (Boehringer, Mannheim, West Germany) were each added to 6 ml of PEG/PBS. The appropriate antibody dilution was determined for each batch of antisera.

Hyland Omega Lipid Fraction Control Serum (Hyland Diagnostics, Malvern, Philadelphia, USA), Lot No 4610G001 was used as the standard, with an assigned value of 1.45 g/l for apoA1 and 0.75 g/l for apoB. These values were determined using an immunonephelometric assay. The measurements were performed on an automated DISC-120 Hyland nephelometer. The incubation time was two hours. Serum dilutions of 200-fold were prepared with Apovax detergent and mixed with polyclonal antisera raised in the rabbit. Hyland Omega control serum was used as a secondary standard, calibrated against purified apoA1 for apoA1 and against low density lipoprotein for apoB. Serum from the Center for Disease Control was used as control material.

Working standards were prepared by diluting the stock standard to cover the assay range 0.2-3.0 g/l for apoA1 and 0.1-2.0 g/l for apoB.

Immunoneph Reference Material (Lot No. 0530/091) (Immuno Ltd, Dunton Green, Sevenoaks, Kent) was used as control. Values of 1.05 g/l and 1.00 g/l were assigned respectively to apoA1 and apoB.

ASSAY PROCEDURE

Serum (20 μ l) plasma treated with edetic acid or control material, or both, were diluted with 380 μ l of phosphate buffered saline using a Dilutrend automatic pipette (Boehringer, Mannheim). Dilutions were made directly into Cobas Bio sample cups. These were thoroughly mixed using a "whirlmixer" prior to positioning on the sample disc. The disc was placed on the sample module and a cuvette rotor installed. Diluted apoA1 or apoB antisera were placed in the reagent boat. Assay variables were selected (table) and run. A standard curve was plotted from which values for samples and controls were interpolated.

Results

OPTIMAL PEG CONCENTRATION

The effect of PEG 6000 on enhancement of the antigen-antibody reactions for apoA1 and apoB is shown in figs 1 and 2. Above PEG concentrations of 60 g/l sensitivity decreases and curve shape deteriorates. A PEG concentration of 50 g/l was selected for both assays.

EFFECT OF TWEEN 20

In the absence of Tween 20 apoA1 and apoB show completely different rates of antigen-antibody complex formation in both standard and sample. The addition of Tween 20 to the reaction mixture seems to strengthen the enhanced reaction rate and sensitivity previously observed with PEG. Blank absorbance values are also much lower in the presence of Tween. Standard curves for both apolipoproteins incorporating PEG (50 g/l) and Tween 20 (2 g/l) in phosphate buffered saline are shown in figs 3 and 4.

EFFECT OF TURBIDITY

The effect of turbidity in the reaction mixture was assessed on samples of sera to which Intralipid had been added. Serum triglyceride concentrations were between 2.2 and 44.0 mmol/l. There was no significant change in the absorbance for either apolipoprotein reaction mixture in the absence of antisera during the time course of the reaction. In the presence of antisera there was no significant variation in the rate of the reaction below a triglyceride concentration of 20 mmol/l.

Table Assay variables for apolipoproteins A1 and B

Variable	Setting	
	ApoA1	ApoB
alpha	+	+
1 Units	DA	DA
2 Calculation factor	1000	1000
3 Standard 1	0	0
4 Standard 2	0	0
5 Standard 3	0	0
6 Limit	0	0
7 Temperature (°C)	25.0	25.0
8 Type of analysis	5	5
9 Wavelength (nm)	340	340
10 Sample volume (μl)	5	30
11 Diluent volume (μl)	20	50
12 Reagent volume (μl)	200	200
13 Incubation time (minutes)	0	0
14 Start reagent volume (μl)	0	0
15 Time of first reading (seconds)	5	5
16 Time interval (seconds)	600	600
17 No of readings	2	2
18 Blanking mode	1	1
19 Printout mode	1	1

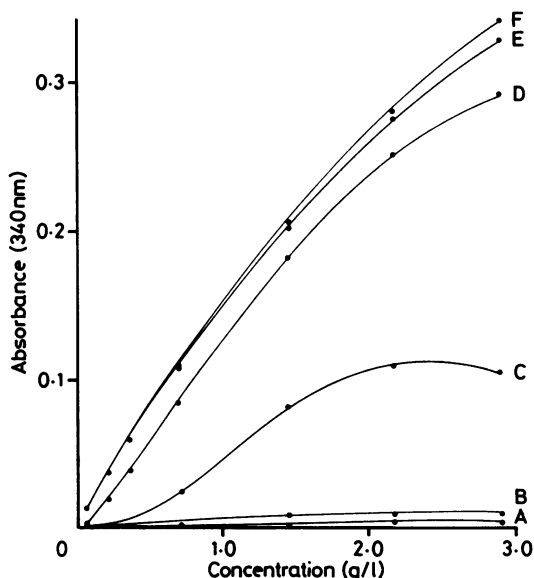


Fig 1 Effect of PEG 6000 on enhancement of antigen-antibody reaction for apoA1. PEG concentrations: A = 10; B = 20; C = 30; D = 40; E = 50 and F = 60 g/l.

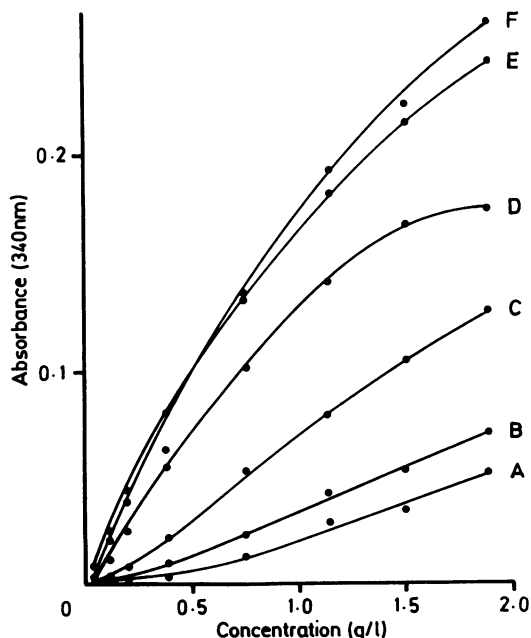


Fig 2 Effect of PEG 6000 on enhancement of antigen-antibody reaction for apoB. PEG concentrations: A = 10; B = 20; C = 30; D = 40; E = 50 and F = 60 g/l.

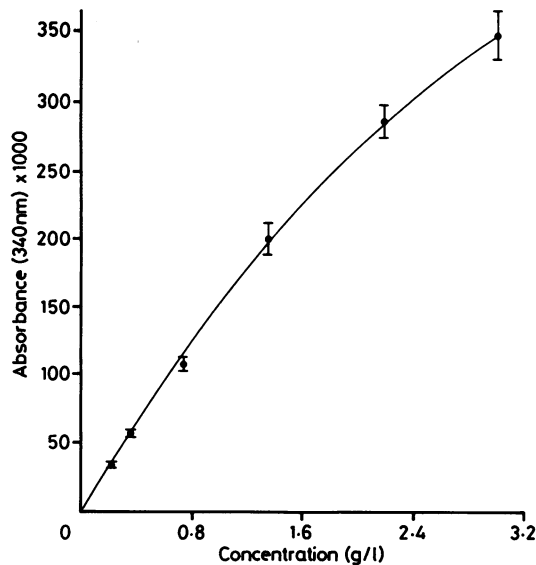


Fig 3 Apolipoprotein A1 standard curve (mean SD).

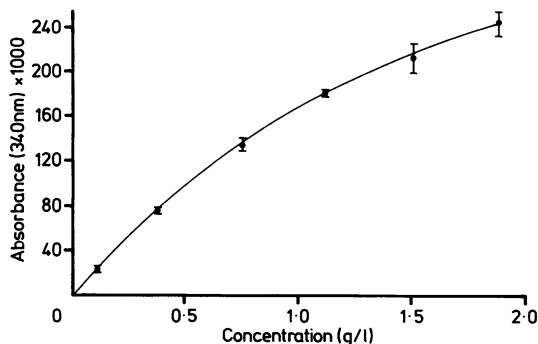


Fig 4 Apolipoprotein B standard curve (mean SD).

SPECIALITY

Antisera tested against appropriate lipoprotein fractions and albumin showed no apparent cross reactivity.

SENSITIVITY

The smallest amount of apolipoprotein that could be detected with 95% confidence was more than 0.05 g/l and 0.02 g/l for apoA1 and apoB, respectively.

RECOVERY

Recovery was assessed on four separate occasions: 50 µl of purified apoA1, 0.14 g/l and apoB, 0.38 g/l, (Sigma Chemical Company Ltd, Poole, Dorset, England), were added to serum; the mean recoveries were 99.1% for apoA1 and 109.5% for apoB.

PRECISION

Intrabatch (n = 19) and interbatch (n = 9) precisions were estimated for three levels of apolipoproteins. Intrabatch precision for both assays ranged from 1.49% to 2.20% and interbatch precision from 2.44% to 3.52%.

Comparison with immunonephelometric assays gave a correlation coefficient r, of 0.85 for apoA1 (n = 123) and 0.94 for apoB (n = 119). The linear regression equations were $y = 1.209x - 0.088$ and $y = 0.78x + 0.121$ for apoA1 and apoB, respectively.

There was a significant correlation between apoA1 and HDL cholesterol $r = 0.796$ (n = 158) and between apoB and total cholesterol $r = 0.831$ (n = 421). The regression equations for apoA1 and apoB were $y = 0.507x + 0.702$ and $y = 0.151x + 0.06$, respectively. There was no significant correlation between apoA1 and total cholesterol $r = 0.162$ (n = 421).

REFERENCE RANGES

Data were obtained on 421 civil servants of both sexes, aged between 35 and 55 years.

The 2.5th and 97.5th percentiles were 0.97–1.90 g/l for apoA1; 0.48–1.31 g/l for apoB, and 0.27–1.02 for the apoB:apoA1 ratio.

Discussion

In the routine clinical chemistry laboratory immunoturbidimetry using centrifugal analysis provides assays for apolipoproteins A1 and B that are economical in terms of both time and resources. A batch of 19 samples can be completed in under an hour using only 20 µl of serum or plasma. Low reagent volumes permit effective conservation of antibody.

Sample preparation is usually unnecessary. Chylomicrons can be removed from samples by ultracentrifugation, but concentrations of triglycerides less than 20 mmol/l do not affect the assay of apolipoproteins A1 and B. It is recommended, however, that chylomicrons are removed from samples with higher triglyceride concentrations and the supernatant used in the assay.

The addition of PEG and the detergent Tween 20 to the reaction mixture avoids the need for a preincubation stage. Both these additives enhance the overall rate of the antigen-antibody reaction and increase sensitivity. Kinetics for standards and samples are similar.

Intrabatch variability was between 2.4% and 3.5% and compares favourably with other methods—for example, the Laurell rocket technique—and also immunonephelometric assays which quote 4% and 7%.^{2,3}

As far as can be determined with recovery experiments the method is also accurate.

Correlation with an immunonephelometric method was good, although there was a bias towards higher results for apoA1 and lower results for apoB by immunonephelometry. This may have been due to variations in sample preparation, different antibodies and standardisation procedures.

The apolipoprotein reference ranges described for immunoturbidimetry are comparable with those published for immunonephelometry.^{4,5}

Although the usefulness of apolipoproteins to predict CHD is still to be established, the apoB:apoA1 ratio may prove to be a useful index. Rising index values above 1.0 may be an early predictor of CHD. Van Stiphout *et al* showed that the apoB:apoA1 ratio in fathers without coronary heart disease was 0.36–1.20.⁶ In those with known coronary atherosclerosis the range was 0.64–1.72.

Immunoturbidimetry provides a precise, accurate, specific and highly sensitive method for measuring apoA1 and apoB. The technique has a further advantage in that it is easily adaptable to instrumentation such as spectrophotometers and other analysers readily available in a routine clinical chemistry laboratory.

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