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Simplified Turbidimetric Determination of Apolipoproteins A-I, A-II and B Using a Microtitre Method

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Summary: A turbidimetric method is described for the determination of apolipoproteins A-I, A-II and B on microtitre plates.

Regression analysis of the resulting values showed a good correlation to apolipoprotein values determined turbidimetrically on Cobas Bio (apolipoprotein A-I, A-II), and those determined by means of radial immuno diffusion (RID) (apolipoprotein A-I: $r = 0.93$, $y = 1.02\,x - 5.0$, $n = 63$; apolipoprotein A-II: $r = 0.90$, $y = 1.07\,x - 5.6$, $n = 44$; apolipoprotein B: $r = 0.92$, $y = 0.95\,x + 9.0$, $n = 58$).

The variation coefficient in the series was 3.5% (apolipoprotein A-I, $n = 21$), 2.5% (apolipoprotein A-II, $n = 20$) and 3.6% (apolipoprotein B, $n = 19$); and the variation coefficient from day to day 3.1% (apolipoprotein A-I, $n = 45$), 4.2% (apolipoprotein A-II, $n = 39$) and 5.3% (apolipoprotein B ($n = 48$).

Introduction

Based on epidemiological (1–4) and clincial (5, 6) studies it is known that a low HDL cholesterol value constitutes a risk factor for coronary heart disease, whereas a high LDL cholesterol value indicates an increased risk of protracting such a disease (7, 8).

Various clinical studies show that the quantification of apolipoproteins improves the predictability of coronary risk (9). There are several methods for determining apolipoproteins: radial immuno diffusion, rocket electrophoresis, radioimmunoassays, enzyme immunoassays, nephelometry, turbidimetry (10). Most of these methods are labour intensive, require special equipment and are hence unsuitable for processing the number of samples required for epidemiological studies.

This present article describes a turbidimetric method for quantification of apolipoproteins on microtitre plates.

Material

Samples

For this study we used serum from normo- and hypertriglyceridaemic patients, which had been collected for the PROCAM-Study (11).

Anti-serum

Anti-serum used for apolipoprotein quantification was obtained from Boehringer, Mannheim (anti-h-apolipoprotein-AI-antiserum, order no. 726 478, anti-h-apolipoprotein-AII-antiserum, order no. 726 486, anti-h-apolipoprotein-B-antiserum, order no. 726 494).

Standards

Apolipoprotein A-I and A-II were measured with the Cobas Bio (Roche) and standardized with the immunoneph reference standard apoproteins human (Immuno, Heidelberg, order no. 43 801 05). Apolipoprotein B measurements, using radial immuno diffusion, were standardized with the immuno reference standard (Immuno, order no. 49 100 05). The apolipoprotein calibration serum (Boehringer, Mannheim, order no. 837 237) or immuno reference standard by Immuno, Heidelberg, are suitable standards for turbidimetric measurements on microtitre plates.

Dilution

For the dilution of antisera for turbidimetric measurements with the microtitre system, we used the anit-h-apolipoprotein antiserum diluent from Boehringer, Mannheim, order no. 779 156.

Microtitre plates

The tests were carried out on flat-bottomed micro-well-dishes 96 (NUNC, Wiesbaden, order no. 269 620). After careful cleaning these dishes can be used several times.

Methods

The apolipoprotein A-I and A-II quantification was performed on the Cobas Bio as previously described (12, 13).

Apolipoprotein B determination with radial immuno diffusion was carried out with the *Mancini* method (14). Anti-apolipoprotein B-antibody (10 µl) was mixed with 20 ml agarose solution (10 g/l) in tris-barbital-lactate-buffer (0.045 mmol/l tris, 0.015 mmol/l barbituric acid, 1.9 mmol/l Na-azide, 0.21 mmol/l Ca-lactate). The sample was 5 µl of diluted serum (1 : 3 in 0.15 mol/l NaCl). The standard was diluted 1 : 1, 1 : 1,5, 1 : 2, 1 : 3, 1 : 4 and 1 : 6 in 0.15 mol/l NaCl. Following an incubation period of 72 hours the ring diameters were evaluated after staining with Coomassie blue R (Serva, Heidelberg).

Turbidimetric tests on microtitre plates

Quantification of apolipoprotein A-I and A-II: The serum was pre-diluted 1 : 60 in 0.15 mol/l NaCl, the standard 1 : 10 – 1 : 320 in geometric sequence. The antiserum was diluted 1 : 11 in antiserum diluent and incubated for 20 minutes at room temperature. For the determination of apolipoprotein A-I, 200 µl of the diluted antibody, and 20 µl diluted serum or diluted standard were pipetted in duplicate onto microtitre plates and incubated at room temperature for 90 minutes. For apolipoprotein A-II, the procedure was similar, but using 30 µl diluted serum. After careful shaking of the dish, turbidity was measured at 340 nm against a basic value (200 µl antibody dilution + 20 µl or 30 µl of 0.15 mol/l NaCl):

Quantification of apolipoprotein B

The serum was prediluted 1 : 30 in 0.15 mol/l NaCl, the standard 1 : 10 – 1 : 160 in geometric sequence. The antiserum was diluted 1 : 11 in antibody diluent and incubated for 20 minutes at room temperature. 200 µl of antibody dilution, 30 µl of serum and standard dilution were pipetted in duplicate on microtitre plates and incubated for 120 minutes at room temperature. Evaluation as for apolipoprotein A-I and A-II.

Results and Discussion

Comparison of the turbidimetric microtitre method (y) and turbidimetric method on the Cobas Bio (x) for apolipoprotein A-I and A-II

Regression analysis of paired values resulted in a good correlation of both methods: apolipoprotein A-I $r = 0.93$, $y = 1.02x - 5.0$, $n = 63$ (fig. 1); apolipoprotein A-II $r = 0.90$, $y = 1.07x - 5.6$, $n = 44$ (fig. 2).

Comparison of the turbidimetric microtitre method and radial immuno diffusion for apolipoprotein B

Regression analysis of turbidimetric values (y) and radial immuno diffusion values (x) resulted in a good correlation of the paired values ($r = 0.92$, $y = 0.95x + 9.8$, $n = 58$) (fig. 3).

Precision of the microtitre method

The variation coefficient for the precision in the series was 3.5% ($\bar{x} \pm$ SD: 1.22 ± 0.043 g/l, $n = 21$) for apolipoprotein A-I, 2.5% ($\bar{x} \pm$ SD: 0.319 ± 0.03 g/l, $n = 20$) for apolipoprotein A-II, 3.6% ($\bar{x} \pm$ SD: 1.01 ± 0.036 g/l, $n = 19$) for apolipoprotein B. The vari-

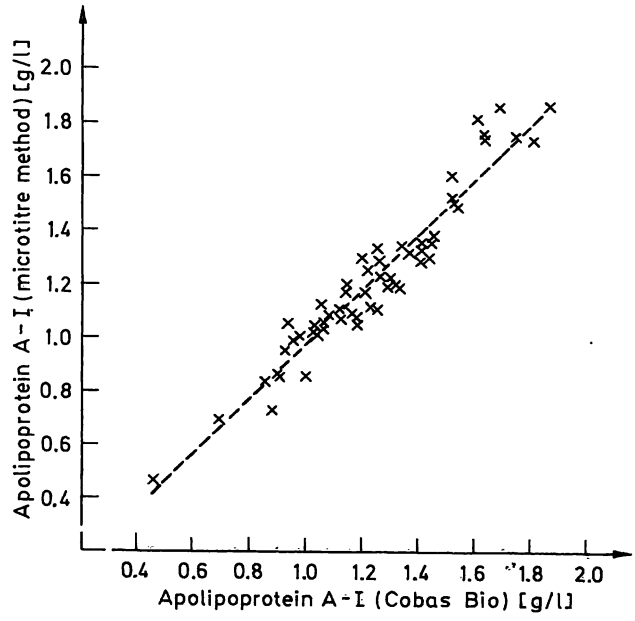

Fig. 1. Regression analysis of apolipoprotein A-I values analysed by turbidimetry on Cobas Bio vs. microtitre method
$r = 0.93$, $y = 1.02\,x - 5.0$, $n = 63$

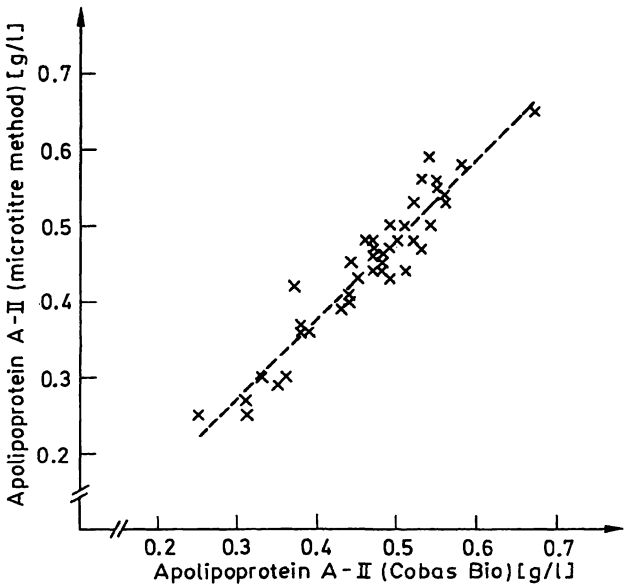

Fig. 2. Regression analysis of apolipoprotein A-II values analysed by turbidimetry on Cobas Bio vs. microtitre method
r = 0.90, y = 1.07 x − 5.6, n = 44

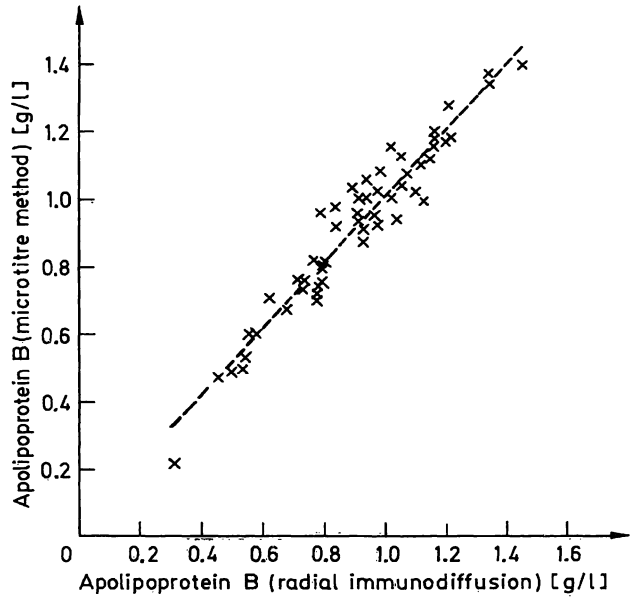

Fig. 3. Regression analysis of apolipoprotein B values analysed by radial immuno diffusion vs turbidimetry on microtitre plates
r = 0.92, y = 0.95 x + 9.8, n = 58

ation coefficient for day-to-day precision was 3.1% ($\bar{x} \pm$ SD: 1.32 ± 0.048 g/l, n = 45) for apolipoprotein A-I, 4.2% ($\bar{x} \pm$ SD: 0.32 ± 0.013 g/l, n = 39) for apolipoprotein A-II and 5.3% ($\bar{x} \pm$ SD: 0.76 ± 0.04 g/l, n = 48) for apolipoprotein B.

Linearity of the microtitre method

In order to test for linearity, standard material in 0.15 mol/l NaCl was increasingly diluted and measured. We found linearity in the region 0.1–4.5 g/l for apolipoprotein A-I, 0.05–2.00 g/l for apolipoprotein A-II and 0.1–3.00 g/l for apolipoprotein B. Working ranges for this method are 0.23–3.70 g/l for apolipoprotein A-I, 0.07–1.05 g/l for apolipoprotein A-II and 0.14–2.24 g/l for apolipoprotein B.

Influence of hypertriglyceridaemia and bilirubin concentration

It was shown that triacylglycerol concentrations up to 8 g/l and bilirubin up to 85 µmol/l had no influence on the precision of the results.

The method introduced here for the quantification of apolipoproteins offers several advantages over previously used methods: It uses far less reagents than the Cobas Bio and allows a faster throughput of samples. Two ml of antiserum on one microtitre dish are sufficient to process 42 samples together with standards within 2 hours. The apolipoprotein B determination in particular is considerably faster with this method than with traditional methods, such as radial immuno diffusion, rocket electrophoresis or enzyme immunoassays. The precision and accuracy of this method correspond to the standards of other methods which was demonstrated by the participation in a control trial of the CDC for apolipoprotein A-I and B.

A simplification of the method can be achieved by using an automated pipetting device. As ELISA test methods are increasingly used, most modern laboratories are equipped with a photometer for microtitre plates, so that the turbidimetric quantification of apolipoproteins on microtitre plates constitutes a cost and time saving alternative to methods used so far.

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